frontiers | Research Topics

Treatment of infectious diseases with bioactive compounds from medicinal plants: Their mechanisms and applications

Edited by
Iván J. Montenegro, Alessandra Russo, Carlos L. Cespedes-Acuña, Alejandro Madrid and Joan Villena García

Published in
Frontiers in Pharmacology

ISSN 1664-8714
ISBN 978-2-83252-057-4
DOI 10.3389/978-2-83252-057-4

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Treatment of infectious diseases with bioactive compounds from medicinal plants: Their mechanisms and applications

Topic editors

Iván J. Montenegro — Universidad de Valparaíso, Chile
Alessandra Russo — University of Catania, Italy
Carlos L. Cespedes-Acuña — University of Bio Bio Chillan Chile, Chile
Alejandro Madrid — Universidad de Playa Ancha, Chile
Joan Villena García — Universidad de Valparaíso, Chile

Citation

Montenegro, I. J., Russo, A., Cespedes-Acuña, C. L., Madrid, A., García, J. V., eds. (2023). *Treatment of infectious diseases with bioactive compounds from medicinal plants: Their mechanisms and applications*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83252-057-4

Table of contents

05 Isoquercetin as an Anti-Covid-19 Medication: A Potential to Realize
Majambu Mbikay and Michel Chrétien

18 Thymoquinone's Antiviral Effects: It is Time to be Proven in the Covid-19 Pandemic Era and its Omicron Variant Surge
Maen Abdelrahim, Abdullah Esmail, Noor Al Saadi, Eva Zsigmond, Ebtesam Al Najjar, Doaa Bugazia, Hadeel Al-Rawi, Ayat Alsaadi and Ahmed O. Kaseb

28 Promising Role of Emodin as Therapeutics to Against Viral Infections
Qingqing Shao, Tong Liu, Wenjia Wang, Tianli Liu, Ximing Jin and Zhuo Chen

39 The Antimicrobial Potential of the Neem Tree *Azadirachta indica*
Marina R. Wylie and D. Scott Merrell

55 Effects of Traditional Chinese Medicine and its Active Ingredients on Drug-Resistant Bacteria
Jimin Li, Shanshan Feng, Xin Liu, Xu Jia, Fengling Qiao, Jinlin Guo and Shanshan Deng

69 Modeling Kaempferol as a Potential Pharmacological Agent for COVID-19/PF Co-Occurrence Based on Bioinformatics and System Pharmacological Tools
Yong Jiang, Yi-Zi Xie, Chen-Wen Peng, Kai-Nan Yao, Xue-Ying Lin, Shao-Feng Zhan, Hong-Fa Zhuang, Hui-Ting Huang, Xiao-Hong Liu, Xiu-Fang Huang and Hang Li

86 A Five-Dimensional Network Meta-Analysis of Chinese Herbal Injections for Treating Acute Tonsillitis Combined With Western Medicine
Peiying Huang, Yin Li, Bixuan Huang, Shuai Zhao, Li Chen, Hansu Guan, Yan Chen, Yuchao Feng, Xiaoyan Huang, Yi Deng, Sisi Lei, Qihua Wu, Haobo Zhang, Zhongyi Zeng, Linsheng Zeng and Bojun Chen

99 Anti-inflammatory activities of Qingfei oral liquid and its influence on respiratory microbiota in mice with ovalbumin-induced asthma
Jun Zheng, Qian Wu, Liang Zhang, Ya Zou, Meifen Wang, Li He and Sheng Guo

117 Fuzheng Huayu Recipe and its active compounds inhibited HBeAg production by promoting TOMM34 gene expression in HBV-infected hepatocytes
Lu Xing, Rui Zeng, Kai Huang, Jingbo Xue, Hongliang Liu, Zhimin Zhao, Yuan Peng, Xudong Hu and Chenghai Liu

132 Polyphenolic promiscuity, inflammation-coupled selectivity: Whether PAINs filters mask an antiviral asset
Rick Sheridan and Kevin Spelman

149 Formulation of water-soluble *Buddleja globosa* Hope extracts and characterization of their antimicrobial properties against *Pseudomonas aeruginosa*
Nicolas Araya, Martín A. Leiva-Soto, Maria V. Bruna, Almendra Castro-Munoz, Beatriz Behrend-Keim, Daniel Moraga-Espinoza and Tania F. Bahamondez-Canas

frontiers in Pharmacology

REVIEW
published: 02 March 2022
doi: 10.3389/fphar.2022.830205

Isoquercetin as an Anti-Covid-19 Medication: A Potential to Realize

Majambu Mbikay and Michel Chrétien*

Functional Endoproteolysis Laboratory, Montreal Clinical Research Institute, Montreal, QC, Canada

Isoquercetin and quercetin are secondary metabolites found in a variety of plants, including edible ones. Isoquercetin is a monoglycosylated derivative of quercetin. When ingested, isoquercetin accumulates more than quercetin in the intestinal mucosa where it is converted to quercetin; the latter is absorbed into enterocytes, transported to the liver, released in circulation, and distributed to tissues, mostly as metabolic conjugates. Physiologically, isoquercetin and quercetin exhibit antioxidant, anti-inflammatory, immuno-modulatory, and anticoagulant activities. Generally isoquercetin is less active than quercetin *in vitro* and *ex vivo*, whereas it is equally or more active *in vivo*, suggesting that it is primarily a more absorbable precursor to quercetin, providing more favorable pharmacokinetics to the latter. Isoquercetin, like quercetin, has shown broad-spectrum antiviral activities, significantly reducing cell infection by influenza, Zika, Ebola, dengue viruses among others. This ability, together with their other physiological properties and their safety profile, has led to the proposition that administration of these flavonols could prevent infection by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), or arrest the progression to severity and lethality of resulting coronavirus disease of 2019 (Covid-19). *In silico* screening of small molecules for binding affinity to proteins involved SARS-CoV-2 life cycle has repeatedly situated quercetin and isoquercetin near to top of the list of likely effectors. If experiments in cells and animals confirm these predictions, this will provide additional justifications for the conduct of clinical trials to evaluate the prophylactic and therapeutic efficacy of these flavonols in Covid-19.

Keywords: isoquercetin, quercetin, antiviral, coronavirus, SARS—CoV—2, COVID—19

OPEN ACCESS

Edited by:
Joan Villena García,
Universidad de Valparaíso, Chile

Reviewed by:
Ana Clara Aprotosoaie,
Grigore T. Popa University of Medicine and Pharmacy, Romania
Jaime A. Yáñez,
Norbert Wiener Private University, Peru

****Correspondence:***
Majambu Mbikay
majambu.mbikay@ircm.qc.ca

Specialty section:
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

Received: *06 December 2021*
Accepted: *26 January 2022*
Published: *02 March 2022*

Citation:
Mbikay M and Chrétien M (2022) Isoquercetin as an Anti-Covid-19 Medication: A Potential to Realize. Front. Pharmacol. 13:830205. doi: 10.3389/fphar.2022.830205

INTRODUCTION

Flavonoids form a widely diverse group of plant secondary metabolites which contribute to plant growth and survival in many ways, including germination and protection from environmental stresses (Kumar et al., 2018). As a common structural feature, they are made of 15 carbon atoms arranged in two phenolic rings (A and B rings) connected by a three-carbon chain in a C6-C3-C6 configuration. In flavonols, the three-carbon chain form a heterocyclic ketone ring (C ring) carrying a hydroxyl group on C3. Quercetin [IUPAC name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one] is a flavonol; distinctively, it carries additional hydroxyl groups at C5 on the A ring as well as at C3′ and C4′ on the B ring (**Figure 1**).

Quercetin derivatives have one or more of these hydroxyl groups modified, often by sugars (e.g., glucose, galactose, rhamnose, arabinose, xylose), but also by methyl, sulfate, acetate, or phosphate

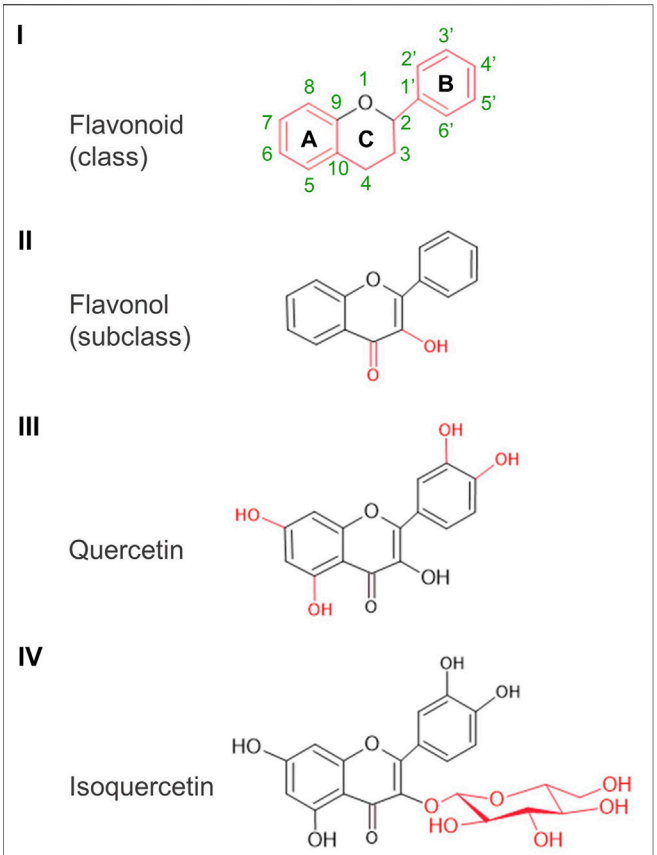


FIGURE 1 | Basic structures: from flavonoid to isoquercetin. Class, subclass, and particular molecular features are illustrated in red. Atom numbering of the A, B, and C rings indicated for the basic structure of flavonoids applies to all the other structures.

groups. In its February 2020 report, the PubChem database counted 679 such derivatives[1]. They are ubiquitously and variably distributed in the plant kingdom, including in edible plant parts such as leafy vegetables, fruits, grapes, spices, and teas.

Quercetin and its derivatives have been the object of intense scientific investigation in the last three decades as evidenced by the exponential growth in the number of scientific articles reported in Google Scholar. From less than 500/decade before the 1980s, these articles number close to 10,000 in the last decade (2010–2019) (**Figure 2**). The vast majority of studies were conducted with unmodified quercetin (quercetin aglycone). Derivatives which have also attracted the attention of investigators include quercetin-3-rhamoglucoside (rutin), quercetin-3-glucoside (isoquercetin or isoquercitrin), quercetin-3-rhamnoside (quercitrin), and 3' methyl-quercetin (isorhamnetin).

In spite of the plethora of preclinical studies demonstrating the therapeutic potential of quercetins against various pathologies, including viral infections, efforts to convert this nutraceutical into a pharmaceutical for therapeutic use in humans has been frustrated by its poor bioavailability after ingestion. Many alternative forms of quercetin with a better metabolic outcome have been investigated, among them the natural mono-glycosylated isoquercetin and the manufactured poly-glycosylated enzymatically-modified isoquercetin (EMIQ) (Makino et al., 2009).

In this review, we focus on the distinctiveness of isoquercetin from quercetin aglycone. We first describe the general physiological properties of quercetin; then, relying mostly on studies that compare the two compounds in parallel, we examine how glycosylation influences these properties as well as the potential of isoquercetin as a better broad-spectrum antiviral for prophylactic and therapeutic use against SARS-CoV-2 infection and the resulting Covid-19 disease.

QUERCETIN RESTORES OXIDATIVE AND INFLAMMATORY HOMEOSTASIS

Quercetin Against Oxidative Distress

Normal cellular metabolism involves reduction/oxidation (redox) reactions which result in the formation of free radical-generating atoms and molecules. Depending on whether the free radical is based on oxygen or nitrogen atoms, these metabolic byproducts are collectively known as reactive oxygen species [ROS, e.g., superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydroxyl ($^{\bullet}OH$)] or reactive nitrogen species [RNS, e.g., nitric oxide ($NO^{\bullet}$), nitrogen oxide ($NO_2^{\bullet}$)], respectively (Gupta et al., 2016). Examples of ROS/RNS-generating metabolic pathways include the electron transport chain of glucose oxidative phosphorylation which leads to the production of ATP in mitochondria (Zhao et al., 2019), the unfolded protein response (UPR) in the endoplasmic reticulum (ER) catalyzed by resident oxidoreductases, and the respiratory burst mediated in phagocytes in reaction to the presence of endogenous or exogenous 'abnormal' molecules (El-Benna et al., 2016).

All cells possess an endogenous antioxidant system that reduces these reactive species. Major components of the system involves glutathione (GSH) and associated reducing enzymes [e.g., glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx) and superoxide dismutase (SOD)] and the protein cysteine thiol–disulfide exchange catalyzed by, among other enzymes, protein disulfide isomerase (PDI) (Gupta et al., 2016). When this system is overwhelmed, ROS/RNS react with biological macromolecules (e.g., lipids, proteins, carbohydrates, and nucleic acids), generating oxidized and dysfunctional varieties such as lipid peroxides, protein carbonyls, oxidized lipoproteins, In the words of Helmut Sies, the discoverer of hydrogen peroxide (H_2O_2), physiological ROS is associated with "oxidative eustress," pathological ROS with "oxidative distress" (Sies, 2020).

Quercetin can efficiently counter oxidative distress. Its potent antioxidant activity of quercetin derives from the very low redox potential afforded by its multiple hydroxyl groups which allow it to donate electrons and protons to and capture electrons from ROS/RNS and other oxidized molecules. Quercetin also sustains the endogenous glutathione-based

[1]https://pubchem.ncbi.nlm.nih.gov/

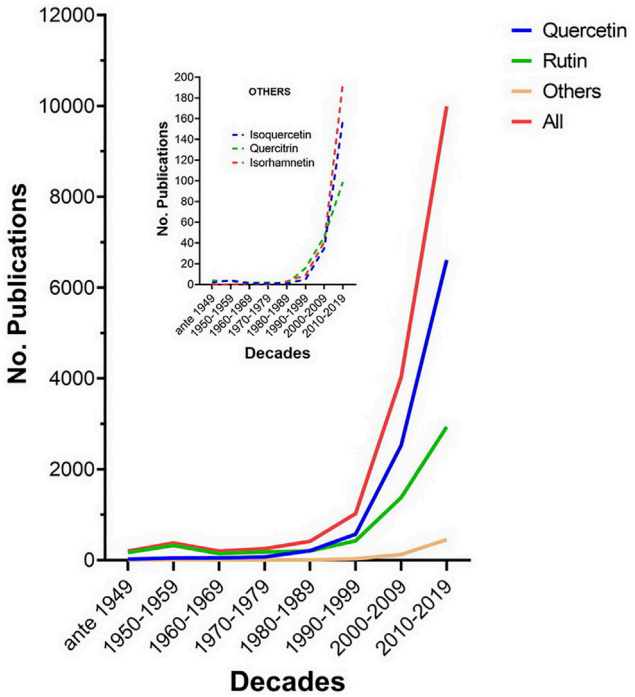


FIGURE 2 | Publications per decade on quercetin and its major derivatives. Data were retrieved from Google Scholar using as separate keywords on the title page the terms quercetin aglycone, rutin, quercitrin, isorhamnetin, and isoquercetin (or isoquercitrin or quercetin-3-glucoside). The number of hits for quercetin aglycone, rutin, other derivatives, and the sum total were classified by decade. Other derivatives include quercitrin, isoquercetin, and isorhamnetin; their hits per decade are displayed in the inset.

antioxidant system, stimulating reductases and inhibiting oxidases (Boadi et al., 2016). Moreover, by chelating transition metals (e.g., Fe^{2+}, Cu^{2+}), quercetin restricts their participation in oxidative processes and in ROS formation (Boots et al., 2008).

Quercetin Against Inflammation

Inflammation involves a cascade of signaling events extending from plasma cell membranes to increased expression and nuclear translocation of transcriptional factors [e.g., nuclear factor kappa B (NF-κB), activating protein-1 (AP-1), nuclear factor-like 2 (Nrf-2)], to activation of a variety of genes for proinflammatory molecules, among them, the cytokines [e.g., tumor necrosis factor α (TNF-α)], interferons (INF), interleukins (IL), and chemokines. An immune response, when moderate and transient, is beneficial for tissue homeostasis. It is modulated by a balance between the two subsets of $CD4^+$ T helper cells, Th1 cells which produce the proinflammatory TNFα and INF-γ, as well as IL-2 and 12 and Th2 cells which produce the anti-inflammatory IL-4, 5, 10, and 13. (Chatterjee, 2016). Quercetin is known to modulate the Th1/Th2 balance towards an overall cytokine profile that can induce a more effective and beneficial (i.e., non-pathogenic) immune response (Park et al., 2009; Tanaka et al., 2020).

Quercetin Disrupts the Oxidation-Inflammation Feedback Loop

In the disease state, oxidative stress and inflammatory stress often feed on one another: on one hand, oxidized macromolecules are recognized by the innate immune system as damage-associated molecular patterns (DAMPs) (Chen et al., 2018); on the other hand, the stimulated immune system produces more free radicals to accelerate the destruction of cells exhibiting these patterns. This oxidation-inflammation feedback loop (OIFL) is the indiscriminate hallmark of a vast array of pathologies, including cancer, diabetes, atherosclerosis, hypertension, Alzheimer's disease, and infections (Liguori et al., 2018; Furman et al., 2019). Quercetin appears to act as an "OIFL disruptor". This ability might underlie many of its purported health benefits. Indeed, a number of preclinical studies have shown that quercetin can mitigate all the above-cited pathologies (Salehi et al., 2020).

ISOQUERCETIN IS QUERCETIN MODIFIED

Isoquercetin is generally considered a "pro-quercetin" in the sense that its biological activities follow its conversion to quercetin. This is largely true when isoquercetin is taken orally, but not when it is administered parenterally. Indeed, injected intravenously into rats, isoquercetin could be detected unaltered in 24-h urine (Choudhury et al., 1999). The question in this case is whether its physiologic activity is a true replication of that of its aglycone relative. Isoquercetin carries a single glucose moiety on carbon three of the C ring of quercetin backbone, losing an OH group (**Figure 1**). This modification not only renders it more hydrophilic and about 4-fold more soluble in water (~206 μM) than quercetin (~50 μM) (Makino et al., 2009), but also makes its B ring non-planar with the other two. These changes may influence its interaction of membrane lipid bilayers, its absorption and metabolism, as well as its physiological properties. As illustrated in the following paragraphs, *in vitro* and *ex vivo*, the glycosylated quercetin exhibits reduction of many of these properties compared to its aglycone relative; however, when taken orally, it provides a significant pharmacokinetic and physiological advantage, as it is better absorbed in the intestines and rapidly converted into the more active quercetin aglycone and its metabolites. The influence and impact of quercetin C3 glycosylation are described below.

On Membrane Interactions

The first point of contact of quercetin or isoquercetin with the eukaryotic cell is the plasma membrane. The latter is made of a phospholipids bilayer to which are associated, incrusted or anchored, various lipids (e.g., cholesterol, triacylglycerols) and proteins (e.g., receptors, enzymes); and which is organized in dynamic functional microdomains (e.g., lipid rafts, caveolae, coated pits, ion channels). From and *via* the plasma membrane is initiated and propagated the intracellular transduction of signals that determine cellular physiology, including gene expression. It has been known since the 1970s that some pathologies are associated with significant alterations of membrane

physicochemical properties (i.e., composition, fluidity, microviscosity, permeability). (Cooper, 1977; Scott, 1982). Some investigators attribute the pleotropic bioactivity of flavonoids, including quercetin derivatives, to their alterations of these properties (Tsuchiya, 2015). For example, by fluorescence polarization measurements on biomimetic membranes, quercetin affects lipid bilayer in biphasic fashion, fluidizing it at low concentrations (<2.4 μM) and rigidifying at higher concentrations (>5 μM) (Tsuchiya et al., 2002). At 10 μM, quercetin and isoquercetin differ in their impact on membrane fluidity: the former reduces it because of its greater hydrophobicity and deeper penetration within the lipid bilayer, whereas the latter, being more hydrophilic, does not (Tsuchiya, 2010). These differential interactions can variably affect the dynamics of membrane receptors, enzymes and other signaling molecules. Membrane fluidization is strongly associated with cancerous cell metastasis (Nicolson, 2015); inflammatory cell excitation (Calder, 2012), lipid peroxidation (Sergent et al., 2005), platelet aggregation (Vlasic et al., 1993), and infection by enveloped viruses (Harada, 2005). Thus, increased membrane rigidity afforded to cells by quercetins and other flavonoids may partly account for their broad spectrum antineoplastic, anti-inflammatory, antioxidant, antithrombotic, and antiviral properties.

On Antioxidant Capacity and Activity

The antioxidant ability of a compound is assessed *in vitro* in term of capacity and activity: the former measures the end-point radical scavenging efficiency and potency; the latter the scavenging kinetics. These properties are strongly influenced by the steric structure of the compound as well as by the reaction solvent and its pH. It is generally measured on free radical-generating substrates such 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ABTS) (Apak, 2019).

In cell-free assays, isoquercetin exhibits lower antioxidant capacity relative to quercetin in phosphate-buffered saline (PBS) at pH ≤ 6, and in methanol, but greater DPPH-measured activity in methanol (Xiao et al., 2021). This was also observed by Park et al. (2021) using the same flavonols. Interestingly, these investigators reported that, in HT22 mouse hippocampal cells, quercetin was about 10-fold more efficient than isoquercetin at reducing cellular ROS formation and the apoptosis that resulted from the treatment of these cells with 4 mM glutamate. Moreover, a combination of the two flavonols at concentrations that were individually ineffective against apoptosis, fully restored the viability of the glutamate-treated HT22 cells (Park et al., 2021), suggesting synergy between them.

Results obtained from cell-based antioxidant assays with these compounds may sometimes differ from those obtained from cell-free assays since, with cells, membrane characteristics and interactions come into play. For example, it have been established that membrane fluidity promotes lipid peroxidation on one hand, and that lipid peroxidation results in membrane rigidity, on the other hand (Borst et al., 2000). Thus, quercetin and isoquercetin may restore "healthy membrane fluidity," by acting both as antioxidants and lipid bilayer-interacting molecules.

On Anti-Inflammatory Property

Ex-vivo, the inflammatory inhibition of flavonoids is commonly evaluated using macrophages—primary or immortalized—stimulated with lipopolysaccharide (LPS) to produce more nitric oxide (NO) as a result of increased expression of inducible nitric oxide synthase (iNOS). Rat peritoneal macrophages pretreated with 100 μM quercetin and isoquercetin before LPS stimulation inhibited NO production by 66% and 48%, respectively, with a corresponding decrease in iNOS expression, indicating that the aglycone has a more potent anti-inflammatory activity (Lee et al., 2008). A differential reduction of these inflammatory marker was also observed in mouse macrophage RAW264.7 cells stimulated with LPS (Choi et al., 2012) or zymosan (Kim et al., 2013); the reduction was associated with inactivation of the NF-κB signaling pathway. In LPS-stimulated in BV2 mouse microglial cells, at 10 μM, quercetin was 6-fold more potent than isoquercetin at inhibiting NO production (Kwon et al., 2004). An alternative model cell system of inflammation-associated liver damage consists of human hepatocellular carcinoma HepG2 cells treated with 5% ethanol. Using this model, it was shown that, while a 1-h pretreatment with 10 μM quercetin or isoquercetin reduced to comparable extent ethanol-induced NO production and iNOS expression, the aglycone form were more effective than the glucoside at inhibiting TNFα secretion, as well as activation and nuclear translocation of the pro-inflammatory Nrf2 transcriptional factor (Lee et al., 2019).

In vivo, inhibition of inflammation by quercetin and isoquercetin seems comparable. For example, when air pouches generated subcutaneously on the backs of rats were injected with carrageenan, an inflammatory response ensued reflected by increased volume of the exudate as well of its content in cells, proteins, TNFα, prostaglandins and macrophage inflammatory protein 2. Injection of quercetin or isoquercetin (10 mg/kg) into the pouch 1 h prior to carrageenan challenge significantly and comparably reduced these inflammatory indices (Morikawa et al., 2003). Similarly, when mice immunologically primed by vaccination with ovalbumin were challenged intranasally with the same compound, they developed an asthma-like inflammatory reaction reflected by an increase, 24 h later, of leukocytes in bronchoalveolar lavage fluid, blood and pulmonary parenchyma. Oral administration of quercetin (10 mg/kg) and isoquercetin (15 mg/kg) similarly reduced eosinophil count in all three biological samples, suggesting they could be equally effective as anti-allergic drugs (Rogerio et al., 2007).

On Anticoagulant Activity

Choi et al. (2016) conducted a battery of *in vitro*, *ex vivo*, and *in vivo* assays to comparatively evaluate the anti-coagulation activity of quercetin and isoquercetin. *In vitro* assays included fibrin clotting, fibrin polymer formation, thrombin activity, Factor Xa activity, and platelet aggregation, coagulation activated partial thromboplastin time (APTT) and prothrombin time (PT); the *ex vivo* assay consisted of measure of APTT and PT on blood collected after i.v. flavonol administration to mice; the *in vivo* assay consisted of evaluating the protection rate against thromboembolism induced by i.v. injection of human

thrombin. By all these assays. The two flavonols were effective anticoagulants; *in vitro*, quercetin was more effective than isoquercetin, except in the Factor Xa inhibition assay; the effect of injected glucoside was stronger than of the aglycone in the *ex vivo* assay; it was slightly weaker in the *in vivo* assay. The greater effectiveness of the aglycone *in vitro* was also observed in an assay using platelet-enriched rat plasma treated with collagen to induce aggregation: 0.5, 1, and 2 mg/ml quercetin inhibited aggregation by 40, 100, and 100%, respectively; while, at the same concentrations, isoquercetin-induced inhibition was <10, 60, and 100%, respectively. (Ko et al., 2018).

Besides interacting directly with fibrin and thrombin, quercetin and isoquercetin can exert their anticoagulant effect through inhibition protein disulfide isomerase (PDI). This ER-resident oxidoreductase is also expressed in platelets and endothelial cells ending up at their surface; its expression, when upregulated by thrombin stimulation or vascular injury, could contribute to thrombogenesis (Xu et al., 2021). Strangely, when the flavonol inhibition of the reductase activity of recombinant PDI was assayed *in vitro* using a spectrometric measure of insulin aggregation in the presence of DTT, the IC_{50} of quercetin, isoquercetin, rutin, and quercetin-3-glucuronide, were >100, 7.1, 6.1, and 5.9 μM, respectively, indicating that these C3 modification in the quercetin derivatives enhanced the anti-PDI activity (Jasuja et al., 2012).

THE PHARMACOKINETIC ADVANTAGE OF ISOQUERCETIN

From Improved Intestinal Absorption

In rats, orally administered isoquercetin is not absorbed and metabolized until it reaches the small intestine, whereas a fraction of quercetin can be taken up by the stomach and secreted into bile (Crespy et al., 2002). Quercetin aglycone is more lipophilic than isoquercetin: thus the aglycone, but not the glucoside, traverses the lipid bilayer and enters cells by passive diffusion as demonstrated by the rapid presence of it and its metabolites on the basolateral side of human intestinal Caco2 cells after application of the flavonols to the apical side (Murota et al., 2000). Penetration of the isoquercetin into cells is apparently facilitated by its hydrophilicity which leads to greater concentration near the intestinal brush border membrane where its sugar is removed by lactase phlorizin hydrolase, producing the aglycone which diffuses through the membrane (Day et al., 2000). The uptake also appears to be actively mediated by sodium-dependent glucose transporter 1 (SGLT-1). Indeed, in an *in vitro* mucosal uptake assay using pieces of rat jejunum, isoquercetin, but not quercetin, significantly inhibited SGLT-1-mediated uptake of a non-metabolisable glucose analogue in a competitive fashion, an inhibition potentiated by the addition of the SGLT-1 blocker phloridzin, indicating that the glucoside utilized the same transporter to enter cells (Ader et al., 2001; Wolffram et al., 2002). The sugar moiety of isoquercetin is removed by mucosal β-glycosylases as shortly as 30-min after perfusion of rat jejunum with isoquercetin, as only the aglycone form, its conjugates (mostly glucuronidated) and metabolites (mostly 3′ and 4′ methylated) could be found in the intestinal lumen and in blood veins (Morand et al., 2000a; Crespy et al., 2001; Chang et al., 2005).

To Improved Pharmacokinetics and Pharmacodynamics

These mechanism of isoquercetin uptake by the intestinal mucosa may explain the 1.5-3-fold greater plasma concentration of quercetin and its metabolites after its ingestion compared to that of quercetin aglycone which has been observed in rats (Hollman et al., 1995; Morand et al., 2000a; Morand et al., 2000b), dogs (Reinboth et al., 2010), pigs (Cermak et al., 2003), and humans (Sesink et al., 2001). Comparing pharmacokinetic parameters after oral administration, isoquercetin yields a maximum plasma concentration C_{max}) of quercetins 1.7 to 10-fold greater and the area under de curves for a given time ($AUC_{0\text{-}t}$) 1.8 to 6-fold greater than quercetin aglycone, depending on species (Lesser et al., 2004; Makino et al., 2009; Reinboth et al., 2010; Stopa et al., 2017) (**Table 1**).

When tissue and plasma concentrations of quercetin metabolites (acid de-glucuronidated/de-sulfated quercetin as well as 3′ and 4′ O-methyl-quercetin) were measured after an 8-day oral gavage of ~40 mmol/kg/d of quercetin and isoquercetin to rats, by comparison, isoquercetin gavage generated 2 to 5 more metabolites in tissues and 2 to 3 more in plasma than quercetin. The order of abundance in tissues was: lung > liver > kidney > heart cerebellum > cortex > hippocampus > striatum. The lung content of metabolites after isoquercetin gavage (10.1 nmol/g) was nearly 2.5 greater than that obtained after quercetin gavage (4.1 nmol/g) (Paulke et al., 2012).

The greater bioavailability of quercetin and its conjugates (which, as discussed below, are biologically active compounds on their own) after oral administration makes it more attractive for harnessing the health benefits attributed to quercetin aglycone.

ISOQUERCETIN IS A BROAD-SPECTRUM ANTIVIRAL

The potential of flavonoids, including quercetin, as broad-spectrum antiviral agents has been widely demonstrated by in cell lines and animal models, as recently reviewed (Badshah et al., 2021). Since isoquercetin, as a pro-quercetin, offers a better pharmacokinetics profile after oral administration, and therefore promises to be more efficacious, we examine here below experimental studies of its antiviral activity, in which the 50% inhibition concentration (IC_{50}) and the 50% cytotoxicity concentration (CC_{50}) were measured and have resulted in selectivity indices (SI = IC_{50}/CC_{50}) of ≥3 (**Table 2**).

Against Influenza Virus

Influenza virus is an enveloped negative-strand RNA virus found in animals (e.g., birds, pigs) and humans and transmissible by air mostly through expiratory aerosol/droplet ejections during cough and sneezing. It causes typical symptoms of viral infections (i.e., fever, headache, muscle and joint pain) accompanied by respiratory discomforts (e.g., sore throat, cough, rhinitis) with

TABLE 1 | Pharmacokinetic parameters of quercetin and isoquercetin after oral administration.

Species	Flavonol[a]	Dose	C_{max}[b]	T_{max}	AUC[a,b] $_{0–12*, 0–24**, 0–\infty***}$	References
Rat	—	μmol/kg bw	μmol/L	h	h × μmol/L*	Makino et al. (2009)
	Quercetin	50	0.26 ± 0.06	—	2.6 ± 0.7	—
	Isoquercetin	50	2.66 ± 0.81	—	15.8 ± 3.6	—
Dog	—	μmol/kg bw	nmol/L	H	min × μmol/L***	Reinboth et al. (2010)
	Quercetin	30	229.2 0.20	3.9 ± 0.5	174.9 ± 19.7	—
	Isoquercetin	30	888.3 ± 71	4.1 ± 0.3	410.2·± 26.7	—
Pig	—	μmol/kg bw	μmol/L	Min	min × μmol/L**	Lesser et al. (2004)
	Quercetin	30	0.518 ± 0.056	102.9 ± 8.0	117.3 ± 18.5	—
	Isoquercetin	30	0.908 ± 0.089	70 ± 7.9	205.5 ± 19.8	—
Man	—	mg/adult	μmol/L	H	h × μmol/L**	Stopa et al. (2017)
	Quercetin	500	0.8	—	3.8	—
	Isoquercetin	500	4.22	—	18.3	—

[a]*Abbreviations: AUC, area under the curve; bw, body weight; IQC, isoquercetin; QC, quercetin aglycone.*

[b]*The quercetin forms titrated were: for rat and man, aglycone, glucuronidated, and methylated quercetins; for rat all (Makino et al., 2009) but methylated quercetins. Data are expressed a mean ±, standard error for rats and pigs, or standard deviation for dogs. -, not reported.*

TABLE 2 | Isoquercetin antiviral efficiency and selectivity.

Virus	Strain/Isolate	Cell line	IC_{50} (μM)	CC_{50} (μM)	SI	References
IAV	Op	MDCK	1.2	46	38	Kim et al. (2010)
ZIKV	PRVABC59	Vero E6	1.2	>100	>83	Wong et al. (2017)
	PF-25013–18	Huh-7	14.0	>200	>14	Gaudry et al. (2018)
	PF-25013–18	A549	15.5	>200	>13	Gaudry et al. (2018)
	PF-25013–18	SH-SY5Y	9.7	>200	>21	Gaudry et al. (2018)
EBOV	Kikwit	Vero E6	5.3	>100	>19	Qiu et al. (2016)
	—	—	IC_{50} (μg/ml)	CC_{50} (μg/ml)	—	—
HSV	Types 1 and 2	Vero	0.4	>200	>500	Hung et al. (2015)
VZV	pOka	PFF	14.5	>20	>1.4	Kim et al. (2020)
HCMV	Towne	PFF	1.9	>20	>10	Kim et al. (2020)

Virus acronyms as well as species and organ origins of cells are described in the text. IC_{50}, 50% inhibitory concentration; CC_{50}, 50% cytotoxic concentration; SI, selectivity index.

possible complication of potentially lethal pneumonia. Although vaccines against it can provide relative protection, they have to be reformulated seasonally to counter new strains that result from the high mutation rate of its genome. Effective synthetic anti-IV drugs have been developed, but the eventual virus resistance to them justifies the search for novel drugs (Javanian et al., 2021).

Isoquercetin has the potential to become such a drug. In an assay using Madin-Darby canine kidney (MDCK) or green monkey kidney Vero cells, isoquercetin was shown to inhibit the replication of influenza A and B viruses (IAV and IBV) with an ED_{50} of 1.2 μM, 40-fold more effectively than quercetin aglycone (ED_{50} 48 μM). While serial passages of the virus in the presence of 2 μM of approved antiviral drugs, such as amantadine (a viral M2 ion channel inhibitor) or oseltamivir (a neuraminidase inhibitor), lead to the emergence of drug-resistant virus, passages in the presence of 2 μM of isoquercetin alone or with the above-cited drugs did not. When mice were intranasally infected with mouse-adapted IAV, the virus was detected in their lungs after 6 days, and the bronchial epithelium showed signs of necrosis. Daily i.p. injection of isoquercetin at 2 mg/kg/d and 10 mg/kg/d, starting 2 days pre-infection, resulted in 3.3-fold and 19.2-fold decrease in lung viral titer, respectively, and in less lung histopathological deterioration (Kim et al., 2010). The greater *in vivo* anti-IAV effectiveness of isoquercetin relative to quercetin was confirmed in a follow-up study (Thapa et al., 2012). Plaque formation by MDCK cells infected with avian H5N1 influenza virus in the presence of as low as 1 ng/ml quercetin and isoquercetin was also shown to be inhibited by 68 and 79%, respectively. (Ibrahim et al., 2013).

Mechanistically, isoquercetin did not inhibit neuraminidase, but blocked polymerase basic protein 2 (PB2) subunit of virus, while reducing oxidative and inflammatory stress as well PDI activity (Kim and Chang, 2018; Nile et al., 2019). Isoquercetin and mostly its quercetin-3-glucuronide were shown to bind PB2 with an affinity of −9.6 and −9.1 kcal/mol, respectively; and to inhibit its activity with a K_i of 3.7 and 0.2 μM, respectively (Gansukh et al., 2021).

Against Zika Virus and Dengue Virus

ZIKV and DENV are single-strand positive RNA flaviviruses transmitted by mosquitoes. Besides systemic morbidity, infection

by ZIKV can lead to neurological complications such as the Guillan-Barré Syndrome, encephalitis and microcephaly of the newborn; infection by DENV to fatal hemorrhagic fever and a shock syndrome (Silva et al., 2020). Because there is no drug to treat diseases caused by these viruses, the possibility that quercetin and its derivatives can counter the underlying infections came as a promising development.

We were the first to show that isoquercetin potently inhibited ZIKV infection and proliferation in Vero cells with an IC_{50} of 1.2 μM and IC_{90} of 1.5 μM, as measured by cytopathic effect and the level of the viral nonstructural protein NS1. Evaluating the *in vivo* anti-ZIKV efficacy of isoquercetin using immuno-compromised mice, we observed that, whereas all untreated mice succumbed to ZIKV infection after 7 days, 80% of mice i.p. injected with isoquercetin at 50 mg/kg/d survived after 7 days; and 50% of them after 30 days (Wong et al., 2017). The inhibitory effectiveness of isoquercetin against ZIKV infection depends in part on cell type. Thus, Gaudry and others (Gaudry et al., 2018) determined the IC_{50} in neuroblastoma SH-SY5Y, hepatocellular carcinoma Huh-7, and lung epithelial A549 cell line to be 9.7, 14.0, and 15.5 μM, respectively. In a series of elegant experiments using A549 cells, these investigators determined that isoquercetin did not affect viral particle integrity or virus attachment to cells, but inhibited virus internalization. Surprisingly, they found quercetin aglycone to be totally ineffective at inhibiting viral infection in these cells. In contrast, the aglycone was shown to be effective in Vero cells with an IC_{50} of 2.3 μM, and to inhibit the viral NS2B-NS3 protease *in vitro* with an ED_{50} of 1.17 μM (Zou et al., 2020).

Ex vivo, isoquercetin appears to be more effective against ZIKV than against DENV infection. DENV-2 infection of Vero cells have been shown to be inhibited by quercetin aglycone with IC_{50} around 30 μM (Zandi et al., 2011). *In vitro*, quercetin and isoquercetin reduced the activity of recombinant NS2B-NS3 protease of DENV-2 and DENV-3 with IC_{50} of 23 and 44 μM, and a K_i of 20 and 37 μM, respectively (De Sousa et al., 2015).

Against Ebola Virus

EBOV is a filovirus which causes a high-fatality disease, called Ebola virus disease (EVD), transmissible through contact with infected biological materials and characterized initially by typical infective symptoms soon followed by severe gastrointestinal symptoms and, in some cases, coagulopathies and vital organ failure (Feldmann et al., 2020). The recent development of anti-EBOV therapeutic vaccines which efficaciously reduce EDV mortality does not preclude the need for alternative medications, in view of the possibility of emergence of immune-evading variants (Tshiani Mbaya et al., 2021).

Isoquercetin could be one such alternative. In 2016, we were the first to report that this flavonol could inhibit infection of Vero cells by distinct species of EBOV with an IC_{50} and IC_{90} of 5.3 μM and 9.3 μM, respectively. Intraperitoneal (i.p.) injection of isoquercetin at 50 mg/kg into mice as little as 30 min prior to infection with lethal dose of mouse-adapted EBOV, followed by similar i.p. injections of the drug every other day, protected 90%–100% animals from EDV mortality. This protection was associated with dramatic reduction of viral load in tissues and blood. Initiation of treatment 1 day post-infection led to only 30% protection, indicating that the flavonol is most effective as a prophylactic anti-EBOV drug (Qiu et al., 2016). The proposed mechanisms of the anti-EBOV activity of isoquercetin involves inhibition of viral entry (Qiu et al., 2016) as well as maintenance of the cellular antiviral interferon signaling cascade which can get blocked by the EBOV protein VP24 in the early steps of infection (Fanunza et al., 2020).

Against Coronaviruses

Coronaviruses are enveloped positive RNA-strand viruses of the *Coronaviridae* family which infects animals and men and are transmissible across species. Their genome encodes several proteins, the most druggable being the RNA-dependent RNA polymerase (RdRp), the chymotrypsin-like protease 3 ($3CL^{pro}$), the papain-like protease (PL^{pro}), the envelope spike (S) glycoprotein. Coronavirus that infect humans can cause diseases ranging from mild upper respiratory disease to severe respiratory syndromes. Recent outbreaks of severe syndromes were caused by severe acute respiratory syndrome-coronavirus-1 (SARS-CoV) in 2003, Middle East respiratory syndrome-coronavirus (MERS-CoV) in 2012, and SARS-CoV-2, the cause of the ongoing pandemic of coronavirus disease since 2019 (Covid-19). Although effective anti-SARS-CoV-2 vaccines have been developed and are being deployed around the world, the need for broad-spectrum therapeutic drugs against immune-evading variants of the virus remains nonetheless.

Since the above outbreaks, there has been a flood *in silico* molecular docking analyses and molecular dynamics simulations of flavonoids binding to target viral proteins. In one such study, quercetin and isoquercetin showed strong affinity (dock score −7.7 to −10.3 kcal/mol) for SARS-CoV-2 S protein, RdRp, $3CL^{pro}$ and PL^{pro}, suggesting that these compounds may possess anti-CoV efficacy (Hiremath et al., 2021). Experimental evidence seems to support some of the *in silico*-derived inferences. For example, *in vitro* assays using recombinant $3CL^{pro}$ from SARS-CoV or MERS-CoV and a Förster resonance energy transfer (FRET) substrate have shown that quercetin or isoquercetin can inhibit the hydrolytic activity of these proteases with IC_{50} in the 24–73 μM range (Ryu et al., 2010; Nguyen et al., 2012; Park et al., 2017; Jo et al., 2019); and that quercetin can inhibit recombinant SARS-CoV PL^{pro}, with an IC_{50} of 8.6 μM (Park et al., 2017). FRET assays were also used to screen a 150 compound-library of small molecules for inhibition of recombinant SARS-CoV-2 $3CL^{pro}$: with an inhibition constant (Ki) of ~7 μM, quercetin was found to be the most potent inhibitor. Furthermore, by isothermal titration calorimetry, it was determined that the flavonol binds to the protease with a dissociation constant (K_d) of 2.7 and 10 μM, in the presence of 0 and 150 mM NaCl, respectively (Abian et al., 2020). Quercetin was found to inhibit SARS-CoV-2 replication in Vero cells with an IC_{50} of 192 μM (Mangiavacchi et al., 2021).

Against Other Viruses

Isoquercetin has also been shown to inhibit herpesviruses *ex vivo*: herpes simplex virus type 1 (HSV-1) and HSV-2 (Abou-Karam and Shier, 1992; Chen et al., 2011) as well as varicella-zoster virus (VZV) and human cytomegalovirus (HCMV) with IC_{50} of 15 and 2 μM, respectively (Kim et al., 2020).

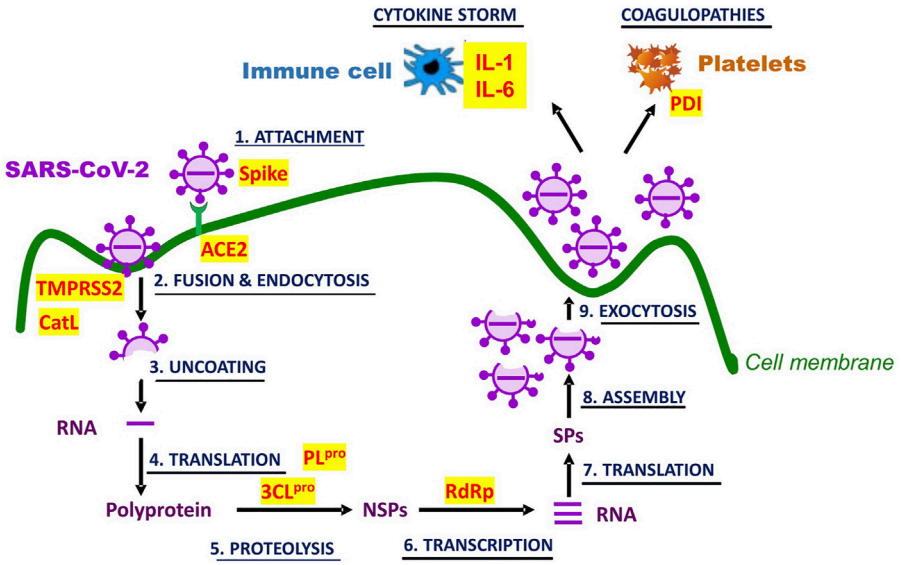


FIGURE 3 | Molecular and physiological targets of quercetin and isoquercetin in Covid-19. SARS-CoV-2 infection and life cycle are graphically represented. Potential targets for inhibition by the flavonols are written in red and highlighted in yellow. Their full identity and the role in Covid-19 pathogenesis are described in the text. NSPs stand for non-structural proteins, and SPs for structural proteins.

ISOQUERCETIN AGAINST COVID-19

Pathophysiology of Covid-19 in Brief

Covid-19 is initiated through infection of airway epithelial cells by SARS-CoV-2. The entry of the virus follows attachment of the S glycoprotein of the viral envelope to angiotensin converting enzyme 2 (ACE2) at the surface of the cells. The attachment and fusion is facilitated by host cell surface proteases such as the proprotein convertase furin, transmembrane protease serine 2 precursor (TMPRSS2) as well as endo/lysosomal cysteine protease cathepsins L (CatL). After endocytosis, the virus is uncoated in endosomes and its RNA released in the cytoplasm where it is used by the host translational machinery to proteins necessary for the replication and multiplication of the virus (e.g., RdRp, $3CL^{pro}$, and PL^{pro}), causing damage to the airway epithelium and resulting in respiratory distress. The progeny viruses are released in the bloodstream, whence they infect all organs expressing the ACE2 receptor, including the liver, the intestine, the kidney, the heart, and the brain (Harrison et al., 2020; Zhao et al., 2021).

Physiologically, tissue damages induce a robust inflammatory response involving recruitment of helper cells, macrophages and monocytes, increased secretion of proinflammatory cytokines (e.g., INF-γ, TNFα, IL1β, IL-2, IL-6, IL-12) and chemokines (e.g., MCP-1), increased membrane permeability, and increased expression of adhesion molecules. The inflammatory response is very probably accompanied by reciprocally-sustaining oxidative stress. This oxidation-inflammation feedback loop could be one of the causes of the immune hyper-responsiveness dubbed "cytokine storm". The "storm," acting on platelets and vascular endothelium induces coagulopathies (e.g., venous thromboembolism, disseminated intravascular coagulation) which contribute to fatal multi-organ injury and failure (Harrison et al., 2020; Savla et al., 2021).

Isoquercetin Targets During Covid-19 Pathogenesis

Isoquercetin and/or quercetin aglycone, could act on several targets to oppose SARS-CoV-2 infection and halt the pathological course of Covid-19. Possible targets are molecular and physiological (**Figure 3**). Among molecular targets, there is not only the viral $3CL^{pro}$ and PL^{pro} whose inhibition by quercetins has been experimentally demonstrated (**Section 5.2**), but also the host ACE2, TMPRSS2, and CatL. To investigate interaction of flavonols with the ACE2 receptor Zhan et al. (2021) fixed the membrane fraction of HEK293 cells overexpressing human ACE2 ($HEK293\text{-}ACE2^{h}$) to carboxymethylcellulose (CMC); of this resin, they make columns which they used in liquid chromatography to show that quercetin and its methylated metabolite isorhamnetin specifically bound to $ACE2^{h}$-CMC. In a surface plasmon resonance assay, they also determined that quercetin and isorhamnetin bound to ACE2 with K_d of 5.9 and 2.5 μM, respectively. They further tested whether the binding could be of consequence for SARS-CoV-2 Spike pseudotyped virus infection of $HEK293\text{-}ACE2^{h}$ cells and observed that, at 50 μM, only isorhamnetin inhibited infection by 48%. As for the other host proteases, CatL was shown to be inhibited by quercetin and isoquercetin with an IC_{50} of 26.3 μM and 115 μM, respectively (Ramalho et al., 2015); on the other hand, by *in silico* analysis, TMPRSS2 can bind quercetin with an affinity of −−7.7 kcal/mol (Alzaabi et al., 2021), suggesting that its protease activity might be diminished by this flavonol. Although quercetin interaction with each one of these molecular targets is

moderate (in the micromolar range), the multiplicity of targets may accentuate the antiviral effectiveness of the flavonol.

Physiological targets may include pathogenic manifestations of viral infections which quercetins are known to counter, most damaging among them being oxidative stress, hyperactive inflammation, and coagulopathies. As of now, the improvement conferred by quercetins in these manifestations are inferred mostly from preclinical studies of infections by virus other than SARS-CoV-2. Quercetin protection against inflammation-induced coagulopathy and multi-organ injury was examined in a rabbit model generated by continuous infusion of 100 μg/kg/h LPS for 6 h: the treatment caused inflammation (↑TNF-α), increased coagulability (↑APTT, ↑PT, ↓fibrinogen, ↓protein C, ↓antithrombin III), as well as kidney (↑blood urea nitrogen), and liver (↑alanine aminotransferase) dysfunctions. Intravenous administration of quercetin (0.5, 1.0, and 2.0 mg/kg/h) dose-dependently and significantly attenuated these alterations of hemostatic and functional parameters (Yu et al., 2013).

Concerns on the Use of Quercetin and Isoquercetin as Anti-Covid-19 Drugs

From experimental studies with cells and animals, there is ample evidence that quercetin aglycone possesses physiological activities that could promote or restore health. Its safety at therapeutic doses has been established in animals and humans (Harwood et al., 2007), leading to its qualification by the US Drug and Food Administration (FDA) as a Generally Recognized as Safe (GRAS) compound (US FDA, 2010). Isoquercetin shows an equally favorable safety profile (Hobbs et al., 2018). In phase 1 clinical trials, no drug-linked severe adverse effects were reported with quercetin up to 5 g/d (Lu et al., 2016) and isoquercetin up to 1 g/d (Buonerba et al., 2018; Zwicker et al., 2019).

Some of the most cited arguments against its utility as a pharmaceutical in humans have been its poor absorption and bioavailability due in part to its sequestration by serum albumin (Fiorani et al., 2003), its rapid metabolization, and its interindividual variability as illustrated by the wide C_{max} coefficient of variation (CV: 37–96%) of quercetin and its metabolites in the plasma of healthy volunteers who had ingested ~1.1 g quercetin aglycone (Guo et al., 2014).

Oral isoquercetin exhibits a significantly improved bioavailability by nearly 5-fold over quercetin aglycone (Stopa et al., 2017) (**Table 1**). Moreover, isoquercetin has a 17-fold weaker affinity for albumin than the aglycone form (Martini et al., 2008), suggesting that it is less likely to be sequestered by albumin or other binding proteins in the intestinal mucosa and in blood. Interestingly, Fiorani et al. (2002), Fiorani et al. (2003) have shown in *ex vivo* assays that quercetin accumulates in red blood cells (up to 0.4 mM), imbedded into membranes or bound to hemoglobin, and that albumin can extract it from these cells, an indication that, *in vivo*, the serum protein could serve as a circulating carrier of the flavonol and its distributor to tissues.

Concerning the rapid metabolization of isoquercetin once deglycosylated, it should be noted that some of its major metabolites such as quercetin-3-glucuronide and isorhamnetin exhibit many of the physiological properties of the aglycone, including antiviral properties (Fan et al., 2011; Terao et al., 2011; Gong et al., 2020). Furthermore, glucuronidated quercetin could also serves as a precursor to the aglycone form, as it is susceptible to deconjugation by cellular β-glucuronidase whose level is up regulated by inflammatory states (Terao et al., 2011). Thus, the effective concentration of quercetin *in vivo* could be far greater than that estimated on the basis its plasma levels only.

Interindividual variability in plasma quercetin after ingestion has been partly attributed to differences of intestinal permeability. Increased permeability has been associated with higher levels of circulating bacterial endotoxin. Plasma quercetin level has been found to positively correlate with that of plasma endotoxin (Guo et al., 2014). Intestinal permeability is strongly influenced by the intestinal microbiome (Chakaroun et al., 2020). Isoquercetin and quercetin have been reported to reshape the microbiota of mice fed a high-fat diet while reducing the metabolic syndrome induced by the diet (Tan et al., 2018; Tan et al., 2021). One can speculate that the beneficial effect of quercetin on the syndrome results in part from the reduction of intestinal permeability due to microbiome reshaping. This reduction probably lowers quercetin absorption while maintaining pharmacokinetics variability due to alternate causes (e.g., genetics, age, lifestyle, health conditions) (Lin et al., 2021). Ultimately, one must wonder whether such variability matters for biological function, especially if the effective concentration in tissues is on the lower side of the concentration range. It worth noting that when quercetin or isoquercetin was fed to rats, mice or pigs, its metabolites accumulated primarily in tissues that are known to be susceptible to SARS-CoV-2 infection, namely lung, liver, intestine, kidney and heart, although the order of abundance per tissue differs among species (De Boer et al., 2005; Paulke et al., 2012; Liu et al., 2014). This accumulation of metabolites in relevant tissues suggests that effective levels against infection by the virus may readily be reached in these sites.

CONCLUSION AND PERSPECTIVE

In this review, we presume that, as a potential anti-SARS-CoV-2 drug, isoquercetin represents a better choice than quercetin aglycone because it is a more absorbable precursor of quercetin, allowing greater bioavailability of the aglycone. Together, these two flavonols represent potentially effective medications for the treatment of COVID-19 patients, the reasons being: 1) their broad-spectrum antiviral activities; 2) their potent activities against SARS-CoV-2-induced symptoms as an antioxidant, anti-inflammatory, immunomodulatory, anticoagulant agent; 3) *in silico* analyses by molecular docking and molecular dynamics simulations, indicating that several proteins involved in SARS-CoV-2 entry and replication exhibit strong affinity for these flavonols; 4) their safety when orally administered in phase 1 clinical trials. Experiments *in vitro* and in animals should corroborate this presumption and elucidate their mechanisms of their action.

At the writing of this review, nine phase-2 clinical trials evaluating the efficacy of quercetins against Covid-19 have been registered in the National Institute of Health database.[2].

[2]https://clinicaltrials.gov

Four trials are listed as completed; the results of one trial have been published in a peer-reviewed journal (Di Pierro et al., 2021). In this trial, a formulation called quercetin phytosome® containing, as additive, the absorption-enhancing sunflower lecithin (Riva et al., 2019), was given orally (1.7 g/day for 7 days and 1 g/day for the subsequent 14 days) along with recommended standard care to middle-aged patients (n = 21) in the early stage of Covid-19; an equal number of patients of similar average age and disease stage was given standard care only. Besides clinical symptoms, RT-PCR-detectable positivity for SARS-CoV-2 RNA in nasopharyngeal swabs was monitored weekly. This positivity declined far more rapidly in the quercetin-treated group (p = 0.0002) and this was associated with greater symptomatic improvement (p = 0.012), suggesting that quercetin may promote viral clearance in SARS-CoV-2-infected subjects and accelerate their recovery. The positive results of this formulation need to be corroborated by larger studies with more extended array of clinical and laboratory parameters. They call for the evaluation of isoquercetin formulations also as potential anti-Covid-19 medication.

AUTHOR CONTRIBUTIONS

MM and MC contributed the writing of the manuscript and the design of the figures. Both authors read and approved the submitted version.

FUNDING

This work was supported by the grants from the Aclon Foundation, the Richard and Edith Strauss Foundation, La Fondation J-Louis Levesque, the Foundation Notre Dame De Zeitoun, the Lazaridis Family Foundation, and Power Corporation du Canada.

REFERENCES

Abian, O., Ortega-Alarcon, D., Jimenez-Alesanco, A., Ceballos-Laita, L., Vega, S., Reyburn, H. T., et al. (2020). Structural Stability of SARS-CoV-2 3CLpro and Identification of Quercetin as an Inhibitor by Experimental Screening. *Int. J. Biol. Macromol.* 164, 1693–1703. doi:10.1016/j.ijbiomac.2020.07.235

Abou-Karam, M., and Shier, W. T. (1992). Isolation and Characterization of an Antiviral Flavonoid from Waldsteinia Fragarioides. *J. Nat. Prod.* 55, 1525–1527. doi:10.1021/np50088a022

Ader, P., Blöck, M., Pietzsch, S., and Wolffram, S. (2001). Interaction of Quercetin Glucosides with the Intestinal Sodium/glucose Co-transporter (SGLT-1). *Cancer Lett.* 162, 175–180. doi:10.1016/s0304-3835(00)00645-5

Alzaabi, M. M., Hamdy, R., Ashmawy, N. S., Hamoda, A. M., Alkhayat, F., Khademi, N. N., et al. (2021). Flavonoids Are Promising Safe Therapy against COVID-19. *Phytochem. Rev.*, 1–22. doi:10.1007/s11101-021-09759-z

Apak, R. (2019). Current Issues in Antioxidant Measurement. *J. Agric. Food Chem.* 67, 9187–9202. doi:10.1021/acs.jafc.9b03657

Badshah, S. L., Faisal, S., Muhammad, A., Poulson, B. G., Emwas, A. H., and Jaremko, M. (2021). Antiviral Activities of Flavonoids. *Biomed. Pharmacother.* 140, 111596. doi:10.1016/j.biopha.2021.111596

Boadi, W. Y., Amartey, P. K., and Lo, A. (2016). Effect of Quercetin, Genistein and Kaempferol on Glutathione and Glutathione-Redox Cycle Enzymes in 3T3-L1 Preadipocytes. *Drug Chem. Toxicol.* 39, 239–247. doi:10.3109/01480545.2015.1082135

Boots, A. W., Haenen, G. R., and Bast, A. (2008). Health Effects of Quercetin: from Antioxidant to Nutraceutical. *Eur. J. Pharmacol.* 585, 325–337. doi:10.1016/j.ejphar.2008.03.008

Borst, J. W., Visser, N. V., Kouptsova, O., and Visser, A. J. (2000). Oxidation of Unsaturated Phospholipids in Membrane Bilayer Mixtures Is Accompanied by Membrane Fluidity Changes. *Biochim. Biophys. Acta* 1487, 61–73. doi:10.1016/s1388-1981(00)00084-6

Buonerba, C., De Placido, P., Bruzzese, D., Pagliuca, M., Ungaro, P., Bosso, D., et al. (2018). Isoquercetin as an Adjunct Therapy in Patients with Kidney Cancer Receiving First-Line Sunitinib (QUASAR): Results of a Phase I Trial. *Front. Pharmacol.* 9, 189. doi:10.3389/fphar.2018.00189

Calder, P. C. (2012). Long-chain Fatty Acids and Inflammation. *Proc. Nutr. Soc.* 71, 284–289. doi:10.1017/s0029665112000067

Cermak, R., Landgraf, S., and Wolffram, S. (2003). The Bioavailability of Quercetin in Pigs Depends on the Glycoside Moiety and on Dietary Factors. *J. Nutr.* 133, 2802–2807. doi:10.1093/jn/133.9.2802

Chakaroun, R. M., Massier, L., and Kovacs, P. (2020). Gut Microbiome, Intestinal Permeability, and Tissue Bacteria in Metabolic Disease: Perpetrators or Bystanders? *Nutrients* 12, 1082. doi:10.3390/nu12041082

Chang, Q., Zuo, Z., Chow, M. S., and Ho, W. K. (2005). Difference in Absorption of the Two Structurally Similar Flavonoid Glycosides, Hyperoside and Isoquercitrin, in Rats. *Eur. J. Pharm. Biopharm.* 59, 549–555. doi:10.1016/j.ejpb.2004.10.004

Chatterjee, S. (2016). "Oxidative Stress, Inflammation, and Disease," in *Oxidative Stress and Biomaterials*. Editors T. Dziubla and D. A. Butterfield (Elsevier), 35–58. doi:10.1016/b978-0-12-803269-5.00002-4

Chen, X., Wang, Z., Yang, Z., Wang, J., Xu, Y., Tan, R. X., et al. (2011). Houttuynia Cordata Blocks HSV Infection through Inhibition of NF-κB Activation. *Antivir. Res.* 92, 341–345. doi:10.1016/j.antiviral.2011.09.005

Chen, Y., Zhou, Z., and Min, W. (2018). Mitochondria, Oxidative Stress and Innate Immunity. *Front. Physiol.* 9, 1487. doi:10.3389/fphys.2018.01487

Choi, S.-J., Tai, B. H., Cuong, N. M., Kim, Y.-H., and Jang, H.-D. (2012). Antioxidative and Anti-inflammatory Effect of Quercetin and its Glycosides Isolated from Mampat (Cratoxylum Formosum). *Food Sci. Biotechnol.* 21, 587–595. doi:10.1007/s10068-012-0075-4

Choi, J. H., Kim, K. J., and Kim, S. (2016). Comparative Effect of Quercetin and Quercetin-3-O-β-D-Glucoside on Fibrin Polymers, Blood Clots, and in Rodent Models. *J. Biochem. Mol. Toxicol.* 30, 548–558. doi:10.1002/jbt.21822

Choudhury, R., Srai, S. K., Debnam, E., and Rice-Evans, C. A. (1999). Urinary Excretion of Hydroxycinnamates and Flavonoids after Oral and Intravenous Administration. *Free Radic. Biol. Med.* 27, 278–286. doi:10.1016/s0891-5849(99)00054-4

Cooper, R. A. (1977). Abnormalities of Cell-Membrane Fluidity in the Pathogenesis of Disease. *N. Engl. J. Med.* 297, 371–377. doi:10.1056/NEJM197708182970707

Crespy, V., Morand, C., Besson, C., Manach, C., Démigné, C., and Rémésy, C. (2001). Comparison of the Intestinal Absorption of Quercetin, Phloretin and Their Glucosides in Rats. *J. Nutr.* 131, 2109–2114. doi:10.1093/jn/131.8.2109

Crespy, V., Morand, C., Besson, C., Manach, C., Demigne, C., and Remesy, C. (2002). Quercetin, but Not its Glycosides, Is Absorbed from the Rat Stomach. *J. Agric. Food Chem.* 50, 618–621. doi:10.1021/jf010919h

Day, A. J., Cañada, F. J., Díaz, J. C., Kroon, P. A., Mclauchlan, R., Faulds, C. B., et al. (2000). Dietary Flavonoid and Isoflavone Glycosides Are Hydrolysed by the Lactase Site of Lactase Phlorizin Hydrolase. *FEBS Lett.* 468, 166–170. doi:10.1016/s0014-5793(00)01211-4

De Boer, V. C., Dihal, A. A., Van Der Woude, H., Arts, I. C., Wolffram, S., Alink, G. M., et al. (2005). Tissue Distribution of Quercetin in Rats and Pigs. *J. Nutr.* 135, 1718–1725. doi:10.1093/jn/135.7.1718

De Sousa, L. R., Wu, H., Nebo, L., Fernandes, J. B., Da Silva, M. F., Kiefer, W., et al. (2015). Flavonoids as Noncompetitive Inhibitors of Dengue Virus NS2B-NS3 Protease: Inhibition Kinetics and Docking Studies. *Bioorg. Med. Chem.* 23, 466–470. doi:10.1016/j.bmc.2014.12.015

Di Pierro, F., Iqtadar, S., Khan, A., Ullah Mumtaz, S., Masud Chaudhry, M., Bertuccioli, A., et al. (2021). Potential Clinical Benefits of Quercetin in the Early Stage of COVID-19: Results of a Second, Pilot, Randomized, Controlled and Open-Label Clinical Trial. *Int. J. Gen. Med.* 14, 2807–2816. doi:10.2147/ijgm.s318949

El-Benna, J., Hurtado-Nedelec, M., Marzaioli, V., Marie, J. C., Gougerot-Pocidalo, M. A., and Dang, P. M. (2016). Priming of the Neutrophil Respiratory Burst: Role in Host Defense and Inflammation. *Immunol. Rev.* 273, 180–193. doi:10.1111/imr.12447

Fan, D., Zhou, X., Zhao, C., Chen, H., Zhao, Y., and Gong, X. (2011). Anti-inflammatory, Antiviral and Quantitative Study of Quercetin-3-O-β-D-Glucuronide in Polygonum Perfoliatum L. *Fitoterapia* 82, 805–810. doi:10.1016/j.fitote.2011.04.007

Fanunza, E., Iampietro, M., Distinto, S., Corona, A., Quartu, M., Maccioni, E., et al. (2020). Quercetin Blocks Ebola Virus Infection by Counteracting the VP24 Interferon-Inhibitory Function. *Antimicrob. Agents Chemother.* 64, e00530-20. doi:10.1128/AAC.00530-20

Feldmann, H., Sprecher, A., and Geisbert, T. W. (2020). Ebola. *N. Engl. J. Med.* 382, 1832–1842. doi:10.1056/NEJMra1901594

Fiorani, M., De Sanctis, R., De Bellis, R., and Dachà, M. (2002). Intracellular Flavonoids as Electron Donors for Extracellular Ferricyanide Reduction in Human Erythrocytes. *Free Radic. Biol. Med.* 32, 64–72. doi:10.1016/s0891-5849(01)00762-6

Fiorani, M., Accorsi, A., and Cantoni, O. (2003). Human Red Blood Cells as a Natural Flavonoid Reservoir. *Free Radic. Res.* 37, 1331–1338. doi:10.1080/10715760310001615998

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., et al. (2019). Chronic Inflammation in the Etiology of Disease across the Life Span. *Nat. Med.* 25, 1822–1832. doi:10.1038/s41591-019-0675-0

Gansukh, E., Nile, A., Kim, D. H., Oh, J. W., and Nile, S. H. (2021). New Insights into Antiviral and Cytotoxic Potential of Quercetin and its Derivatives - A Biochemical Perspective. *Food Chem.* 334, 127508. doi:10.1016/j.foodchem.2020.127508

Gaudry, A., Bos, S., Viranaicken, W., Roche, M., Krejbich-Trotot, P., Gadea, G., et al. (2018). The Flavonoid Isoquercitrin Precludes Initiation of Zika Virus Infection in Human Cells. *Int. J. Mol. Sci.* 19, 1093. doi:10.3390/ijms19041093

Gong, G., Guan, Y. Y., Zhang, Z. L., Rahman, K., Wang, S. J., Zhou, S., et al. (2020). Isorhamnetin: A Review of Pharmacological Effects. *Biomed. Pharmacother.* 128, 110301. doi:10.1016/j.biopha.2020.110301

Guo, Y., Mah, E., and Bruno, R. S. (2014). Quercetin Bioavailability Is Associated with Inadequate Plasma Vitamin C Status and Greater Plasma Endotoxin in Adults. *Nutrition* 30, 1279–1286. doi:10.1016/j.nut.2014.03.032

Gupta, P., Lakes, A., and Dziubla, T. (2016). "A Free Radical Primer," in *Oxidative Stress and Biomaterials*. Editors T. Dziubla and D. A. Butterfield (Elsevier), 1–33. doi:10.1016/b978-0-12-803269-5.00001-2

Harada, S. (2005). The Broad Anti-viral Agent Glycyrrhizin Directly Modulates the Fluidity of Plasma Membrane and HIV-1 Envelope. *Biochem. J.* 392, 191–199. doi:10.1042/bj20051069

Harrison, A. G., Lin, T., and Wang, P. (2020). Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. *Trends Immunol.* 41, 1100–1115. doi:10.1016/j.it.2020.10.004

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., and Lines, T. C. (2007). A Critical Review of the Data Related to the Safety of Quercetin and Lack of Evidence of *In Vivo* Toxicity, Including Lack of Genotoxic/carcinogenic Properties. *Food Chem. Toxicol.* 45, 2179–2205. doi:10.1016/j.fct.2007.05.015

Hiremath, S., Kumar, H. D. V., Nandan, M., Mantesh, M., Shankarappa, K. S., Venkataravanappa, V., et al. (2021). In Silico docking Analysis Revealed the Potential of Phytochemicals Present in Phyllanthus Amarus and Andrographis Paniculata, Used in Ayurveda Medicine in Inhibiting SARS-CoV-2. *3 Biotech.* 11, 44. doi:10.1007/s13205-020-02578-7

Hobbs, C. A., Koyanagi, M., Swartz, C., Davis, J., Kasamoto, S., Maronpot, R., et al. (2018). Comprehensive Evaluation of the Flavonol Anti-oxidants, Alpha-Glycosyl Isoquercitrin and Isoquercitrin, for Genotoxic Potential. *Food Chem. Toxicol.* 113, 218–227. doi:10.1016/j.fct.2017.12.059

Hollman, P. C., De Vries, J. H., Van Leeuwen, S. D., Mengelers, M. J., and Katan, M. B. (1995). Absorption of Dietary Quercetin Glycosides and Quercetin in Healthy Ileostomy Volunteers. *Am. J. Clin. Nutr.* 62, 1276–1282. doi:10.1093/ajcn/62.6.1276

Hung, P. Y., Ho, B. C., Lee, S. Y., Chang, S. Y., Kao, C. L., Lee, S. S., et al. (2015). Houttuynia Cordata Targets the Beginning Stage of Herpes Simplex Virus Infection. *PLoS One* 10, e0115475. doi:10.1371/journal.pone.0115475

Ibrahim, A. K., Youssef, A. I., Arafa, A. S., and Ahmed, S. A. (2013). Anti-H5N1 Virus Flavonoids from Capparis Sinaica Veill. *Nat. Prod. Res.* 27, 2149–2153. doi:10.1080/14786419.2013.790027

Jasuja, R., Passam, F. H., Kennedy, D. R., Kim, S. H., Van Hessem, L., Lin, L., et al. (2012). Protein Disulfide Isomerase Inhibitors Constitute a New Class of Antithrombotic Agents. *J. Clin. Invest.* 122, 2104–2113. doi:10.1172/jci61228

Javanian, M., Barary, M., Ghebrehewet, S., Koppolu, V., Vasigala, V., and Ebrahimpour, S. (2021). A Brief Review of Influenza Virus Infection. *J. Med. Virol.* 93, 4638–4646. doi:10.1002/jmv.26990

Jo, S., Kim, H., Kim, S., Shin, D. H., and Kim, M. S. (2019). Characteristics of Flavonoids as Potent MERS-CoV 3C-like Protease Inhibitors. *Chem. Biol. Drug Des.* 94, 2023–2030. doi:10.1111/cbdd.13604

Kim, Y., and Chang, K. O. (2018). Protein Disulfide Isomerases as Potential Therapeutic Targets for Influenza A and B Viruses. *Virus. Res.* 247, 26–33. doi:10.1016/j.virusres.2018.01.010

Kim, Y., Narayanan, S., and Chang, K. O. (2010). Inhibition of Influenza Virus Replication by Plant-Derived Isoquercetin. *Antivir. Res.* 88, 227–235. doi:10.1016/j.antiviral.2010.08.016

Kim, B. H., Choi, J. S., Yi, E. H., Lee, J. K., Won, C., Ye, S. K., et al. (2013). Relative Antioxidant Activities of Quercetin and its Structurally Related Substances and Their Effects on NF-κB/CRE/AP-1 Signaling in Murine Macrophages. *Mol. Cell* 35, 410–420. doi:10.1007/s10059-013-0031-z

Kim, C. H., Kim, J. E., and Song, Y. J. (2020). Antiviral Activities of Quercetin and Isoquercitrin against Human Herpesviruses. *Molecules* 25, 2379. doi:10.3390/molecules25102379

Ko, E. Y., Nile, S. H., Jung, Y.-S., and Keum, Y. S. (2018). Antioxidant and Antiplatelet Potential of Different Methanol Fractions and Flavonols Extracted from Onion (Allium cepa L.). *3 Biotech.* 8, 155. doi:10.1007/s13205-018-1184-4

Kumar, V., Suman, U., Rubal, S. K., and Yadav, S. K. (2018). "Flavonoid Secondary Metabolite: Biosynthesis and Role in Growth and Development in Plants," in *Recent Trends and Techniques in Plant Metabolic Engineering*. Editors K. S. Yadav, K. Vinay, and S. P. Singh (Spinger, Singapore: Springer), 19–45. doi:10.1007/978-981-13-2251-8_2

Kwon, Y. S., Kim, S. S., Sohn, S. J., Kong, P. J., Cheong, I. Y., Kim, C. M., et al. (2004). Modulation of Suppressive Activity of Lipopolysaccharide-Induced Nitric Oxide Production by Glycosidation of Flavonoids. *Arch. Pharm. Res.* 27, 751–756. doi:10.1007/bf02980144

Lee, S., Park, H. S., Notsu, Y., Ban, H. S., Kim, Y. P., Ishihara, K., et al. (2008). Effects of Hyperin, Isoquercitrin and Quercetin on Lipopolysaccharide-Induced Nitrite Production in Rat Peritoneal Macrophages. *Phytother Res.* 22, 1552–1556. doi:10.1002/ptr.2529

Lee, S., Lee, J., Lee, H., and Sung, J. (2019). Relative Protective Activities of Quercetin, Quercetin-3-Glucoside, and Rutin in Alcohol-Induced Liver Injury. *J. Food Biochem.* 43, e13002. doi:10.1111/jfbc.13002

Lesser, S., Cermak, R., and Wolffram, S. (2004). Bioavailability of Quercetin in Pigs Is Influenced by the Dietary Fat Content. *J. Nutr.* 134, 1508–1511. doi:10.1093/jn/134.6.1508

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., et al. (2018). Oxidative Stress, Aging, and Diseases. *Clin. Interv. Aging* 13, 757–772. doi:10.2147/CIA.S158513

Lin, Y. S., Thummel, K. E., Thompson, B. D., Totah, R. A., and Cho, C. W. (2021). Sources of Interindividual Variability. *Methods Mol. Biol.* 2342, 481–550. doi:10.1007/978-1-0716-1554-6_17

Liu, L., Tang, Y., Gao, C., Li, Y., Chen, S., Xiong, T., et al. (2014). Characterization and Biodistribution *In Vivo* of Quercetin-Loaded Cationic Nanostructured Lipid Carriers. *Colloids Surf. B Biointerfaces* 115, 125–131. doi:10.1016/j.colsurfb.2013.11.029

Lu, N. T., Crespi, C. M., Liu, N. M., Vu, J. Q., Ahmadieh, Y., Wu, S., et al. (2016). A Phase I Dose Escalation Study Demonstrates Quercetin Safety and Explores Potential for Bioflavonoid Antivirals in Patients with Chronic Hepatitis C. *Phytother Res.* 30, 160–168. doi:10.1002/ptr.5518

Makino, T., Shimizu, R., Kanemaru, M., Suzuki, Y., Moriwaki, M., and Mizukami, H. (2009). Enzymatically Modified Isoquercitrin, Alpha-Oligoglucosyl Quercetin 3-O-Glucoside, Is Absorbed More Easily Than Other Quercetin

Glycosides or Aglycone after Oral Administration in Rats. *Biol. Pharm. Bull.* 32, 2034–2040. doi:10.1248/bpb.32.2034

Mangiavacchi, F., Botwina, P., Menichetti, E., Bagnoli, L., Rosati, O., Marini, F., et al. (2021). Seleno-Functionalization of Quercetin Improves the Non-covalent Inhibition of Mpro and its Antiviral Activity in Cells against SARS-CoV-2. *Int. J. Mol. Sci.* 22, 7048. doi:10.3390/ijms22137048

Martini, S., Bonechi, C., and Rossi, C. (2008). Interaction of Quercetin and its Conjugate Quercetin 3-O-Beta-D-Glucopyranoside with Albumin as Determined by NMR Relaxation Data. *J. Nat. Prod.* 71, 175–178. doi:10.1021/np070285u

Morand, C., Manach, C., Crespy, V., and Remesy, C. (2000a). Quercetin 3-O-Beta-Glucoside Is Better Absorbed Than Other Quercetin Forms and Is Not Present in Rat Plasma. *Free Radic. Res.* 33, 667–676. doi:10.1080/10715760000301181

Morand, C., Manach, C., Crespy, V., and Remesy, C. (2000b). Respective Bioavailability of Quercetin Aglycone and its Glycosides in a Rat Model. *Biofactors* 12, 169–174. doi:10.1002/biof.5520120127

Morikawa, K., Nonaka, M., Narahara, M., Torii, I., Kawaguchi, K., Yoshikawa, T., et al. (2003). Inhibitory Effect of Quercetin on Carrageenan-Induced Inflammation in Rats. *Life Sci.* 74, 709–721. doi:10.1016/j.lfs.2003.06.036

Murota, K., Shimizu, S., Chujo, H., Moon, J. H., and Terao, J. (2000). Efficiency of Absorption and Metabolic Conversion of Quercetin and its Glucosides in Human Intestinal Cell Line Caco-2. *Arch. Biochem. Biophys.* 384, 391–397. doi:10.1006/abbi.2000.2123

Nguyen, T. T., Woo, H. J., Kang, H. K., Nguyen, V. D., Kim, Y. M., Kim, D. W., et al. (2012). Flavonoid-mediated Inhibition of SARS Coronavirus 3C-like Protease Expressed in Pichia pastoris. *Biotechnol. Lett.* 34, 831–838. doi:10.1007/s10529-011-0845-8

Nicolson, G. L. (2015). Cell Membrane Fluid-Mosaic Structure and Cancer Metastasis. *Cancer Res.* 75, 1169–1176. doi:10.1158/0008-5472.can-14-3216

Nile, S. H., Kim, D. H., Nile, A., Park, G. S., Gansukh, E., and Kai, G. (2020). Probing the Effect of Quercetin 3-glucoside from Dianthus Superbus L against Influenza Virus Infection- *In Vitro* and In Silico Biochemical and Toxicological Screening. *Food Chem. Toxicol.* 135, 110985. doi:10.1016/j.fct.2019.110985

Park, H. J., Lee, C. M., Jung, I. D., Lee, J. S., Jeong, Y. I., Chang, J. H., et al. (2009). Quercetin Regulates Th1/Th2 Balance in a Murine Model of Asthma. *Int. Immunopharmacol.* 9, 261–267. doi:10.1016/j.intimp.2008.10.021

Park, J. Y., Yuk, H. J., Ryu, H. W., Lim, S. H., Kim, K. S., Park, K. H., et al. (2017). Evaluation of Polyphenols from Broussonetia Papyrifera as Coronavirus Protease Inhibitors. *J. Enzyme Inhib. Med. Chem.* 32, 504–515. doi:10.1080/14756366.2016.1265519

Park, H. J., Kim, H. N., Kim, C. Y., Seo, M. D., and Baek, S. H. (2021). Synergistic protection by Isoquercitrin and Quercetin against Glutamate-Induced Oxidative Cell Death in HT22 Cells via Activating Nrf2 and HO-1 Signaling Pathway: Neuroprotective Principles and Mechanisms of Dendropanax Morbifera Leaves. *Antioxidants (Basel)* 10, 554. doi:10.3390/antiox10040554

Paulke, A., Eckert, G. P., Schubert-Zsilavecz, M., and Wurglics, M. (2012). Isoquercitrin Provides Better Bioavailability Than Quercetin: Comparison of Quercetin Metabolites in Body Tissue and Brain Sections after Six Days Administration of Isoquercitrin and Quercetin. *Pharmazie* 67, 991–996. doi:10.1691/ph.2012.2050

Qiu, X., Kroeker, A., He, S., Kozak, R., Audet, J., Mbikay, M., et al. (2016). Prophylactic Efficacy of Quercetin 3-β-O-D-Glucoside against Ebola Virus Infection. *Antimicrob. Agents Chemother.* 60, 5182–5188. doi:10.1128/AAC.00307-16

Ramalho, S. D., De Sousa, L. R., Burger, M. C., Lima, M. I., Da Silva, M. F., Fernandes, J. B., et al. (2015). Evaluation of Flavonols and Derivatives as Human Cathepsin B Inhibitor. *Nat. Prod. Res.* 29, 2212–2214. doi:10.1080/14786419.2014.1002404

Reinboth, M., Wolffram, S., Abraham, G., Ungemach, F. R., and Cermak, R. (2010). Oral Bioavailability of Quercetin from Different Quercetin Glycosides in Dogs. *Br. J. Nutr.* 104, 198–203. doi:10.1017/S000711451000053X

Riva, A., Ronchi, M., Petrangolini, G., Bosisio, S., and Allegrini, P. (2019). Improved Oral Absorption of Quercetin from Quercetin Phytosome®, a New Delivery System Based on Food Grade Lecithin. *Eur. J. Drug Metab. Pharmacokinet.* 44, 169–177. doi:10.1007/s13318-018-0517-3

Rogerio, A. P., Kanashiro, A., Fontanari, C., Da Silva, E. V., Lucisano-Valim, Y. M., Soares, E. G., et al. (2007). Anti-inflammatory Activity of Quercetin and Isoquercitrin in Experimental Murine Allergic Asthma. *Inflamm. Res.* 56, 402–408. doi:10.1007/s00011-007-7005-6

Ryu, Y. B., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, J. Y., Kim, D., et al. (2010). Biflavonoids from Torreya Nucifera Displaying SARS-CoV 3CL(pro) Inhibition. *Bioorg. Med. Chem.* 18, 7940–7947. doi:10.1016/j.bmc.2010.09.035

Salehi, B., Machin, L., Monzote, L., Sharifi-Rad, J., Ezzat, S. M., Salem, M. A., et al. (2020). Therapeutic Potential of Quercetin: New Insights and Perspectives for Human Health. *ACS Omega* 5, 11849–11872. doi:10.1021/acsomega.0c01818

Savla, S. R., Prabhavalkar, K. S., and Bhatt, L. K. (2021). Cytokine Storm Associated Coagulation Complications in COVID-19 Patients: Pathogenesis and Management. *Expert Rev. Anti Infect. Ther.* 19, 1397–1413. doi:10.1080/14787210.2021.1915129

Scott, J. A. (1982). Membrane Fluidity as an index of Pathology. *Med. Hypotheses* 9, 223–228. doi:10.1016/0306-9877(82)90139-6

Sergent, O., Pereira, M., Belhomme, C., Chevanne, M., Huc, L., and Lagadic-Gossmann, D. (2005). Role for Membrane Fluidity in Ethanol-Induced Oxidative Stress of Primary Rat Hepatocytes. *J. Pharmacol. Exp. Ther.* 313, 104–111. doi:10.1124/jpet.104.078634

Sesink, A. L., O'leary, K. A., and Hollman, P. C. (2001). Quercetin Glucuronides but Not Glucosides Are Present in Human Plasma after Consumption of Quercetin-3-Glucoside or Quercetin-4'-Glucoside. *J. Nutr.* 131, 1938–1941. doi:10.1093/jn/131.7.1938

Sies, H. (2020). Findings in Redox Biology: From H2O2 to Oxidative Stress. *J. Biol. Chem.* 295, 13458–13473. doi:10.1074/jbc.X120.015651

Silva, N. M., Santos, N. C., and Martins, I. C. (2020). Dengue and Zika Viruses: Epidemiological History, Potential Therapies, and Promising Vaccines. *Trop. Med. Infect. Dis.* 5, 150. doi:10.3390/tropicalmed5040150

Stopa, J. D., Neuberg, D., Puligandla, M., Furie, B., Flaumenhaft, R., and Zwicker, J. I. (2017). Protein Disulfide Isomerase Inhibition Blocks Thrombin Generation in Humans by Interfering with Platelet Factor V Activation. *JCI Insight* 2, e89373. doi:10.1172/jci.insight.89373

Tan, S., Caparros-Martin, J. A., Matthews, V. B., Koch, H., O'gara, F., Croft, K. D., et al. (2018). Isoquercetin and Inulin Synergistically Modulate the Gut Microbiome to Prevent Development of the Metabolic Syndrome in Mice Fed a High Fat Diet. *Sci. Rep.* 8, 10100. doi:10.1038/s41598-018-28521-8

Tan, Y., Tam, C. C., Rolston, M., Alves, P., Chen, L., Meng, S., et al. (2021). Quercetin Ameliorates Insulin Resistance and Restores Gut Microbiome in Mice on High-Fat Diets. *Antioxidants (Basel)* 10, 1251. doi:10.3390/antiox10081251

Tanaka, Y., Furuta, A., Asano, K., and Kobayashi, H. (2020). Modulation of Th1/Th2 Cytokine Balance by Quercetin *In Vitro*. *Medicines (Basel)* 7, 46. doi:10.3390/medicines7080046

Terao, J., Murota, K., and Kawai, Y. (2011). Conjugated Quercetin Glucuronides as Bioactive Metabolites and Precursors of Aglycone *In Vivo*. *Food Funct.* 2, 11–17. doi:10.1039/c0fo00106f

Thapa, M., Kim, Y., Desper, J., Chang, K. O., and Hua, D. H. (2012). Synthesis and Antiviral Activity of Substituted Quercetins. *Bioorg. Med. Chem. Lett.* 22, 353–356. doi:10.1016/j.bmcl.2011.10.119

Tshiani Mbaya, O., Mukumbayi, P., and Mulangu, S. (2021). Review: Insights on Current FDA-Approved Monoclonal Antibodies against Ebola Virus Infection. *Front. Immunol.* 12, 721328. doi:10.3389/fimmu.2021.721328

Tsuchiya, H., Nagayama, M., Tanaka, T., Furusawa, M., Kashimata, M., and Takeuchi, H. (2002). Membrane-rigidifying Effects of Anti-cancer Dietary Factors. *Biofactors* 16, 45–56. doi:10.1002/biof.5520160301

Tsuchiya, H. (2010). Structure-dependent Membrane Interaction of Flavonoids Associated with Their Bioactivity. *Food Chem.* 120, 1089–1096. doi:10.1016/j.foodchem.2009.11.057

Tsuchiya, H. (2015). Membrane Interactions of Phytochemicals as Their Molecular Mechanism Applicable to the Discovery of Drug Leads from Plants. *Molecules* 20, 18923–18966. doi:10.3390/molecules201018923

US FDA (2010). *Agency Response Letter GRAS Notice No. GRN 000341*. Newton, MA: Quercegen Pharma LLC.

Vlasic, N., Medow, M. S., Schwarz, S. M., Pritchard, K. A., Jr., and Stemerman, M. B. (1993). Lipid Fluidity Modulates Platelet Aggregation and Agglutination *In Vitro*. *Life Sci.* 53, 1053–1060. doi:10.1016/0024-3205(93)90258-5

Wolffram, S., Blöck, M., and Ader, P. (2002). Quercetin-3-glucoside Is Transported by the Glucose Carrier SGLT1 across the brush Border Membrane of Rat Small Intestine. *J. Nutr.* 132, 630–635. doi:10.1093/jn/132.4.630

Wong, G., He, S., Siragam, V., Bi, Y., Mbikay, M., Chretien, M., et al. (2017). Antiviral Activity of Quercetin-3-β-O-D-Glucoside against Zika Virus Infection. *Virol. Sin* 32, 545–547. doi:10.1007/s12250-017-4057-9

Xiao, Z., He, L., Hou, X., Wei, J., Ma, X., Gao, Z., et al. (2021). Relationships between Structure and Antioxidant Capacity and Activity of Glycosylated Flavonols. *Foods* 10, 849. doi:10.3390/foods10040849

Xu, X., Chiu, J., Chen, S., and Fang, C. (2021). Pathophysiological Roles of Cell Surface and Extracellular Protein Disulfide Isomerase and Their Molecular Mechanisms. *Br. J. Pharmacol.* 178, 2911–2930. doi:10.1111/bph.15493

Yu, P. X., Zhou, Q. J., Zhu, W. W., Wu, Y. H., Wu, L. C., and Lin, X. (2013). Effects of Quercetin on LPS-Induced Disseminated Intravascular Coagulation (DIC) in Rabbits. *Thromb. Res.* 131, e270–e273. doi:10.1016/j.thromres.2013.03.002

Zandi, K., Teoh, B. T., Sam, S. S., Wong, P. F., Mustafa, M. R., and Abubakar, S. (2011). Antiviral Activity of Four Types of Bioflavonoid against Dengue Virus Type-2. *Virol. J.* 8, 560. doi:10.1186/1743-422X-8-560

Zhan, Y., Ta, W., Tang, W., Hua, R., Wang, J., Wang, C., et al. (2021). Potential Antiviral Activity of Isorhamnetin against SARS-CoV -2 Spike Pseudotyped Virus *In Vitro*. *Drug Dev. Res.* 82, 1124–1130. doi:10.1002/ddr.21815

Zhao, R. Z., Jiang, S., Zhang, L., and Yu, Z. B. (2019). Mitochondrial Electron Transport Chain, ROS Generation and Uncoupling (Review). *Int. J. Mol. Med.* 44, 3–15. doi:10.3892/ijmm.2019.4188

Zhao, M. M., Yang, W. L., Yang, F. Y., Zhang, L., Huang, W. J., Hou, W., et al. (2021). Cathepsin L Plays a Key Role in SARS-CoV-2 Infection in Humans and Humanized Mice and Is a Promising Target for New Drug Development. *Signal. Transduct. Target. Ther.* 6, 134. doi:10.1038/s41392-021-00558-8

Zou, M., Liu, H., Li, J., Yao, X., Chen, Y., Ke, C., et al. (2020). Structure-activity Relationship of Flavonoid Bifunctional Inhibitors against Zika Virus Infection. *Biochem. Pharmacol.* 177, 113962. doi:10.1016/j.bcp.2020.113962

Zwicker, J. I., Schlechter, B. L., Stopa, J. D., Liebman, H. A., Aggarwal, A., Puligandla, M., et al. (2019). Targeting Protein Disulfide Isomerase with the Flavonoid Isoquercetin to Improve Hypercoagulability in Advanced Cancer. *JCI Insight* 4, e125851. doi:10.1172/jci.insight.125851

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Pharmacology

REVIEW
published: 05 April 2022
doi: 10.3389/fphar.2022.848676

Thymoquinone's Antiviral Effects: It is Time to be Proven in the Covid-19 Pandemic Era and its Omicron Variant Surge

Maen Abdelrahim[1,2,3]*, *Abdullah Esmail*[1,4,5], *Noor Al Saadi*[6], *Eva Zsigmond*[1], *Ebtesam Al Najjar*[5], *Doaa Bugazia*[7], *Hadeel Al-Rawi*[8], *Ayat Alsaadi*[9] and *Ahmed O. Kaseb*[10]*

[1]Houston Methodist Cancer Center, Houston Methodist Hospital, Houston, TX, United States, [2]Cockrell Center for Advanced Therapeutic Phase I Program, Houston Methodist Research Institute, Houston, TX, United States, [3]Weill Cornell Medical College, Institute of Academic Medicine, Houston, TX, United States, [4]Houston Methodist Research Institute, Houston, TX, United States, [5]Faculty of Medicine and Health Sciences, University of Science and Technology, Sanaa, Yemen, [6]Faculty of Medicine, Xavier University School of Medicine Aruba, Oranjestad, Aruba, [7]Faculty of Medicine, University of Tripoli, Tripoli, Libya, [8]Faculty of Medicine, University of Jordan, Amman, Jordan, [9]Department of Biology and Chemistry, Buffalo State College, Buffalo, NY, United States, [10]Department of Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

OPEN ACCESS

Edited by:
Carlos L. Cespedes-Acuña, University of Bio-Bio, Chile

Reviewed by:
Raju Dash, Bangladesh Council of Scientific and Industrial Research (BCSIR), Bangladesh
José Guillermo Avila Acevedo, Universidad Nacional Autónoma de México, Mexico

***Correspondence:**
Maen Abdelrahim
mabdelrahim@houstonmethodist.org
Ahmed O. Kaseb
akaseb@mdanderson.org

Specialty section:
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

Received: 04 January 2022
Accepted: 17 March 2022
Published: 05 April 2022

Citation:
Abdelrahim M, Esmail A, Al Saadi N, Zsigmond E, Al Najjar E, Bugazia D, Al-Rawi H, Alsaadi A and Kaseb AO (2022) Thymoquinone's Antiviral Effects: It is Time to be Proven in the Covid-19 Pandemic Era and its Omicron Variant Surge. Front. Pharmacol. 13:848676. doi: 10.3389/fphar.2022.848676

The COVID-19 pandemic has impacted every country in the world. With more than 400 million cases and more than 5.5 million deaths. The FDA either approved or authorized the emergency use for three vaccines against COVID-19. The treatment options of COVID-19 are very limited. Multiple complementary and alternative medicine modalities were suggested to be efficacious in the treatment of COVID-19 such as Thymoquinone. The effects of Thymoquinone have been examined and multiple studies indicate a promising beneficial effect. However, the current body of research is limited in terms of its scope, quality, and quantity. While higher-quality studies are required, physicians do not routinely recommend the use of marketed supplements of natural products, including Thymoquinone for COVID-19. Given the numerous suggested positive effects of Thymoquinone, including anti-inflammatory and antimicrobial properties, additional research is required to confirm or refute these promising benefits. Complementary and alternative medicine is an area that requires additional evidence-based practice and research to confirm effects observed in clinical practice.

Keywords: COVID-19, pandemic, Coronavirus, Thymoquinone, PAXLOVID, molnupiravir, COVID-19 vaccines and anti-viral agents, Omicron variant

INTRODUCTION

The COVID-19 pandemic has impacted almost every country in the world. With more than 400 million cases and around 5.5 million deaths, finding a treatment is a priority (World Health Organization, 2021a). However, the necessity of finding a treatment has led to the adoption of non-evidence-based practices. Hydroxychloroquine was one of the first medications to be proposed as a possible treatment for COVID-19. Additionally, multiple complementary and alternative medicine strategies have been suggested as possible treatments of COVID-19 (Ang et al., 2020; Badakhsh et al., 2021).

In addition to the recently discovered COVID-19, six unique strains of human coronaviruses have been identified (Elfiky, 2020; Hui et al., 2020). Coronaviruses are around 30 kb enclosed, positive-sense single-stranded RNA viruses. They infect a wide variety of hosts (Channappanavar et al., 2014). Coronaviruses are classified into four genera based on their genetic structure: α, β, γ, and δ. Only mammals are infected by the α and β coronaviruses (Rabi et al., 2020). The common cold and croup are caused by human coronaviruses such as 229E and NL63, which belong to the alpha coronavirus family. β Coronaviruses, on the other hand, include SARS-CoV, OC43, Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 are the most dangerous and are responsible for roughly 800 fatalities each year. According to the WHO, SARS HCoV has a 10% fatality rate, whereas MERS HCoV has a 36% mortality rate (World Health Organization, 2021b).

Since the emergence of COVID, physicians and researchers have struggled to effectively treat the novel coronavirus. More recently medications of varying levels of effectivity have been implicated in the treatment of COVID 19. These include steroids, antiviral drug Remdesivir (RDV), and monoclonal antibody (mAb) (Beigel et al., 2020; Deb et al., 2021). The mAbs are thought to help reduce the viral load by blocking virus entrance into cells by binding to viral spikes and therefore preventing virus attachment to cell surface receptors (Deb et al., 2021). The mAbs may potentially target host cell receptors or co-receptors, rendering the host cells' binding sites inaccessible to SARS-CoV-2. Alternatively, mAbs can act as immunosuppressive agents, limiting immune-mediated damage (Deb et al., 2021). Most recently, *Molnupiravir* as early treatment has shown a reduced risk of hospitalization or death in at-risk, unvaccinated adults with Covid-19 (Jayk Bernal et al., 2021). furthermore, *Pfizer* released phase 2/3 results from the *PAXLOVID* trial, confirming the novel COVID-19 oral antiviral treatment's robust efficacy in reducing the risk of hospitalization or death by 89% (within 3 days of symptom onset) and 88% (within 5 days of symptom onset) compared to placebo; no deaths in non-hospitalized, high-risk adults with COVID-19 compared to placebo (Archive, 2021), in addition, *PAXLOVID* has been authorized by the United States Food and Drug Administration (Ashraf et al., 2021) as emergency use authorization and became the first oral antiviral authorized by FDA for treatment of COVID-19 (U.S. Food and Drug Administration, 2021a).

Three COVID-19 vaccines, *Pfizer-BioNTech*, *Moderna*, and *Johnson & Johnson's Janssen*, were either approved or authorized for emergency use by the FDA (U.S. Food and Drug Administration, 2021b; U.S. Food and Drug Administration, 2021c; U.S. Food and Drug Administration, 2021d). The vaccines were effective in 90% of people regardless of age, gender, and underlying health issues. Furthermore, effectiveness was demonstrated in a subsequent analysis that included people with and without evidence of past SARS-CoV-2 infections (Oliver et al., 2020). Discomfort at the injection site, muscle pain, chills, joint pain, fatigue, headache, and fever were described as common adverse events of the COVID19 vaccinations (Centers for Disease Control and Prevention, 2021a; Abdelrahim and Esmail, 2021). Adverse effects following the second dose included pain at the injection site, muscle pain, chills, joint pain, tiredness, headache, and fever often lasted several days more than the first dose (Abdelrahim and Esmail, 2021; U.S. Food and Drug Administration, 2021e).

Multiple complementary and alternative medicine modalities where there suggested to be efficacious in the treatment of COVID-19. Suggested treatment options in the scientific literature include *Thymoquinone* and its natural source *Nigella sativa* (Bouchentouf and Noureddine, 2020). *Thymoquinone* is a component of many plants, with *Nigella sativa* being its primary natural source. *Nigella sativa* is used complementary or alternative medicine for its many proposed effects by different cultures and traditions (Goyal et al., 2017). The molecular formula of *Thymoquinone* is $C_{10}H_{12}O_2$, whereas it is $C_{18}H_{28}ClN_3O_5S$ for *Hydroxychloroquine sulfate*. Thus, it is unlikely for both chemical structures to have similar effects **Figure1**. **Table 1** shows the different plants that contain *Thymoquinone*. *Thymoquinone* was found to have possible effects on certain biological functions (Khader and Eckl, 2014) and this led to some interest in studying the anti-microbial properties of *Thymoquinone* (Forouzanfar et al., 2014). Studies on the anti-viral effects of *Thymoquinone* are limited in literature; however, multiple *in-vitro* and *in-vivo* studies suggest some therapeutic potential. Moreover, Salim and Nour (Bouchentouf and Noureddine, 2020) have recently demonstrated that compounds other than *Thymoquinone* within the *Nigella sativa* plant may play a role in targeting COVID-19.

This review is a timely review of *Thymoquinone's* properties as an anti-viral agent. Similar reports are needed to keep the medical community updated regarding the efficacy of various alternative medicines so that medical professionals can inform and educate patients. This review aims to illustrate the role *Thymoquinone* effect in the immunological response to COVID-19 and other viral infections. In addition, we hope to shed the light on the potential drug development and the clinical utility of *Thymoquinone* to treat COVID-19 patients which is an era of unmet need for the time being.

CORONAVIRUS OVERVIEW

In the downstream areas of Open Reading Frame 1 (ORF 1), all coronaviruses have particular genes that encode proteins for viral replication, nucleocapsid development, and spike creation (Elfiky, 2020). The glycoprotein spikes on coronaviruses' outer surface are essential for the virus's attachment and penetration into host cells. The MERS-coronavirus requires dipeptidyl peptidase 4 (DPP4), whereas the HCoV-NL63 and SARS-coronaviruses require angiotensin-converting enzyme 2 (ACE2) as a major receptor (Gralinski and Menachery, 2020). The cell-surface Heat Shock Protein A5 (HSPA5), also known as GRP78 or BiP, has been found to be identified by the viral spike proteins of SARS-Cov-2 (Datau et al., 2010). SARS-CoV-2 employs the same ACE2 cell receptor and method for entrance into host cells as SARS-CoV, these details were

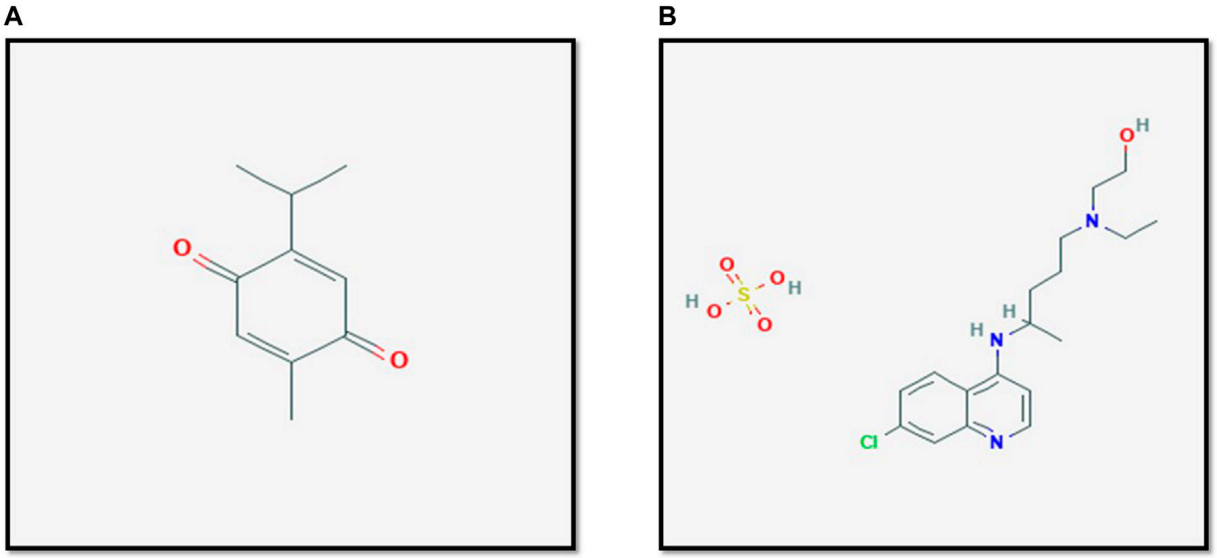


FIGURE 1 | The structure of Thymoqunione **(A)** and Hydroxychloroquine **(B)**. The molecular formula of Hydroxychloroquine sulfate is $C_{18}H_{28}ClN_3O_5S$, whereas it is $C_{10}H_{12}O_2$ for Thymoquinone. Thus, it is unlikely for both chemical structures to have similar effects. Figures are adapted from PubChem (National. Center for Biotechnology Information, 2021a; National. Center for Biotechnology Information, 2021b).

TABLE 1 | Plants containing *Thymoquinone*.

Plants Containing *Thymoquinone*
Nigella sativa
Satureja Hortenis
Eupatorium Cannabinum
Juniperus Communis
Monarda Didyma
Monarda Media
Monarda Menthifolia
Thymus Pilegioides
Thymus Serpyllum
Thymus Vulgaris
Urejamontana

validated in a fluorescence experiments (Gralinski and Menachery, 2020; Shereen et al., 2020; Xu et al., 2020).

Attachment, penetration, biosynthesis, maturity, and release are the five phases in a virus's life cycle within the host. Viruses enter host cells by endocytosis or membrane fusion after binding to host receptors (attachment) (penetration). The components of the virus are subsequently released into the host cells, and viral RNA is taken into the nucleus for replication. Viral proteins are made from viral mRNA (biosynthesis). Finally, new virus particles (maturation) are produced and discharged. Spike (S), membrane (M), envelop (E), and nucleocapsid (N) are the four structural proteins found in Coronaviruses (N) (Walls et al., 2020). The spike protein is a transmembrane trimetric glycoprotein that protrudes from the viral surface and controls coronavirus diversification and host tropism. Spike proteins are made up of two functional subunits: the S1 subunit is in charge of binding to the host cell receptor, and the S2 subunit is in charge of fusing the viral and cellular membranes. The ACE2 receptor has already been identified as a functioning SARS-CoV receptor (Li et al., 2003). The spike protein for SARS-CoV-2 interacts with ACE2 according to structural and functional investigations (Chen et al., 2020; Letko et al., 2020; Walls et al., 2020). ACE2 is highly expressed in the lungs, heart, ileum, kidneys, and bladder (Zou et al., 2020). ACE2 is a highly expressed epithelial cell of the lungs. Following SARS-attachment CoV-2's to the host protein, the spike protein is cleaved by proteases. The S1 and S2 subunits remain non-covalently linked after cleavage at the S1/S2 cleavage site, and the distal S1 subunit aids in the prefusion stabilization of the membrane-anchored S2 subunit (Walls et al., 2020). Following cleavage at the S2 site, the spike protein is probably activated for membrane fusion with irreversible conformational changes.

Antigen presentation by dendritic cells (DCs) and macrophages trigger T cell responses against coronaviruses. DCs and macrophages can phagocytize virus-infected apoptotic cells (Fujimoto et al., 2000). DCs and macrophages, for example, can phagocytize virus-infected apoptotic epithelial cells, resulting in antigen presentation to T cells. In addition to ACE2, SARS-CoV may bind to dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) and DC-SIGNR (L-SIGN) and DC-SIGNR-related protein (DC-SIGNR, L-SIGN) (Jeffers et al., 2004; Marzi et al., 2004; Yang et al., 2004). Dendritic cells and macrophages both express high numbers of DC-SIGN. To present viral antigens to T cells, these antigen-presenting cells go to the draining lymph nodes. T lymphocytes, both $CD4^+$ and $CD8^+$, play an important function. $CD4^+$ T cells stimulate B cells to produce virus-specific antibodies, whereas $CD8^+$ T cells destroy virally infected cells. Regarding to coronavirus variants, the scientists track all variants, however,

some are classified as variants to be monitored, variants of interest, variants of high consequence, and variants of concern such as *Omicron*–B.1.1.29 and *Delta*–B.1.617.2 (Centers for Disease Control and Prevention, 2021b; Karim and Karim, 2021). Some variants spread more easily and quickly than others, for example, the *Omicron* variant may spread more easily than other variants, including Delta (Centers for Disease Control and Prevention, 2021a). These classifications are based on the ease with which the variant spreads, the severity of the symptoms, how well the variant responds to treatments, and how well immunizations protect against the variant.

SCIENTIFIC REPORT

Several synthetic compounds initially thought to have shown promise in COVID-19 therapy, including hydroxychloroquine and chloroquine phosphate (Cortegiani et al., 2020; Gao et al., 2020) and newer antiviral drugs like lopinavir (Yao et al., 2020), have subsequently been shown to have little or no effect on hospitalized COVID- 19 patients, as indicated by overall mortality, initiation of ventilation and duration of hospital stay (Pan et al., 2021). On the other hand, *Remdesivir* (Holshue et al., 2020; Wang et al., 2020) clinical data suggest efficacy in treating COVID-19 and is the first FDA-approved COVID-19 therapy (Lamb, 2020).

The creation of innovative antiviral medications may be driven by traditional herbal medicines and purified natural ingredients. For example, Emetine an isoquinoline alkaloid isolated from *Cephaelis ipecacuanha* is an effective amoebicidal drug. Similarly, the drug quinine is derived from *Cinchona* tree bark. Other common drugs derived from natural compounds include *aspirin*, *morphine*, and *paclitaxel*, an antineoplastic drug (Ganjhu et al., 2015). Between 1981 and 2014, half of all medications approved were derived from or resembled a natural component (Newman and Cragg, 2016).

According to scientific investigations, *Nigella sativa* (Family Ranunculaceae) is developing as a therapeutic plant with a wide range of pharmacological potential. *Nigella sativa*, often known as black seed, is native to Southern Europe, North Africa, and Southwest Asia. It is also cultivated in other regions of the world, including the Eastern Mediterranean and India (Khare, 2004). *Nigella sativa* is a commonly used medicinal herb in several traditional medical systems across the world, including Unani and Tibb, Ayurveda, and Siddha. The seeds and oil of the plant have a long history of use as both medicinal and sustenance (Ahmad et al., 2013). The star of this study, *Thymoquinone* is one such product derived from *Nigella sativa*. *Thymoquinone* has been investigated for its potential anti-inflammatory, anti-microbial, and anti-tumor effects (Beigel et al., 2020) (Bouchentouf and Noureddine, 2020). Most of these studies have been performed *in vitro* or animal-based models. However, very few studies have been able to establish clear clinical evidence of therapeutic effects.

Experiments demonstrate that *Thymoquinone* inhibits the growth of a variety of bacteria. Different extracts of *Nigella sativa* showed possible effects on multiple bacteria, including extracts that contained *Thymoquinone* alone. Chaieb et al. (2011) showed that *Thymoquinone* was effective against seven out of sixteen tested bacteria. These bacteria were mainly Gram-positive bacteria. Other studies have confirmed that *Thymoquinone* has the most potent effect on Gram-positive bacteria (Kokoska et al., 2008). Additionally, *in-vivo* studies using animal models have also suggested possible positive effects. In an acute pyelonephritis model, treatment with *Thymoquinone* (a dose of 10 mg/kg) was given before bacterial inoculation of E-Coli and *Thymoquinone* was also repeated every 24 h. Histological examination exhibited a reduction in oxidative damage and a nephron-protective effect due to *Thymoquinone* treatment (Evirgen et al., 2011).

The effects of *Thymoquinone* have also been studied in fungal infections. Both *in vitro* and *in vivo* studies have suggested that *Thymoquinone* may play a possible therapeutic role in the treatment of different fungal infections, such as dermatophytes and *candida* (Aljabre et al., 2005). Like in anti-bacterial studies, anti-fungal experiments also have inconsistencies in dosing, the type of extracts, as well as a lack of clinical proof.

In direction of antiviral effect, *Thymoquinone's* antiviral effectiveness against various viral infections has been supported by several studies, according to its multiple positive benefits, including antioxidant, anti-inflammatory, and immunomodulatory properties, as well as the possibility of direct viral elimination, and these studies reported that the viral loads in the liver and spleen were dramatically reduced, which correlated with increased IFN- production and CD4 (+) T cell response (Forouzanfar et al., 2014; Sommer et al., 2020; Salem and Hossain, 2000). In addition, multiple studies have suggested the same concept of that *Thymoquinone* has antiviral properties specifically in regards to cytomegalovirus (CMV), human immunodeficiency virus (Archive, 2021), and influenza. In contrast to other anti-microbial studies, these reports included clinical outcomes which highlight the possible efficacy of *Thymoquinone* as a therapeutic agent for HIV (Onifade et al., 2015). Two patients who were ineligible for the highly active antiretroviral therapy (HAART) achieved seroconversion with *Thymoquinone*. One of the patients was a 27-year-old pregnant female, who achieved seroconversion and no vertical transmission. In another study, *Thymoquinone* was shown to decrease hyperinsulinemia associated with HAART therapy (Chandra et al., 2009).

These promising studies support the need for further investigation. **Table 2** summarizes some selected examples of the antiviral effects of *Thymoquinone.*

In addition to *Thymoquinone,* anti-viral effects of compounds found in the oils of the *Nigella sativa* plant have also been examined, as illustrated by *in-vitro* and animal studies of the murine CMV (Salem and Hossain, 2000). *Thymoquinone* treatment with or without curcumin led to reduced symptoms and viral shedding in animals infected by the H9N2 virus, a form of avian influenza that affects poultry and in more rare cases humans (Cortegiani et al., 2020). Other extracts have been shown

TABLE 2 | Selected examples of the anti-viral effects of *Thymoquinone* (TQ) and *Nigella sativa* extracts.

Virus	Type of study	Comments and outcomes	References
CMV	Animal	- Study has been done using Murine CMV - Possible *in-vitro* effect in inhibition of CMV - Increase in interferon-gamma and macrophages number	Salem and Hossain, (2000)
Human immunodeficiency virus	Case reports	- 27-year-old pregnant female ineligible for HAART, achieved seroconversion and no vertical transmission	Onifade et al. (2015)
Human immunodeficiency virus	Animal	- Decrease in HAART-related hyperinsulinemia in treated rats	Chandra et al. (2009)

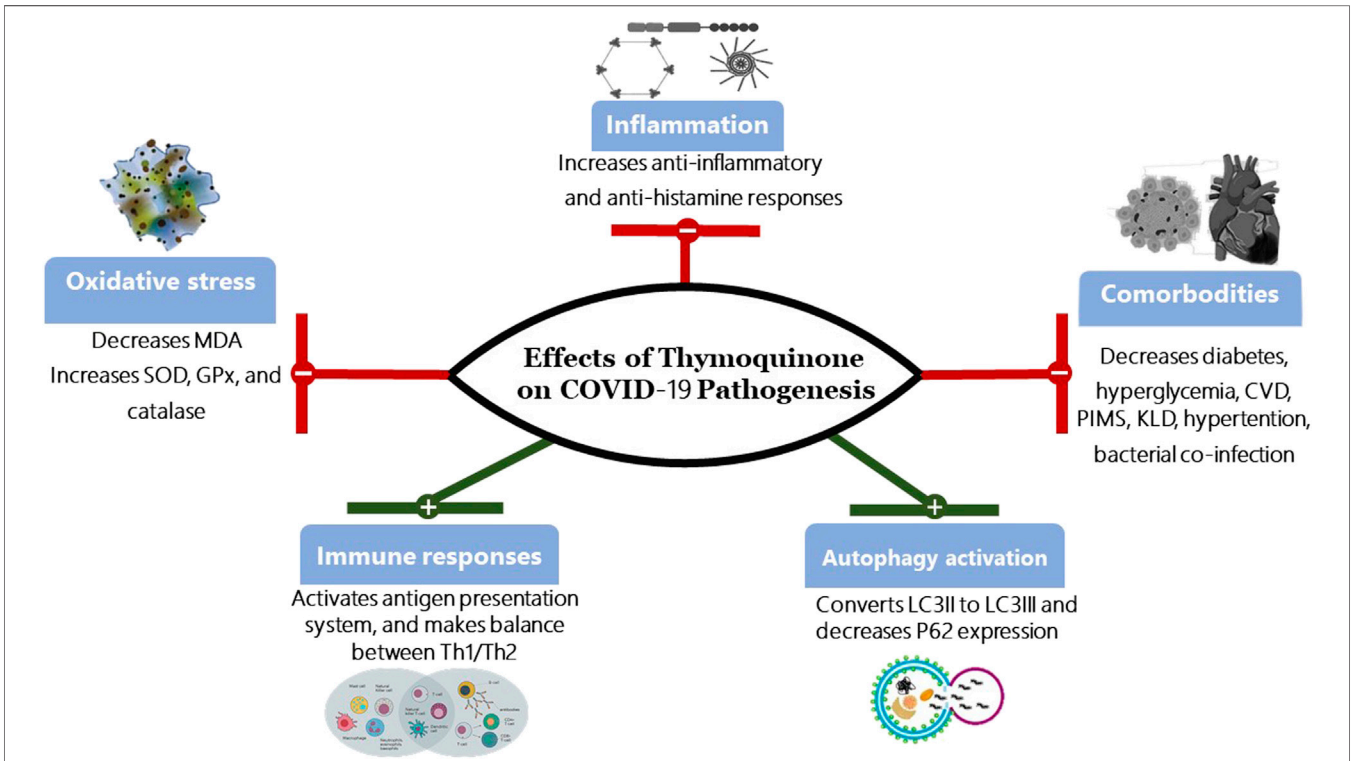


FIGURE 2 | Effects of *Thymoquinone* on COVID-19 Pathogenesis. SOD, Superoxide dismutase; GPx, Glutathione peroxidase; MDA, Malonaldehyde Th1, type I helper T lymphocytes; Th2, type II helper T cells; CVD, Cardiovascular disease; PIMS, Paediatric Inflammatory Multisystem Syndrome; KLD, Kawasaki-like diseases; LC3, Microtubule-associated protein 1A/1B-light chain 3; P62, protein 62.

to have probable effects in the treatment of hepatitis C (Barakat et al., 2013).

Interestingly, one study has demonstrated that extracts from multiple plants or their scientific extraction, including *Thymoquinone* (*Nigella sativa*), Anthemis hyalina, and Citrus sinensismay influence the outcomes of coronavirus infections. All three extracts showed possible therapeutic benefit, with Anthemis hyaline, having the most effect (Ulasli et al., 2014). A recent study (under pre-print review) has shown that compounds, other than *Thymoquinone*, extracted from *Nigella sativa* regulate molecular docking (Bouchentouf and Noureddine, 2020). Molecular docking is promising in silico method for screening diverse drugs for their antiviral potential by comparing their binding affinities to various viral or host cell receptor proteins. Various viral proteins involved in viral entry, such as spike proteins, and replication, such as viral proteases, are molecular targets of SARS-CoV-2 (Senger et al., 2020). Additionally, a double-blind randomized controlled trial discovered that *Nigella sativa* extracts reduced inflammatory cytokine response in a patient with rheumatoid arthritis (Hadi et al., 2016). These results are promising in the case of COVID-19 due to the fact that infected patients are in a state of chronic inflammation and at risk of developing cytokine release syndrome. These

observations were suggestive of a potential role for *Thymoquinone* in the treatment of COVID-19 **Figure 2**.

Abdel-Fattah et al. (2000) found that since *Thymoquinone* has antinociceptive effects by indirectly activating the supraspinal μ1- and κ-opioid receptor subtypes, it may prevent SARS-CoV-2 entrance into pneumocytes *via* ACE2. Multiple investigations have found that opioid receptors and ACE have overlapping inhibitory chemicals, for example, Rahman (2020) speculated that *Thymoquinone* might also block ACE2. Takai et al. (1996) additionally proposed that brain endogenous angiotensin II, by its antagonistic interaction with the endogenous opioid system, was implicated in central nociceptive pathways. Furthermore, Lantz et al. (1991) showed that opioid-active peptides, such as hemorphins, have an inhibitory effect on ACE. The above line of evidence suggests that opioid receptors and ACE share similar inhibitory molecules and as such, in publication, Rahman indicated that *Thymoquinone* may also block ACE2 (Rahman, 2020).

In a collaborative research project, Codex Bio Labs tested black seed oil and *Thymoquinone* for their effect on viral entry and viral protein translation using Codex's Murine Leukemia Virus (MLV) particles pseudotyped (PP) with the SARS-CoV-2 Spike protein (unpublished data). Various combinations/concentrations of black seed oil and *Thymoquinone* were tested against SARS-CoV-2 MLV pseudovirus particles (pp) by assessing Luciferase activities measured with a Firefly Luciferase Assay Kit (CB-80552-010, Codex BioSolutions Inc.). It was observed that *Thymoquinone* seemed to block viral infection. However, at high concentrations *Thymoquinone* caused cell death indicating cytotoxic effects. To confirm this result, cell growth assays were performed in the presence of *Thymoquinone* with Codex's EnerCount cell growth assay kit which measures ATP levels inside the cells.

Similarly, in a seropositive HIV infected patient treated with *Thymoquinone* (10 ml twice/day for 6 months), Onifade et al. (2013) demonstrated a decrease in viral load to an undetectable level within 3 months, an increase in CD4 count, relief of symptoms, and a sustained sero-reversion following COVID-19 therapy. Another investigation on a seropositive HIV-infected woman who received *Thymoquinone* (*Nigella sativa*) and honey treatment (10 ml thrice/day for 1 year) demonstrated prolonged sero-reversion, which the author attributed to *Thymoquinone's* possible virucidal effect (Onifade et al., 2015).

Akhtar and Riffat (1991) demonstrated the efficacy of a single oral administration of *Thymoquinone* (*Nigella sativa*) as powdered seeds and ethanolic extract (40 mg/kg body weight) in reducing the percentage of fecal eggs per Gram in children who were infected with cestodes.

In a study conducted on Hepatitis C (HCV) patients, Abdel-Moneim et al. were able to demonstrate that extracts of *Nigella sativa* (*Thymoquinone*) and *Zingiber officinale*, alone and together (500 mg of *Nigella sativa* and/or Zingiber officinale twice daily for 1 month), improved liver function and decreased viral load in the HCV patients (Onifade et al., 2013). Decreased viral load and improved liver function were similarly reported in another study by Barakat et al. (2013) where HCV patients received capsules of *Nigella sativa* oil (450 mg) three times a day over 3 months. Furthermore, *Thymoquinone* has been studied for benefits other than anti-inflammatory effects which are beyond the scope of this review (Mostofa et al., 2017; Mohammed and Islam, 2018; Shanmugam et al., 2018; Jehan et al., 2020; Leong et al., 2021; Salehi et al., 2021).

THYMOQUINONE STUDIES IN COVID-19 PATIENTS

Clinical Studies

In an investigator-initiated, 313 COVID-19 positive patients were divided into two groups: mild to moderate (cough, fever, sore throat, nasal congestion, malaise and/or shortness of breath) and severe (fever and/or cough along with pneumonia, severe dyspnea, respiratory distress, tachypnea (>30 breaths/min or hypoxia (SpO2 <90% on room air) however, this was conducted as open-label-placebo and randomized controlled trial, 210 and 103 patients were allocated to the mild/moderate and severe groups, respectively, using the clinical care criteria for COVID-19 implemented by Pakistan's Ministry of National Health Services (Ashraf et al., 2020). Within each of the two groups, the patients were randomly allocated to the treatment group (which received honey + *Thymoquinone* (*Nigella sativa*) [HNS]) or the control group (which received no therapy) (receiving empty capsules). Honey (1 g) and *Nigella sativa* seeds (80 mg) per kg body weight were given orally in 2-3 split doses daily for up to 13 days in the HNS group, whereas the control group got a placebo (empty capsules). The primary outcomes were viral elimination (no RT-PCR for SARS-CoV-2 RNA), clinical symptom relief, and a reduction in Clinical Grading Score (CGS) on day 6. Fever decrease (day 4), C-Reactive protein CRP levels (day 6), the intensity of symptoms (day 8), CGS score (day 10), and death on day 30 were all secondary outcomes. HNS aided with symptom relief and viral clearance, as well as lowering mortality in individuals with moderate and severe illness, according to the findings. COVID-19 symptoms were shown to be relieved earlier in the HNS groups than in the control groups: 4 versus 7 days for moderate patients and 6 versus 13 days for severe disease patients. For both moderate and severe cases, viral elimination (being negative for the SARS-CoV-2 RT-PCR test) occurred 4 days sooner in the HNS group. On day 4, there was a considerable decrease in the severity of fever in the severe patients (OR: 0.21; 95% CI: 0.09–0.46; $p = 0.0001$). On day 6, C- reactive proteins (CRP) levels in both HNS groups reduced dramatically (p 0.0001) when compared to their respective control groups. On day 8, 98.13% of patients in HNS-treated mild cases were asymptomatic, compared to 56.31% in the control group (OR: 0.009; 95% CI: 0.001–0.08; $p < 0.0001$). More patients in the HNS group were asymptomatic in severe instances, whereas more in the control arm experienced mild symptoms (median) (OR: 0.1; 95% CI: 0.04–0.24). On day 10, 96.26% of moderate case-patients with HNS had fully resumed regular activities, compared to 68.93% in the control group (OR: 0.07; 95% CI: 0.02–0.21). The median CGS at day 10 for the severe group demonstrated that HNS treated patients returned to normal activities, whereas control patients remained hospitalized and required oxygen treatment (OR:0.05; 95% CI: 0.02–0.15). Morality after 30 days was 18.87% in the control group and 4%

with HNS treatment (OR: 0.18 95% CI: 0.02–0.92) (Ashraf et al., 2020).

Non-Clinical Studies

A molecular docking and molecular dynamics stimulation study conducted by Elfiky (2021) tested the effect of natural products against the HSPA5 substrate-binding domain. The results showed that active components in cinnamon and seeds of *Nigella sativa* may tightly bind to cell-surface HSPA5 (one of the host cell receptors recognized by the viral spike protein) and could be successful in hindering SARS-CoV-2 spike recognition and attachment.

In an, *in vitro* model of rheumatoid arthritis, Vaillancourt et al. (2011) illustrated that *Thymoquinone* significantly decreases lipopolysaccharide (LPS) -induced proinflammatory cytokines such as interleukin1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), metalloproteinase-13 (MMP-13), COX-2, and prostaglandin E2.

Similarly, Gholamnezhad et al. (2019) reported an anti-inflammatory effect of *Thymoquinone* in allergic lung inflammation. There was a *Thymoquinone*-associated decrease in IL-4, IL-5, and IL-13, but an increase in IFN-γ in BALF and lung homogenates.

As part of an *in-vitro* study, Cobourne-Duval et al. compared LPS/IFNγ-activated BV-2 microglial cells (immortalized murine microglial cell line) with and without *Thymoquinone* treatment in a quantitative proteomic study. The following inflammatory cytokines had considerably increased protein expression in LPS/IFNγ-activated BV-2 cells compared to controls: IL-2 (127%), IL-4 (151%), IL-6 (670%), IL-10 (133%), and IL-17a (127%). When comparing the protein expression levels of the same inflammatory cytokines in *Thymoquinone* treated LPS/IFNγ-activated cells to the protein expression levels in activated cells without *Thymoquinone* treatment, the protein expression levels in *Thymoquinone* treated LPS/IFN-activated cells were significantly reduced ($p < 0.0001$). IL-2, IL-4, IL-6, IL-10, and IL-17a levels were reduced by 38 percent, 19 percent, 83 percent, 23 percent, and 29 percent, respectively, when compared to controls (Cobourne-Duval et al., 2018). Additional findings of the study showed that *Thymoquinone* significantly inhibited the production of various inflammatory cytokines in LPS/IFNγ stimulated BV-2 microglial cells, displaying an inhibitory impact on the expression of several interleukins such as IL-2, IL-4, IL-6, IL-10, and IL-17a (Cobourne-Duval et al., 2018).

DISCUSSION

In pre-clinical studies, *Thymoquinone* has been shown to possess anti-inflammatory properties as well as anti-corona virus properties by blocking viral entry. The acute and sub-acute toxicity of *Thymoquinone* has been examined in various *in-vitro* and *in-vivo* experiments. *Thymoquinone/Nigella sativa* has been studied extensively over many years and has been found to be relatively safe, with very few side effects despite the low level of toxicity that the seed extract and its constituent's exhibit (Abukhader, 2012; Ong et al., 2016). Furthermore, *Black seed* (*Black Cumin* or *Nigella sativa*) has been categorized by the FDA under spices and other natural seasonings/flavorings that are generally recognized as safe for their intended use (409 of the Act Title 21, Chapter I, Subchapter B, Sec. 182.10 Spices and other natural seasonings and flavorings).

A review of the literature on the therapeutic uses of *Thymoquinone/Nigella sativa* shows some promising results but remains inconclusive. There is a scarcity of studies investigating clinical efficacy, especially at higher doses. Furthermore, any results derived from preclinical studies are confounded by the use of varied extracts thus introducing heterogeneity in the product being tested. More rigorous pre-clinical and clinical research studies need to be conducted before *Thymoquinone/Nigella sativa* can be routinely used as an effective complementary or alternative treatment.

In addition to efficacy, alternative medicine must also satisfy safety criteria. For instance, it is a misconception that these substances are always healthy, since, in addition to possible intrinsic adverse effects, marketed preparations may also have additives that can increase the risk of negative side effects. For example, *Thymoquinone* was found to inhibit CYP enzymes, particularly CYP29C, which may lead to possible interactions (Albassam et al., 2018).

Importantly, the regulatory processes governing complementary and alternative medicine preparations are not as strict as for other pharmaceuticals. The devastating health effects of the COVID-19 pandemic have led to the use of a variety of non-evidence-based treatments that are yet to be validated by large, randomized control trials.

CONCLUSION

Although multiple studies indicate promising beneficial effects of *Thymoquinone* in the treatment of various diseases, the current body of research is limited in terms of its scope, quality, and quantity. Physicians are discouraged from recommending the use of marketed supplements of natural products, including *Thymoquinone,* for COVID-19. Given the numerous suggested positive effects of *Thymoquinone,* including its anti-inflammatory, additional research is required to confirm these promising benefits or refute the suggested benefits.

AUTHOR CONTRIBUTIONS

AE and MA wrote the first draft of the manuscript. All authors vouch for the accuracy and contents of the manuscript. All authors approved the final version of the draft.

ACKNOWLEDGMENTS

The assistance provided by Ibrahim N. Muhsen; MD in editing this manuscript and providing some comments to improve this review was greatly appreciated.

REFERENCES

Abdel-Fattah, A. M., Matsumoto, K., and Watanabe, H. (2000). Antinociceptive Effects of Nigella Sativa Oil and its Major Component, Thymoquinone, in Mice. *Eur. J. Pharmacol.* 400 (1), 89–97. doi:10.1016/s0014-2999(00)00340-x

Abdelrahim, M., and Esmail, A. (2021). Regional Lymphadenopathy after COVID-19 Vaccine in a Cancer Patient: A Case Report. *J. Surg. Res.* 4 (4), 4. doi:10.26502/jsr.10020171

Abukhader, M. M. (2012). The Effect of Route of Administration in Thymoquinone Toxicity in Male and Female Rats. *Indian J. Pharm. Sci.* 74 (3), 195–200. doi:10.4103/0250-474X.106060

Ahmad, A., Husain, A., Mujeeb, M., Khan, S. A., Najmi, A. K., Siddique, N. A., et al. (2013). A Review on Therapeutic Potential of Nigella Sativa: A Miracle Herb. *Asian Pac. J. Trop. Biomed.* 3 (5), 337–352. doi:10.1016/S2221-1691(13)60075-1

Akhtar, M. S., and Riffat, S. (1991). Field Trial of Saussurea Lappa Roots against Nematodes and Nigella Sativa Seeds against Cestodes in Children. *J. Pak Med. Assoc.* 41 (8), 185–187.

Albassam, A. A., Ahad, A., Alsultan, A., and Al-Jenoobi, F. I. (2018). Inhibition of Cytochrome P450 Enzymes by Thymoquinone in Human Liver Microsomes. *Saudi Pharm. J.* 26 (5), 673–677. doi:10.1016/j.jsps.2018.02.024

Aljabre, S. H., Randhawa, M. A., Akhtar, N., Alakloby, O. M., Alqurashi, A. M., and Aldossary, A. (2005). Antidermatophyte Activity of Ether Extract of Nigella Sativa and its Active Principle, Thymoquinone. *J. Ethnopharmacol* 101 (1-3), 116–119. doi:10.1016/j.jep.2005.04.002

Ang, L., Song, E., Lee, H. W., and Lee, M. S. (2020). Herbal Medicine for the Treatment of Coronavirus Disease 2019 (COVID-19): A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J. Clin. Med.* 9 (5), 4–8. doi:10.3390/jcm9051583

Archive, P. R. (2021). Pfizer Announces Additional Phase 2/3 Study Results Confirming Robust Efficacy of Novel COVID-19 Oral Antiviral Treatment Candidate in Reducing Risk of Hospitalization or Death. Available from: https://www.pfizer.com/news/press-release/press-release-detail/pfizer-announces-additional-phase-23-study-results.

Ashraf, S., Ashraf, S., Akmal, R., Ashraf, M., Kalsoom, L., Maqsood, A., et al. (2021). Prophylactic Potential of Honey and Nigella Sativa L. Against Hospital and Community-Based SARS-CoV-2 Spread: A Structured Summary of a Study Protocol for a Randomised Controlled Trial. *Trials* 22 (1), 618. doi:10.1186/s13063-021-05510-3

Ashraf, S., Ashraf, S., Ashraf, M., Imran, M. A., Kalsoom, L., Siddiqui, U. N., et al. (2020). Honey and Nigella Sativa against COVID-19 in Pakistan (HNS-COVID-PK): A Multi-center Placebo-Controlled Randomized Clinical Trial. *medRxiv* 2020, 17–19. doi:10.1101/2020.10.30.20217364

Badakhsh, M., Dastras, M., Sarchahi, Z., Doostkami, M., Mir, A., and Bouya, S. (2021). Complementary and Alternative Medicine Therapies and COVID-19: a Systematic Review. *Rev. Environ. Health* 36 (3), 443–450. doi:10.1515/reveh-2021-0012

Barakat, E. M., El Wakeel, L. M., and Hagag, R. S. (2013). Effects of Nigella Sativa on Outcome of Hepatitis C in Egypt. *World J. Gastroenterol.* 19 (16), 2529–2536. doi:10.3748/wjg.v19.i16.2529

Beigel, J. H., Tomashek, K. M., Dodd, L. E., Mehta, A. K., Zingman, B. S., Kalil, A. C., et al. (2020). Remdesivir for the Treatment of Covid-19 - Final Report. *N. Engl. J. Med.* 383 (19), 1813–1826. doi:10.1056/NEJMoa2007764

Bouchentouf, M., and Noureddine, M. (2020). identification-of-compounds-from-nigella-sativa-as-new-potential-inhibitors-of-2019-novel-coronasvirus-covid-19-molecular-docking. *Angiosperms*.

Centers for Disease Control and Prevention (2021a). Pfizer-BioNTech COVID-19 Vaccine Overview and Safety 2021. Available from: https://www.cdc.gov/coronavirus/2019-ncov/vaccines/different-vaccines.html.

Centers for Disease Control and Prevention (2021b). Variants of the Virus,Omicron Variant: What You Need to Know. updated 2021. Available from: https://www.cdc.gov/coronavirus/2019-ncov/variants/about-variants.html.

Chaieb, K., Kouidhi, B., Jrah, H., Mahdouani, K., and Bakhrouf, A. (2011). Antibacterial Activity of Thymoquinone, an Active Principle of Nigella Sativa and its Potency to Prevent Bacterial Biofilm Formation. *BMC Complement. Altern. Med.* 11 (1), 29. doi:10.1186/1472-6882-11-29

Chandra, S., Murthy, S. N., Mondal, D., and Agrawal, K. C. (2009). Therapeutic Effects of Nigella Sativa on Chronic HAART-Induced Hyperinsulinemia in Rats. *Can. J. Physiol. Pharmacol.* 87 (4), 300–309. doi:10.1139/Y09-014

Channappanavar, R., Zhao, J., and Perlman, S. (2014). T Cell-Mediated Immune Response to Respiratory Coronaviruses. *Immunol. Res.* 59 (1-3), 118–128. doi:10.1007/s12026-014-8534-z

Chen, Y., Guo, Y., Pan, Y., and Zhao, Z. J. (2020). Structure Analysis of the Receptor Binding of 2019-nCoV. *Biochem. Biophys. Res. Commun.* 525 (1), 135–140. doi:10.1016/j.bbrc.2020.02.071

Cobourne-Duval, M. K., Taka, E., Mendonca, P., and Soliman, K. F. A. (2018). Thymoquinone Increases the Expression of Neuroprotective Proteins while Decreasing the Expression of Pro-inflammatory Cytokines and the Gene Expression NFκB Pathway Signaling Targets in LPS/IFNγ -activated BV-2 Microglia Cells. *J. Neuroimmunol* 320, 87–97. doi:10.1016/j.jneuroim.2018.04.018

Cortegiani, A., Ingoglia, G., Ippolito, M., Giarratano, A., and Einav, S. (2020). A Systematic Review on the Efficacy and Safety of Chloroquine for the Treatment of COVID-19. *J. Crit. Care* 57, 279–283. doi:10.1016/j.jcrc.2020.03.005

Datau, E. A., Wardhana, W., Surachmanto, E. E., Pandelaki, K., Langi, J. A., and Fias, F. (2010). Efficacy of Nigella Sativa on Serum Free Testosterone and Metabolic Disturbances in central Obese Male. *Acta Med. Indones* 42 (3), 130–134.

Deb, P., Molla, M. M. A., and Saif-Ur-Rahman, K. M. (2021). An Update to Monoclonal Antibody as Therapeutic Option against COVID-19. *Biosaf Health* 3 (2), 87–91. doi:10.1016/j.bsheal.2021.02.001

Elfiky, A. A. (2020). Anti-HCV, Nucleotide Inhibitors, Repurposing against COVID-19. *Life Sci.* 248, 117477. doi:10.1016/j.lfs.2020.117477

Elfiky, A. A. (2021). Natural Products May Interfere with SARS-CoV-2 Attachment to the Host Cell. *J. Biomol. Struct. Dyn.* 39 (9), 3194–3203. doi:10.1080/07391102.2020.1761881

Evirgen, O., Gökçe, A., Ozturk, O. H., Nacar, E., Onlen, Y., Ozer, B., et al. (2011). Effect of Thymoquinone on Oxidative Stress in Escherichia Coli-Induced Pyelonephritis in Rats. *Curr. Ther. Res. Clin. Exp.* 72 (5), 204–215. doi:10.1016/j.curtheres.2011.09.002

Forouzanfar, F., Bazzaz, B. S., and Hosseinzadeh, H. (2014). Black Cumin (Nigella Sativa) and its Constituent (Thymoquinone): a Review on Antimicrobial Effects. *Iran J. Basic Med. Sci.* 17 (12), 929–938.

Fujimoto, I., Pan, J., Takizawa, T., and Nakanishi, Y. (2000). Virus Clearance through Apoptosis-dependent Phagocytosis of Influenza A Virus-Infected Cells by Macrophages. *J. Virol.* 74 (7), 3399–3403. doi:10.1128/jvi.74.7.3399-3403.2000

Ganjhu, R. K., Mudgal, P. P., Maity, H., Dowarha, D., Devadiga, S., Nag, S., et al. (2015). Herbal Plants and Plant Preparations as Remedial Approach for Viral Diseases. *Virusdisease* 26 (4), 225–236. doi:10.1007/s13337-015-0276-6

Gao, J., Tian, Z., and Yang, X. (2020). Breakthrough: Chloroquine Phosphate Has Shown Apparent Efficacy in Treatment of COVID-19 Associated Pneumonia in Clinical Studies. *Biosci. Trends* 14 (1), 72–73. doi:10.5582/bst.2020.01047

Gholamnezhad, Z., Shakeri, F., Saadat, S., Ghorani, V., and Boskabady, M. H. (2019). Clinical and Experimental Effects of Nigella Sativa and its Constituents on Respiratory and Allergic Disorders. *Avicenna J. Phytomed* 9 (3), 195–212.

Goyal, S. N., Prajapati, C. P., Gore, P. R., Patil, C. R., Mahajan, U. B., Sharma, C., et al. (2017). Therapeutic Potential and Pharmaceutical Development of Thymoquinone: A Multitargeted Molecule of Natural Origin. *Front. Pharmacol.* 8, 656. doi:10.3389/fphar.2017.00656

Gralinski, L. E., and Menachery, V. D. (2020). Return of the Coronavirus: 2019-nCoV. *Viruses* 12 (2), 1-6. doi:10.3390/v12020135

Hadi, V., Kheirouri, S., Alizadeh, M., Khabbazi, A., and Hosseini, H. (2016). Effects of Nigella Sativa Oil Extract on Inflammatory Cytokine Response and Oxidative Stress Status in Patients with Rheumatoid Arthritis: a Randomized, Double-Blind, Placebo-Controlled Clinical Trial. *Avicenna J. Phytomed* 6 (1), 34–43.

Holshue, M. L., DeBolt, C., Lindquist, S., Lofy, K. H., Wiesman, J., Bruce, H., et al. (2020). First Case of 2019 Novel Coronavirus in the United States. *N. Engl. J. Med.* 382 (10), 929–936. doi:10.1056/NEJMoa2001191

Hui, D. S., I Azhar, E., Madani, T. A., Ntoumi, F., Kock, R., Dar, O., et al. (2020). The Continuing 2019-nCoV Epidemic Threat of Novel Coronaviruses to Global Health - the Latest 2019 Novel Coronavirus Outbreak in Wuhan, China. *Int. J. Infect. Dis.* 91, 264–266. doi:10.1016/j.ijid.2020.01.009

Jayk Bernal, A., Gomes da Silva, M. M., Musungaie, D. B., Kovalchuk, E., Gonzalez, A., Delos Reyes, V., et al. (2021). Molnupiravir for Oral Treatment of Covid-19 in Nonhospitalized Patients. *New Engl. J. Med.* 386 (6), 4–9. doi:10.1056/NEJMoa2116044

Jeffers, S. A., Tusell, S. M., Gillim-Ross, L., Hemmila, E. M., Achenbach, J. E., Babcock, G. J., et al. (2004). CD209L (L-SIGN) Is a Receptor for Severe Acute Respiratory Syndrome Coronavirus. *Proc. Natl. Acad. Sci. U S A.* 101 (44), 15748–15753. doi:10.1073/pnas.0403812101

Jehan, S., Zhong, C., Li, G., Zulqarnain Bakhtiar, S., Li, D., and Sui, G. (2020). Thymoquinone Selectively Induces Hepatocellular Carcinoma Cell Apoptosis in Synergism with Clinical Therapeutics and Dependence of P53 Status. *Front. Pharmacol.* 11, 555283. doi:10.3389/fphar.2020.555283

Karim, S. S. A., and Karim, Q. A. (2021). Omicron SARS-CoV-2 Variant: a New Chapter in the COVID-19 Pandemic. *The Lancet* 398 (10317), 2126–2128. doi:10.1016/s0140-6736(21)02758-6

Khader, M., and Eckl, P. M. (2014). Thymoquinone: an Emerging Natural Drug with a Wide Range of Medical Applications. *Iran J. Basic Med. Sci.* 17 (12), 950–957.

Khare, C. P. (2004). *Indian Medicinal Plants*. 1 ed. New York: Springer-Verlag.

Kokoska, L., Havlik, J., Valterova, I., Sovova, H., Sajfrtova, M., and Jankovska, I. (2008). Comparison of Chemical Composition and Antibacterial Activity of Nigella Sativa Seed Essential Oils Obtained by Different Extraction Methods. *J. Food Prot.* 71 (12), 2475–2480. doi:10.4315/0362-028x-71.12.2475

Lamb, Y. N. (2020). Remdesivir: First Approval. *Drugs* 80 (13), 1355–1363. doi:10.1007/s40265-020-01378-w

Lantz, I., Glämsta, E. L., Talbäck, L., and Nyberg, F. (1991). Hemorphins Derived from Hemoglobin Have an Inhibitory Action on Angiotensin Converting Enzyme Activity. *FEBS Lett.* 287 (1-2), 39–41. doi:10.1016/0014-5793(91)80011-q

Leong, X. F., Choy, K. W., and Alias, A. (2021). Anti-Inflammatory Effects of Thymoquinone in Atherosclerosis: A Mini Review. *Front. Pharmacol.* 12, 758929. doi:10.3389/fphar.2021.758929

Letko, M., Marzi, A., and Munster, V. (2020). Functional Assessment of Cell Entry and Receptor Usage for SARS-CoV-2 and Other Lineage B Betacoronaviruses. *Nat. Microbiol.* 5 (4), 562–569. doi:10.1038/s41564-020-0688-y

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting Enzyme 2 Is a Functional Receptor for the SARS Coronavirus. *Nature* 426 (6965), 450–454. doi:10.1038/nature02145

Marzi, A., Gramberg, T., Simmons, G., Möller, P., Rennekamp, A. J., Krumbiegel, M., et al. (2004). DC-SIGN and DC-SIGNR Interact with the Glycoprotein of Marburg Virus and the S Protein of Severe Acute Respiratory Syndrome Coronavirus. *J. Virol.* 78 (21), 12090–12095. doi:10.1128/JVI.78.21.12090-12095.2004

Mohammed, A., and Islam, M. S. (2018). Spice-Derived Bioactive Ingredients: Potential Agents or Food Adjuvant in the Management of Diabetes Mellitus. *Front. Pharmacol.* 9, 893. doi:10.3389/fphar.2018.00893

Mostofa, A. G. M., Hossain, M. K., Basak, D., and Bin Sayeed, M. S. (2017). Thymoquinone as a Potential Adjuvant Therapy for Cancer Treatment: Evidence from Preclinical Studies. *Front. Pharmacol.* 8, 295. doi:10.3389/fphar.2017.00295

National. Center for Biotechnology Information (2021a). PubChem Compound Summary for CID 10281, Thymoquinone. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Thymoquinone (Accessed Dec 25, 2021).

National. Center for Biotechnology Information (2021b). PubChem Compound Summary for CID 12947, Hydroxychloroquine Sulfate. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Hydroxychloroquine-sulfate (Accessed Dec 25, 2021).

Newman, D. J., and Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79 (3), 629–661. doi:10.1021/acs.jnatprod.5b01055

Oliver, S. E., Gargano, J. W., Marin, M., Wallace, M., Curran, K. G., Chamberland, M., et al. (2020). The Advisory Committee on Immunization Practices' Interim Recommendation for Use of Pfizer-BioNTech COVID-19 Vaccine - United States, December 2020. *MMWR Morb Mortal Wkly Rep.* 69 (50), 1922–1924. doi:10.15585/mmwr.mm6950e2

Ong, Y. S., Saiful Yazan, L., Ng, W. K., Noordin, M. M., Sapuan, S., Foo, J. B., et al. (2016). Acute and Subacute Toxicity Profiles of Thymoquinone-Loaded Nanostructured Lipid Carrier in BALB/c Mice. *Int. J. Nanomedicine* 11, 5905–5915. doi:10.2147/IJN.S114205

Onifade, A. A., Jewell, A. P., and Adedeji, W. A. (2013). Nigella Sativa Concoction Induced Sustained Seroreversion in HIV Patient. *Afr. J. Tradit Complement. Altern. Med.* 10 (5), 332–335. doi:10.4314/ajtcam.v10i5.18

Onifade, A. A., Jewell, A. P., and Okesina, A. B. (2015). Seronegative Conversion of an HIV Positive Subject Treated with *Nigella sativa* and Honey. *Afr. J. Infect. Dis.* 9 (2), 47–50. doi:10.4314/ajid.v9i2.6

Pan, H., Pan, H., Peto, R., Henao-Restrepo, A. M., Preziosi, M. P., Sathiyamoorthy, V., et al. (2021). Repurposed Antiviral Drugs for Covid-19 - Interim WHO Solidarity Trial Results. *N. Engl. J. Med.* 384 (6), 497–511. doi:10.1056/NEJMoa2023184

Rabi, F. A., Al Zoubi, M. S., Kasasbeh, G. A., Salameh, D. M., and Al-Nasser, A. D. (2020). SARS-CoV-2 and Coronavirus Disease 2019: What We Know So Far. *Pathogens* 9 (3), 1–8. doi:10.3390/pathogens9030231

Rahman, M. T. (2020). Potential Benefits of Combination of Nigella Sativa and Zn Supplements to Treat COVID-19. *J. Herb Med.* 23, 100382. doi:10.1016/j.hermed.2020.100382

Salehi, B., Quispe, C., Imran, M., Ul-Haq, I., Živković, J., Abu-Reidah, I. M., et al. (2021). Nigella Plants – Traditional Uses, Bioactive Phytoconstituents, Preclinical and Clinical Studies. *Front. Pharmacol.* 12, 4-10. doi:10.3389/fphar.2021.625386

Salem, M. L., and Hossain, M. S. (2000). Protective Effect of Black Seed Oil from Nigella Sativa against Murine Cytomegalovirus Infection. *Int. J. Immunopharmacol* 22 (9), 729–740. doi:10.1016/s0192-0561(00)00036-9

Senger, M. R., Evangelista, T. C. S., Dantas, R. F., Santana, M. V. D. S., Gonçalves, L. C. S., de Souza Neto, L. R., et al. (2020). COVID-19: Molecular Targets, Drug Repurposing and New Avenues for Drug Discovery. *Mem. Inst. Oswaldo Cruz* 115, e200254. doi:10.1590/0074-02760200254

Shanmugam, M. K., Ahn, K. S., Hsu, A., Woo, C. C., Yuan, Y., Tan, K. H. B., et al. (2018). Thymoquinone Inhibits Bone Metastasis of Breast Cancer Cells through Abrogation of the CXCR4 Signaling Axis. *Front. Pharmacol.* 9, 1294. doi:10.3389/fphar.2018.01294

Shereen, M. A., Khan, S., Kazmi, A., Bashir, N., and Siddique, R. (2020). COVID-19 Infection: Origin, Transmission, and Characteristics of Human Coronaviruses. *J. Adv. Res.* 24, 91–98. doi:10.1016/j.jare.2020.03.005

Sommer, A. P., Försterling, H-D., and Naber, K. G. (2020). Thymoquinone: Shield and Sword against SARS-CoV-2. *Precision Nanomedicine* 3 (3), 541–548. doi:10.33218/001c.12984

Takai, S., Song, K., Tanaka, T., Okunishi, H., and Miyazaki, M. (1996). Antinociceptive Effects of Angiotensin-Converting Enzyme Inhibitors and an Angiotensin II Receptor Antagonist in Mice. *Life Sci.* 59 (21), PL331–6. doi:10.1016/0024-3205(96)00527-9

Ulasli, M., Gurses, S. A., Bayraktar, R., Yumrutas, O., Oztuzcu, S., Igci, M., et al. (2014). The Effects of Nigella Sativa (Ns), Anthemis Hyalina (Ah) and Citrus Sinensis (Cs) Extracts on the Replication of Coronavirus and the Expression of TRP Genes Family. *Mol. Biol. Rep.* 41 (3), 1703–1711. doi:10.1007/s11033-014-3019-7

U.S. Food and Drug Administration (2021a). Coronavirus (COVID-19) Update: FDA Authorizes First Oral Antiviral for Treatment of COVID-19 2021. Available from: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19.

U.S. Food and Drug Administration (2021b). Janssen COVID-19 Vaccine. [Available from: https://www.fda.gov/media/146303/download.

U.S. Food and Drug Administration (2021c). Moderna COVID-19 Vaccine. Available from: https://www.fda.gov/media/144636/download.

U.S. Food and Drug Administration (2021d). Pfizer-BioNTech COVID-19 Vaccine. Available from: https://www.fda.gov/media/144412/download.

U.S. Food and Drug Administration (2021e). Pfizer-BioNTech COVID-19 Vaccine;Common Side Effects. Available from: https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/pfizer-biontech-covid-19-vaccine.

Vaillancourt, F., Silva, P., Shi, Q., Fahmi, H., Fernandes, J. C., and Benderdour, M. (2011). Elucidation of Molecular Mechanisms Underlying the Protective Effects of Thymoquinone against Rheumatoid Arthritis. *J. Cel Biochem* 112 (1), 107–117. doi:10.1002/jcb.22884

Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 181 (2), 281–e6. doi:10.1016/j.cell.2020.02.058

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and Chloroquine Effectively Inhibit the Recently Emerged Novel Coronavirus (2019-nCoV) *In Vitro*. *Cell Res* 30 (3), 269–271. doi:10.1038/s41422-020-0282-0

World Health Organization (2021a). WHO Coronavirus (COVID-19) Dashboard. Available from: https://covid19.who.int/.

World Health Organization (2021b). Middle East Respiratory Syndrome Coronavirus (MERS-CoV) 2016. Available from: https://www.who.int/health-topics/middle-east-respiratory-syndrome-coronavirus-mers#tab=tab_1.

Xu, X., Chen, P., Wang, J., Feng, J., Zhou, H., Li, X., et al. (2020). Evolution of the Novel Coronavirus from the Ongoing Wuhan Outbreak and Modeling of its Spike Protein for Risk of Human Transmission. *Sci. China Life Sci.* 63 (3), 457–460. doi:10.1007/s11427-020-1637-5

Yang, Z. Y., Huang, Y., Ganesh, L., Leung, K., Kong, W. P., Schwartz, O., et al. (2004). pH-Dependent Entry of Severe Acute Respiratory Syndrome Coronavirus Is Mediated by the Spike Glycoprotein and Enhanced by Dendritic Cell Transfer through DC-SIGN. *J. Virol.* 78 (11), 5642–5650. doi:10.1128/JVI.78.11.5642-5650.2004

Yao, T. T., Qian, J. D., Zhu, W. Y., Wang, Y., and Wang, G. Q. (2020). A Systematic Review of Lopinavir Therapy for SARS Coronavirus and MERS Coronavirus-A Possible Reference for Coronavirus Disease-19 Treatment Option. *J. Med. Virol.* 92 (6), 556–563. doi:10.1002/jmv.25729

Zou, X., Chen, K., Zou, J., Han, P., Hao, J., and Han, Z. (2020). Single-cell RNA-Seq Data Analysis on the Receptor ACE2 Expression Reveals the Potential Risk of Different Human Organs Vulnerable to 2019-nCoV Infection. *Front. Med.* 14 (2), 185–192. doi:10.1007/s11684-020-0754-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

MINI REVIEW
published: 04 May 2022
doi: 10.3389/fphar.2022.902626

Promising Role of Emodin as Therapeutics to Against Viral Infections

*Qingqing Shao[1], Tong Liu[1], Wenjia Wang[1], Tianli Liu[1], Ximing Jin[1] and Zhuo Chen[1,2]**

[1]Institute of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]Department of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Emodin is an anthraquinone derivative that is widely present in natural plants and has a wide spectrum of pharmacological effects, such as antibacterial, anti-inflammatory, anti-fibrotic and anticancer and so on. Through reviewing studies on antiviral effect of emodin in the past decades, we found that emodin exhibits ability of inhibiting the infection and replication of more than 10 viruses *in vitro* and *in vivo*, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), coxsackievirus B (CVB), hepatitis B virus (HBV), influenza A virus (IAV), SARS-CoV, viral haemorrhagic septicaemia rhabdovirus (VHSV), enterovirus 71 (EV71), dengue virus serotype 2 (DENV-2) and Zika virus (ZIKV). Therefore, this review aims to summarize the antiviral effect of emodin, in order to provide reference and hopes to support the further investigations.

Keywords: emodin, virus infection, HSV-2, HCMV (human cytomegalovirus), COVID-19

OPEN ACCESS

Edited by:
Iván J. Montenegro, Universidad de Valparaíso, Chile

Reviewed by:
Ilija Barukcic, Independent Researcher, Jever, Germany

****Correspondence:***
Zhuo Chen
chenz@tjh.tjmu.edu.cn

Specialty section:
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

Received: *23 March 2022*
Accepted: *20 April 2022*
Published: *04 May 2022*

Citation:
Shao Q, Liu T, Wang W, Liu T, Jin X and Chen Z (2022) Promising Role of Emodin as Therapeutics to Against Viral Infections. Front. Pharmacol. 13:902626. doi: 10.3389/fphar.2022.902626

INTRODUCTION

Emodin (1,3,8-trihydroxy-6-methylanthraquinone, C15H10O5) is an anthraquinone derivative, which has been identified in 17 families of natural plants (Zheng et al., 2021), including *Rheum palmatum*, *Polygonum cuspidatum*, *Polygonum multiflorum* (Ahn et al., 2016; Li et al., 2016), *Cassiae semen* (Yang et al., 2019), etc. These herbs have long been used as antibacterial, anti-inflammatory, anti-fibrotic and anticancer, anti-aging, anti-hyperlipidaemia, antidiabetic, neuroprotective, hepatoprotective, antioxidant, laxative and hypotensive activities and treatment of infection medicine in China (Peng et al., 2013; Lin L et al., 2015; Dong et al., 2017; Xiang et al., 2020). As a component of these medicinal materials, emodin also has the same medicinal effects for various diseases, including asthma, atopic dermatitis, osteoarthritis, diabetes and diabetic complications, atherosclerosis, Alzheimer's disease, hepatic disease, constipation and several types of cancers and so on (Zheng et al., 2014; Dong et al., 2016). In recent years, there has been increasing evidence indicating that emodin has good antiviral properties and is commonly used in the treatment and prevention of epidemics caused by viruses.

Abbreviations: HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; CVB, coxsackievirus B; HBV, hepatitis B virus; IAV, influenza A virus; VHSV, viral haemorrhagic septicaemia rhabdovirus; EV71, enterovirus 71; DENV-2, dengue virus serotype 2; ZIKV, Zika virus; ARDS, acute respiratory distress syndrome; SARS, severe acute respiratory syndrome; GH, genital herpes; MTD, mean time death; NPC, nasopharyngeal carcinoma; ROS, reactive-oxygen species; VMC, viral myocarditis; HFMD, Hand, Foot and Mouth Disease; EC50, 50% effective concentration; TLR, Toll-like receptor.

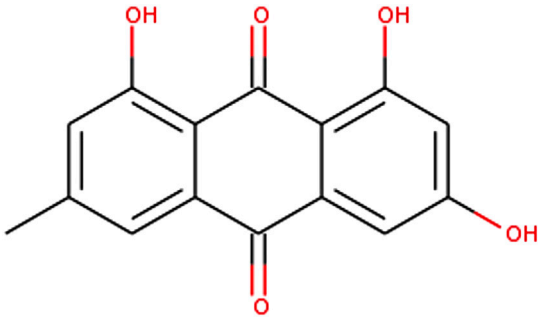


FIGURE 1 | Chemical structure of emodin.

Virus-infection diseases pose a significant treatment burden owing to their characteristics of recurrency and resulting complications. For example, seasonal influenza A virus (IAV) infection of patients with metabolic diseases may lead to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Bei et al., 2021), and HSV causes lifelong infections by establishing latency in neurons which promote recurrent disease and new infections when the immune system is weak (Ma et al., 2021). All of these features present difficulties for antiviral treatment, and we all know that the development of vaccines against many viruses is still in progress. Thus, there is a great need to explore novel drugs which can suppress different kinds of viruses and can antiviral infection *via* multiple perspectives. The efficacy of traditional Chinese medicine (TCM) in treating viral infectious diseases has been demonstrated in a number of public health events, including severe acute respiratory syndrome (SARS) in 2003 and coronavirus disease (COVID-19) in 2019. Similarly, herbal medicines and their active ingredients have been shown to have significant antiviral effects (Shao et al., 2021). There are some evidences (including in silico study and cell experiments) indicating that emodin might be effective therapy factor against SARS-CoV and COVID-19 (Ho et al., 2007; Schwarz et al., 2011; Batista et al., 2019; Basu et al., 2020; Caruso et al., 2020; Boozari and Hosseinzadeh, 2021; Nawrot-Hadzik et al., 2021; Rolta et al., 2021), suggesting that it may be an effective new antiviral agent. Through collection and collation of related literature, we found that emodin also has good antiviral effects on other viruses, so the aim of this review is to collect and present the current experimental evidence for the antivirus efficacy and underlying mechanisms of emodin, with a view to informing the development of new antiviral drugs.

BIOLOGICAL ACTIVITY OF EMODIN

As an anthraquinone derivative, emodin's basic chemical structure is an anthracene ring (tricyclic aromatic) with two ketone groups in position C9 and C10 (Monisha et al., 2016). The chemical structure of emodin is depicted in **Figure 1**. In general, emodin exerts its pharmacological activity at concentrations of a few tens of μM (Zheng et al., 2021). In pharmacokinetic experiments, after intragastric administration, emodin was quickly absorbed from the gastrointestinal tract and then rapidly metabolized to form its glucuronide, and the parent form of emodin was barely detectable *in vivo* (Shia et al., 2010). In rats' experiment, biliary excretion of emodin reached a maximum at approximately 6 h; 70% of the biliary activity was in the form of conjugated emodin. Urinary excretion was 18 and 22% at 24 and 72 h, respectively, free emodin and emodin acid were principal metabolites found in the pooled urine (Mueller et al., 1999; Monisha et al., 2016). Although emodin is known to be rapidly soluble in DMSO, ethanol or alkaline solutions, it is practically insoluble in water (Zheng et al., 2021). In addition to this, the significant first-pass elimination effect of emodin in the liver and intestine determines its low oral bioavailability (Teng et al., 2012). In recent years, a numerous of studies aimed to overcome these shortcomings and much efforts have been achieved. To date, various methods have been investigated to enhance solubility of emodin, including physical or chemical modifications and the use of solubilisers or surfactants. A thermo-reversible gel based on poloxamer is a very attractive formulation for topical administration through the body surface to reduce metabolism and increase the solubility of emodin. Verified that thermoreversible poloxamer gel containing emodin indeed improved emodin solubility Ban et al. (2017). In addition, emodin-nicotinamide (EM-NCT) cocrystal form could improved emodin's aqueous solubility, dissolution rate, and stability (Park et al., 2019). As for bioavailability, demonstrated that as a bioenhancer, piperine can enhance the bioavailability of emodin by inhibiting its glucuronidation Di et al. (2015). Furthermore, Two cocrystals of emodin (EM) with berberine chloride (BER), EM-BER 1) and 2 EM-BER-EtOH 2) has higher C-max and AUC compared with pure emodin, indicating higher bioavailability of that (Deng et al., 2018).

ANTIVIRAL ACTIVITY OF EMODIN

Anti-Herpes Simplex Virus Activities of Emodin

HSV including two subtypes, HSV-1 and HSV-2, which are large double-stranded DNA viruses of the Herpesviridae family and share 83% sequence homology in the protein coding region (Dolan et al., 1998). HSV infection is characterized by lifelong infection with intermittent clinical and subclinical viral reactivation, and it is the leading cause of genital ulcers around the world, named genital herpes (GH). Both HSV-1 and HSV-2 infection can cause GH, while HSV-2 is the main culprit, which infected 491.5 million people in 2016 worldwide (James et al., 2020). GH leading to great burden to individuals and to public health, since people infected with virus suffering painful, frequent genital lesions. On the other hand, HSV infection in early pregnancy can be transmitted through the placenta to the embryos, causing miscarriage, fetal malformations or permanent neurological damage, seriously affecting the quality of the birth population (Gupta et al., 2007). And the disruption of the mucosa caused by genital ulcers provides an entry point, thus facilitating HIV infection (Corey et al., 2004). At the same time, current

TABLE 1 | Anti-HSV activities of emodin.

References	Cell lines/ mice	Virus	Mechanism
Andersen et al. (1991)	Vero cell	HSV-1 HSV-2	Emodin inactivates 5×10^7 PFU/ml HSV-1 and 5×10^6 PFU/ml HSV-2
Cohen et al. (1996)	Vero cell	HSV-1	Emodin suppresses 105 PFU/ml HSV-1
Hsiang and Ho, (2008)	Vero cell	HSV-1	Emodin seduces 50% HSV-1 virus yields by inhibiting UL12 activity
Xiong et al. (2011)	HEp-2 cell	HSV-1 HSV-2	Emodin inhibits the replication of 100 TCID50/mL HSV-1 and HSV-2
Xiong et al. (2011)	BALB/c mice	HSV-1 HSV-2	Emodin shows good survival in HSV infected mice and clears HSV from brain, heart, liver and ganglia
Huang et al. (2021)	BALB/c mice	HSV-1	Emodin inhibits the inflammatory response in the brain of mice with herpes virus encephalitis by inhibiting TLR3 expression

treatment has not yet been able to completely eliminate latent HSV infection from the body, resulting in recurrence of the disease, which has a significant impact on the physical and mental health of patients and their quality of life. HSV-1 mainly causes cold sores on the lips, occasionally causes corneal lesions, and can also spread to the central nervous system causing serious diseases (Shukla and Valyi-Nagy, 2022). Antiviral drugs that target HSV viral DNA polymerase neither eradicate latent virus nor decrease the risk, frequency or severity of relapse. Meanwhile, vaccines against HSV are also still in progress and no breakthrough has been made. Therefore, it is essential to develop new antiviral drugs.

It has long been known that emodin shows antiviral effect on HSV-1 and HSV-2 (**Table 1**). In Vero cells, 10 μg/ml emodin were able to inactivate 5×10^7 PFU/ml HSV-1 and 5×10^6 PFU/ml HSV-2 (Andersen et al., 1991), and 2 μg/ml emodin could completely suppress 10^5 PFU/ml HSV-1 (Cohen et al., 1996), and 21.5 ± 4.4 μM was sufficient to reduce 50% HSV-1 (30 PFU) virus yields without cytotoxic effect (Hsiang and Ho, 2008). While in HEp-2 (human laryngeal carcinoma) cells, emodin inhibited the replication of 100 TCID50/mL HSV-1 and HSV-2, with concentration of 50 μg/ml on HSV-1, and 25 μg/ml on HSV-2 (Xiong et al., 2011).

In *in vivo* experiments, compared with the acyclovir-treated BALB/c mice, the 6.7 g/kg/day emodin group showed good survival in both HSV subtype-infected mice and longer mean time death (MTD) in HSV-1-infected mice. At the same time, emodin could clear HSV from brain, heart, liver and ganglia effectively (Xiong et al., 2011). 0.6 mg of emodin given to the HSV-1 infected male BALB/c mice daily for five consecutive days can effectively inhibit the inflammatory response in the brain of mice with herpesvirus encephalitis, the mRNA expressions of TLR3, TRIF, TRADD, TRAF6, traf3, p38, NEMO, and IRF3 are decreased, and the expressions of IL-6, TNF-α and IFN-β are decreased, indicating that emodin could inhibit the inflammatory response in the brain of mice with herpes virus encephalitis by inhibiting TLR3 expression (Huang et al., 2021).

The alkaline nuclease encoded by the UL12 gene of HSV-1 has endonuclease and exonuclease activities under alkaline pH conditions. Although UL12 is not required for either viral DNA synthesis or packaging, UL12 is required for efficiency of these processes. Therefore, HSV-1 UL12 can be a new target for anti-herpes virus drugs. C-Y Hsiang and T-Y Ho found that emodin could reduce the virus plaque formation in Vero cells by inhibiting UL12 activity, and the inhibitory effect may result from the interaction between emodin and critical catalytic amino acid residues of UL12 by docking analysis (Hsiang and Ho, 2008).

Anti-Human Cytomegalovirus Activities of Emodin

HCMV is a very common herpes virus that infect a high percentage of the world's population. After initial infection, HCMV is latent in the infected cells, thus causing lifelong infection, and usually without obvious clinical symptoms (Goodrum et al., 2012). However, HCMV infection (primary or (re)infection and reactivation) in immunocompromised individuals (e.g., HIV-infected persons, transplant recipients or children with congenital infection) can lead to serious complications (Deng et al., 2021). In addition, HCMV modulates the host immune response and promotes the modification of non-coding RNA and regulatory proteins, leading to an immunosuppressive tumor microenvironment. HCMV can also contribute to tumor survival by affecting cell proliferation and survival, invasion, immune evasion, immunosuppression and the production of angiogenic factors. Therefore, HCMV infection strongly associated with the development of tumors (El Baba and Herbein, 2021). Drug therapy currently approved for the treatment of systemic CMV infection has limited efficacy due to dose-limiting toxicity, and long-term treatment often results in drug resistance. Therefore, more effective and less toxic therapies are urgently needed to combat CMV infection (Alam et al., 2015). Current studies on the treatment of HCMV with emodin are all *in vitro* and the most commonly used cell line is human lung fibroblasts (MRC-5) cells (**Table 2**). Emodin showed antiviral activity against HCMV strain AD-169 ($10^{6.6}$ PFU/ml) with 4.1 μM EC50 and 9.6 μM IC50. At the same time, emodin could effectively inhibit a ganciclovir resistant strain C8805-37 with 3.7 μM EC50 and 12.6 μM IC50 (Barnard et al., 1992). For strain AD169 with an MOI of 0.8, emodin could reduce the infectious yield with an EC50 of 4.9 μM (Alam et al., 2015). These evidences suggest that emodin has the

TABLE 2 | Anti-HCMV activities of emodin.

References	Cell lines/ mice	Virus	Mechanism
Barnard et al. (1992)	MRC-5 cells	strain AD-169 strain C8805-37	Emodin shows antiviral activity against strain AD-169 with 4.1 μM EC50 and 9.6 μM IC50 and inhibits strain C8805-37 with 3.7 μM EC50 and 12.6 μM IC50
Alam et al. (2015)	MRC-5 cells	strain AD169	Emodin reduces the infectious yield with an EC50 of 4.9 μM

potential to combat HCMV infection, but more validation and *in vivo* experiments are needed.

Anti-Epstein-Barr Virus Activities of Emodin

EBV is a gamma human herpesvirus that mainly infects B-cells and epithelial cells. EBV infection is the most common and persistent viral infection in humans, with approximately 95% of the world's population remaining asymptomatic throughout their lives (Young et al., 2016). A small proportion of people infected with EBV present with infectious mononucleosis, which can cause persistent fatigue for up to 6 months and lead to serious neurological, hematological or hepatic complications. EBV was also the first human oncovirus to be identified and it is closely associated with several lymphomas and epithelial cancers especially in immunocompromised individuals, such as Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma (NPC) (Cui and Snapper, 2021). In general, the utilizing of existing antiviral compounds is limited by toxic side effects, poor oral bioavailability and the risk of the emergence of resistant viral strains. There is a need to develop new drugs against EBV and virus-related diseases.

Replication of EBV plays an important role in the pathogenesis of NPC, and the relapse and metastasis in NPC patients remain major causes of mortality. Therefore, inhibition of EBV reactivation is now being considered as a goal for the therapy of NPC. As a natural product, emodin has the effect of anti-virus as well as anti-tumor. *In vitro* and *in vivo* studies have shown that emodin inhibits the expression of EBV lytic proteins Zta, Rta, EAD, and DNase and blocks virion production by repressing the transcription of EBV immediate early genes (Yiu et al., 2014; Wu et al., 2019). At the same time, emodin inhibits the tumorigenic properties induced by repeated EBV reactivation, including micronucleus formation, cell proliferation, migration, and matrigel invasiveness (Wu et al., 2019).

In addition, found that the cell proliferation of Burkitt's lymphoma-derived Raji cells, which are EBV-positive cells, could be suppressed by the Polygonum cuspidatum ethyl acetate subfraction containing emodin (F3a) *via* increasing the intracellular reactive-oxygen species (ROS), activating the apoptosis-related proteins, and increasing the apoptosis percentage Yiu et al. (2021). These results mean that emodin may be a therapeutic drug for EBV-related tumors. Apart from this, EBV nuclear antigen EBNA1, a dimeric protein, which can bind to EBV genome sequences to initiate the process of DNA synthesis, is a potential therapeutic target for the treatment of EBV infection. Molecular docking revealed emodin bound to EBNA1 with high affinities, means that emodin may against HBV infection by inhibiting EBNA1 (Jakhmola et al., 2021). The above mechanisms are summarized in **Table 3**.

Anti-Coxsackievirus B Activities of Emodin

Coxsackieviruses are a group of envelope-free, orthotropic, single-stranded RNA viruses belonging to the small ribonucleic acid virus family of human enterovirus species (Simmonds et al., 2020). Coxsackieviruses are divided into group A (CVA) and group B (CVB). CVBs contain six virus types, CVB1-CVB6, which are common human pathogens associated with a wide range of diseases from gastrointestinal disorders to aseptic meningitis, myocarditis and pancreatitis, particularly in infants and children (Liu and Luo, 2021). Although the structure, molecular biology and associated pathophysiological mechanisms of CVB have been extensively studied, specific inhibitors have not yet been identified and applied to clinical studies. Therefore, research into natural or synthetic compounds is constantly ongoing in order to find suitable candidates for antiviral effectively (Hamdi et al., 2021). Several *in vitro* and *in vivo* studies showed anti-CVB activity of emodin (**Table 4**).

CVB3 is a primary causal agent of viral myocarditis (VMC). Zhang et al. explored the role and mechanism of anti-CVB3 of emodin through three cell lines and in A/J mice. CVB3 infected the immortalized human cardiomyocytes at an MOI of 20 for 24 h, the HeLa cells at an MOI of 10 for 5 h, and the HL-1 cells at an MOI of 10 for 14 h respectively and treated with 20 μM emodin at the same time. *In vivo*, 4-week-old male A/J mice were treated by 40 mg/kg emodin before 5×10^4 pfu CVB3 infection, and the duration of administration lasts for 5 days. The results showed that emodin could inhibit CVB3 replication *in vitro* and in mice through multiple pathways of viral protein translation inhibition. Firstly, emodin suppressed translation initiation of ribosomal protein L32 *via* inhibiting Akt/mTOR (mammalian target of rapamycin) signaling and activating 4EBP1 (eukaryotic initiation factor 4R-binding protein 1). Secondly, emodin inhibited CVB3 VP1 (viral protein 1) synthesis by regulating Akt/mTORC1/p70S6K (p70 S6 kinase), ERK1/2 (extracellular signal-regulated kinase 1/2)/p90RSK (p90 ribosomal S6 kinase) and Ca^{2+}/calmodulin. During this process, eEF2K is a major factor mediating cross-talk of signaling cascades which verified by inhibiting eEF2K with siRNA overexpression or inhibitor A484954. The above mechanisms ware also validated by overexpression and inhibition of Akt (Zhang HM et al., 2016). Apart from this, emodin could decrease the HW/BW ratio, myocardial

TABLE 3 | Anti-EBV activities of emodin.

References	Cell lines/mice	Virus	Mechanism
Wu et al. (2019)	EBV-infected NPC cell lines NA and HA; SCID mice	EBV	Emodin inhibits the tumorigenic properties induced by repeated EBV reactivation, including micronucleus formation, cell proliferation, migration, and matrigel invasiveness
Yiu et al. (2014)	P3HR1 cells	EBV	Emodin inhibits the expression of EBV lytic proteins Zta, Rta, EAD, and Dnase, and blocks virion production by repressing the transcription of EBV immediate early genes
Yiu et al. (2021)	The Burkitt's lymphoma-derived Raji cells	EBV	Polygonum cuspidatum ethyl acetate subfraction containing emodin (F3a) can increases the intracellular ROS, activats the apoptosis-related proteins, and increases the apoptosis percentage

TABLE 4 | Anti-CVB activities of emodin.

References	Cell lines/mice	Virus	Mechanism
Zhang H. M. et al. (2016)	HeLa cells HL-1 cells A/J mice	CVB3	Emodin inhibits viral replication through impairing translational machinery and suppression of viral translation elongation
Lin et al. (2019)	BALB/c mice	CVB3	Emodin decreases the HW/BW ratio, myocardial pathological score, the myocardial MDA level and serum cTnI and BNP levels, the TNF-alpha and IL-6 levels, and increase the myocardial SOD level of VMC mice
Zhang Y. F. et al. (2016)	BALB/c mice	CVB3	Emodin inhibits TLR4 and P38MAPK expression, thereby protecting cardiac tissue
Ding (2020)	mouse BV2 microglia BALB/c mice	CVB3	Emodin inhibits the TLR3 pathway, decreasing levels of TLR3 protein, IL-6, NF-κB and IFN-β, as well as alleviates pathology of virus infected mice
Liu et al. (2013)	Hep-2 cells BALB/c mice	CVB4	Emodin reduces CVB4 entry and replication on Hep-2 cells, increases survival rate, body weight and MTD of CVB4 infected mice, and decreases myocardial virus titers and pathologic scores/lesions, as well as inhibits CVB4-induced apoptosis *in vitro* and *in vivo*
Liu Z. et al. (2015)	HEp-2 cells	CVB5	Emodin could inhibit the replication of CVB5 by regulating cytokine (IFN-γ and TNF-α) expression

pathological score, the myocardial MDA level and serum cTnI and BNP levels, TNF-α and IL-6 levels, and increase the myocardial SOD level of VMC mice (Lin et al., 2019), while attenuating cardiac injury in CVB3-infected BALB/c mice by inhibiting TLR4 and P38MAPK expression, thereby protecting cardiac tissue (Zhang YF et al., 2016).

In addition to myocarditis, CVB3 is also the main culprit in Hand, Foot and Mouth Disease (HFMD), and a high prevalence of CVB3 has been repeatedly found in Chinese patients with HFMD (Ding et al., 2020). Meanwhile, encephalitis in HFMD is a serious threat to children's health and life. Ding et al. explored the effect of emodin on CVB3 infection caused HFMD using mouse BV2 microglia as a cellular model. The results suggested that emodin could inhibit the TLR3 pathway which can recognize virus and initiate innate immune responses to suppress viral infection. Then using three-week-old male BALB/c mice to establish animal model by injecting 20 μl of virus solution directly into the brain, then 80 mg/kg, 40 mg/kg and 20 mg/kg emodin daily intragastrical for 7 days. Emodin displayed notable effects on alleviating pathology, decreasing TLR3 protein in brain tissues and expression levels of IL-6, NF-κB, IFN-β in serum, and the 80 mg/kg emodin group has the best effect of anti-inflammatory (Ding et al., 2020), which suggested emodin may be effective agent to HFMD.

Besides CVB3, emodin also exhibits anti-viral effect to CVB4. CVB4 can cause a broad range of diseases, including myocarditis, pancreatitis, hepatitis, meningoencephalitis, gastroenteritis, necrotizing enterocolitis, pneumonia and even death in neonates. Emodin can reduce CVB4 entry and replication on Hep-2 cells in a concentration- and time-dependent manner, and the 4–6-week BALB/c mice orally treated with different dosages of emodin displayed a dose dependent increase of survival rate, body weight and prolonged MTD, accompanied by significantly decreased myocardial virus titers and pathologic scores/lesions. Moreover, emodin could inhibit CVB4-induced apoptosis *in vitro* and *in vivo* which represents the cardio protection of emodin (Liu et al., 2013).

Coxsackievirus B5 (CVB5) is one of the five most common types of enterovirus (EV) and is associated with encephalitis and myocarditis in immunocompromised children and central nervous system disease in the elderly (Zhong et al., 2009). Therefore, the search for evidence and mechanisms of emodin against CVB5 infection is also warranted. Liu et al. found that emodin had potent inhibitory activities against 100 TCID50/ml CVB5 in HEp-2 cells, with the 50% effective concentration (EC50) ranging from 13.06 to 14.27 μmol/L. It acted as a biological synthesis inhibitor against CVB5 in a concentration- and time-dependent manner. Moreover, emodin could decrease the mRNA expression of IFN-α but enhance TNF-γ expression significantly compared to the model group, suggesting that emodin could inhibit the replication of CVB5 by regulating cytokine (IFN-γ and TNF-α) expression (Liu Z et al., 2015).

Anti-Hepatitis B Virus Activities of Emodin

Chronic HBV infection is a major global health problem and an important cause of complications, including liver failure, development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Liaw and Chu, 2009). There are

TABLE 5 | Anti-HBV activities of emodin.

References	Cell lines/mice	Virus	Mechanism
Dang et al. (2006)	HepG2.2.15 cells	HBV	Emodin inhibits the extracellular HBV DNA, HBsAg and HBeAg, and the inhibition efficacy increases as time passed and peaked after 9 days of treatment
Dang et al. (2009)	HBV transgenic mice	HBV	Emodin and APS Co-treatment could decrease HBV DNA levels, the contents of HBsAg, HBeAg and HBcAg

approximately two billion people worldwide infected by HBV, resulting in approximately one million deaths each year (Polaris Observatory Collaborators, 2018; Chien and Liaw, 2022). The population of infection with HBV is still increasing even though vaccination can prevent HBV, and the currently recognized effective antiviral treatment drugs have disadvantages such as high adverse effects and high prices. In recent years, researchers have been investigated the antiviral activity of various products from plants in order to find effective alternative medicines (Chai et al., 2019).

Emodin may be a new treatment for HBV infection (**Table 5**). The inhibitory effect of emodin on HBV DNA replication and HBsAg secretion is time- and concentration-dependent *in vitro*. The human hepatoma G2.2.15 (HepG2.2.15) cell line stably expresses HBV particles. After exposure to three different concentrations of emodin (12.5 mg/L, 25 mg/L and 50 mg/L) for 3, 6, and 9 days in HepG2.2.15 cells, the inhibition rates of extracellular HBV DNA, HBsAg, and HBeAg were significantly increased. And the inhibition rates increased as time passed and peaked after 9 days of treatment, and 50 mg/L emodin treatment exhibited the best antiviral effect (Dang et al., 2006). In addition, in HBV transgenic mice, 57.59 mg/kg/d emodin and 287.95 mg/kg/d astragalus polysaccharide (APS) Co-treatment for 3 weeks could significantly decrease HBV DNA levels, the contents of HBsAg, HBeAg and HBcAg when compared with control group, which means emodin may function as a complementary factor in the treatment of HBV infection (Dang et al., 2009).

The HBV core protein contains 183 residues that self-assemble to form the viral capsid. In infected cells, the HBV core protein regulates nearly every step of the viral replication process. It is an excellent target for the development of novel, virus-selective and effective antiviral drugs to improve treatment options for HBV infectious diseases. Emodin derivatives showed promising inhibitory characteristics to orientation of capsid assembly by core proteins using molecular docking and dynamic simulation, indicating that viral replication would be inhibited by emodin derivatives (Firdayani et al., 2018).

Anti-Influenza A Virus Activities of Emodin

IAV belongs to one of the three influenza genera (including A, B, and C) of the family Orthomyxoviridae and is a segmented, negative-sense ribonucleic acid virus (Atkin-Smith et al., 2018). Influenza infections have a serious impact on health worldwide, causing almost 3–5 million cases of critical illness and approximately 250,000–500,000 deaths worldwide each year (Gui and Chen, 2021). Currently, the classical antiviral drugs (amantadine, ribavirin or oseltamivir) are widely used in the clinic. Nevertheless, new effective drugs are still needed because of potential toxicities, rapid emergence of antiviral resistance and high prices of existing drugs (Zhi et al., 2019).

Emodin is a highly promising anti-IVA agent (**Table 6**). The current studies on emodin for IAV are based on A549 lung cancer cells, and A549 cells are the most common used cell type of researching anti-IVA effect of emodin. For IAV (PR8), the mechanism of the anti-viral effect of emodin in A549 cells was to activate PPARα/γ and AMPK, decrease fatty acid biosynthesis, and increase intracellular ATP levels. In order to further prove that PPARα/γ and AMPK are the key proteins that inhibit PR8 replication, inhibitors were used, and it was found that the inhibitors of PPARα/γ and AMPK weakened the antiviral effect of emodin (Bei et al., 2021). For H1N1, inhibiting the expression of hemagglutinin and neuraminidase, increasing the expression of interferon beta (IFN-β) through Toll-like receptor 9 (TLR9) are the key ways to inhibit the replication of it (Lin CJ et al., 2015). In *vivo* studies, emodin also significantly protected mice from IAV infection and pneumonia (Bei et al., 2021). Meanwhile, though experiments on A549 cells and C57BL/6J mice, DAI et al. comprehensively explored the mechanism of emodin's antiviral effect to H1N1. The results showed that emodin could significantly inhibit IAV (ST169, H1N1) replication, reduce the expressions of TLR2/3/4/7, MyD88 and TRAF6, decrease phosphorylation of p38/JNK MAPK and nuclear translocation of NF-kB p65, those are crucial to H1N1 infection and replication. Nrf2 signaling pathway is a classic anti-inflammatory pathway, and activation of which can inhibit the activation of TLR pathways. Suppression of Nrf2 *via* siRNA markedly blocked the inhibitory effects of emodin on TLR4, p38/JNK, and NF-kB pathways and on IAV-induced production of IL-1, IL-6 and expression of IAV M2 protein. Meanwhile, Nrf2 signaling pathway is also essential to anti-oxidate. Therefore, emodin could activate the Nrf2 pathway and decreased ROS levels, increased GSH levels and GSH/GSSG ratio, and upregulated the activities of SOD, GR, CAT and GSH-Px. Similarly, Dai et al. also clarified that emodin has a therapeutic effect on H1N1infected acute lung injury (ALI) mice. Emodin increased the survival rate of mice, reduced lung edema, pulmonary viral titer and inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-a), and improved lung histopathological changes (Dai et al., 2017).

Anti-SARS-CoV Activities of Emodin

Coronaviruses belong to the family of Coronaviridae (subfamily Coronaviridae), whose members infect a wide range of hosts, resulting symptoms and illnesses ranging from the common cold to severe and ultimately fatal diseases such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and currently

TABLE 6 | Anti-IVA activities of emodin.

References	Cell lines/mice	Virus	Mechanism
Bei et al. (2021)	A549 cells BALB/c mice	IAV (PR8)	Emodin activates PPARα/γ and AMPK, decrease fatty acid biosynthesis, and increases intracellular ATP levels. Emodin also protects mice from IAV infection and pneumonia
Lin et al. (2019)	A549 cells	H1N1	Emodin inhibits IVA replication *via* regulating TLR-9-induced IFN-β production
Dai et al. (2017)	A549 cells and C57BL/6J mice	H1N1	Emodin can inhibit IAV replication and influenza viral pneumonia by activating Nrf2 signaling and inhibiting TLR4, p38/JNK MAPK and NF-κB pathways

TABLE 7 | Anti-SARS-CoV activities of emodin.

References	Cell lines/mice	Virus	Mechanism
Ho et al. (2007)	Vero E6 cells	SARS-CoV	Emodin blocks the S protein and ACE2 interaction in a dose-dependent manner and inhibits the infectivity of S protein-pseudotyped retrovirus
Schwarz et al. (2011)	Rhabdomyosarcoma cells	SARS-CoV	Emodin inhibits the 3a ion channel
Nawort-Hadzik et al. (2021)	/	SARS-CoV-2	Emodin reveals over 50% inhibition of SARS-CoV-2 Mpro

coronavirus disease 2019 (COVID-19) (Dhama et al., 2020). These diseases pose a great threat to people's lives and health and has a huge impact on the global economy. To date, few measures are available to effectively treat COVID-19. Therefore, there is an urgent need for new drugs to combat the disease or for new treatments for these kinds of extremely dangerous coronavirus diseases (Yu et al., 2021).

In 2003, SARS resulted in progressive respiratory failure and death in close to 10% of infected individuals (Ksiazek et al., 2003), and studies showed that emodin exhibits anti-SARS-CoV potential (**Table 7**). SARS-CoV S protein is a large type I membrane glycoprotein projection from viral envelope, mutations in this gene dramatically affect the virulence, pathogenesis, and host cell tropism. While angiotensin-converting enzyme 2 (ACE2) was identified as a functional receptor for SARS-CoV (W. Li et al., 2003), suggesting that blocking the binding of the S protein with its cellular receptor can prevent virus entry. Recombinant SARS-CoV S protein and S protein pseudotyped retrovirus was used to explore the interaction between S protein and ACE2, it was found that S protein binds to ACE2 in a dose-dependent manner, while emodin blocked the binding of S protein to ACE2 in a dose dependent manner, with IC_{50} value of 200 μM. At the same time, emodin can also inhibit the infectivity of S protein-pseudotyped retrovirus to ACE2-expression Vero E6 cells. The above data suggest that emodin may be considered as a potential lead therapeutic agent in the treatment of SARS (Ho et al., 2007). Apart from this, SARS-CoV has an open reading frame, ORF-3a, that encodes an ion-osmotic channel in infected cells, and the activity of the 3a protein may affect viral release. Rhabdomyosarcoma cells (RD cells) were used to explore the effect of emodin on viral replication *via* the 3a protein. It was found that 100 μM emodin could inhibit the 3a ion channel of the coronaviruses SARS-CoV and HCoV-OC43 and the viral release of HCoV-OC43, with a K1/2 value of about 20 μM (Schwarz et al., 2011). These data demonstrated that S protein, ACE2 and ORF-3a may be the effective targets of emodin anti- SARS-CoV.

17 years later, in December 2019, a novel coronavirus, defined by the World Health Organization (WHO) as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in January 2020, re-emerged, known as COVID-19. The main protease (Mpro, also called 3CLpro) is the nonstructural proteins of the virus, and inhibition of this enzyme could prevent the replication of the virus. Found that emodin revealed over 50% inhibition of SARS-CoV-2 Mpro, suggesting the beneficial effects of emodin on COVID-19 Nawrot-Hadzik et al. (2021). In order to improve the activity of emodin against human coronavirus NL63 (HCoV-NL63) and also to generate a set of initial SAR guidelines, Monika et al. prepared emodin derivatives and found that halogenation of emodin can improve the antiviral activity of which. Moreover, the most active compound was the iodinated emodin analogue E_3I, the anti-HCoV-NL63 activity of which was comparable to that of remdesivir (Horvat et al., 2021). Of course, studies on emodin against COVID-19 to date are all based on *in vitro* experiments and there is limited discussion of its mechanisms, so more researches are needed to demonstrate the important role of emodin could play in this plague.

Other Viruses

In addition to the inhibitory effects of the common viral infections mentioned above, there is evidence that emodin may also exert antiviral effects against other viruses (**Table 8**). VHSV is a negative RNA enveloped virus. Emodin exhibited anti-VHSV-07.71 effect in epithelioma papulosum cyprini (EPC) cell cultures (Alves et al., 2004). Human EV71 is the causative agent for HFMD outbreaking in Asia. Emodin (29.6 μmol/L) effectively inhibited viral replication thus protecting MRC5 cells from EV71-induced cytopathic effects by inhibiting viral maturation and diminishing cell cycle arrest at S phase (Zhong et al., 2017). Apart from that, emodin exhibited significant prophylactic effects

TABLE 8 | Other virus.

References	Cell lines/mice	Virus	Mechanism
Alves et al. (2004)	epithelioma papulosum cyprini (EPC) cell	VHSV	Emodin exhibits anti-VHSV effect in epithelioma papulosum cyprini (EPC) cells
Zhong et al. (2017)	MRC5 cells	EV71	Emodin inhibits viral replication by inhibiting viral maturation and diminishing cell cycle arrest at S phase
Batool et al. (2018)	Baby hamster kidney (BHK-21) cells	DENV-2	Emodin exhibits prophylactic effects against dengue virus serotype 2 (DENV-2) infectivity treated before infection
Batista et al. (2019)	Vero E6 cells	ZIKV	Emodin reduces the infectivity of ZIKV approximately 83.3%

against DENV-2 (two doses, 45 and 90 PFU) infectivity treating before infection (Batool et al., 2018) and reduced the infectivity of ZIKV approximately 83.3% (from 7.8×10^3 PFU/ml to 1.3×10^3 PFU/ml) in Vero E6 cells (Batista et al., 2019).

TOXICITY OF EMODIN

Despite the wide range of pharmacological effects of emodin, we cannot ignore its side effects, especially the toxicity on organs. The liver is one of the main target organs in drug toxicology. Intracellular metabolomic analysis showed that emodin significantly disrupted cellular glutathione and fatty acid metabolism, and the level of emodin-cysteine adduct increased with increasing emodin concentrations, which suggest a cytotoxic effect of emodin on the metabolic pathways of human hepatocytes (Liu X et al., 2015). Meanwhile, Wang et al. investigated the nephrotoxicity of emodin by inducing HK-2 cells (a human proximal tubular epithelial cell line) apoptosis *via* mitochondrial pathway (Wang et al., 2015). In addition to this, emodin has been reported to be reproductively toxic and genotoxic (Li et al., 2010; Oshida et al., 2011; Luo et al., 2015). At the same time, anthraquinones (AQ) such as emodin and chrysophanol exerts the chronic effects on organ weights and over 30 haematological, biochemical and histological parameters, and irreversible pathological changes can be caused at very high doses (4000 mg/kg) (Islam et al., 2015). Therefore, long-term high doses administration should be completely avoided during pregnancy, and it is essential to improve bioavailability and thus reduce the dose administered to achieve some reduction in drug toxicity.

CONCLUSION

Viral infections are characterized by variables and the possibility of lifelong latency, and easy to develop resistance to antiviral drugs. In addition to inhibiting viral replication itself, the treatment of various related diseases caused by virus is also a major challenge. As a common natural product, emodin and its antiviral efficacy and mechanism have been explored and studied for about 30 years. The current researches on emodin are all based on cells and mice, and the viruses that can be suppressed by emodin including HSV-1, HSV-2, HCMV, HBV, CVB (type 3–5), EBV, IVA, SARS-CoV, VHSV, EV71, DENV-2, and ZIKV. The specific anti-virus mechanisms of emodin involved vary from virus to virus, while the common denominator is the ability to suppress the inflammatory response caused by viral infection, such as decreasing the expression of IL-6, TNF-α and IFN-β. Emodin not only exhibits the effect of inhibiting replication and infection of various viruses, but also has a certain recovery effect on tissue damage caused by virus infection. And some of its effects are even comparable to that of the commonly used antiviral drug acyclovir.

The current evidence is enough to show that emodin is a promising antiviral drug, but it is undeniable that more research is needed in the future to explain its antiviral mechanism, as well as human experiments to verify its safety and efficacy and possibility of treating viral infectious diseases as a single agent or in combination with other drugs.

AUTHOR CONTRIBUTIONS

QS: conceptualization, methodology, writing—original draft. TL and WW: software. TL and XJ: project administration. ZC: funding acquisition, writing—review and editing.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 81874483).

REFERENCES

Ahn, S. M., Kim, H. N., Kim, Y. R., Choi, Y. W., Kim, C. M., Shin, H. K., et al. (2016). Emodin from Polygonum Multiflorum Ameliorates Oxidative Toxicity in HT22 Cells and Deficits in Photothrombotic Ischemia. *J. Ethnopharmacol.* 188, 13–20. doi:10.1016/j.jep.2016.04.058

Alam, Z., Al-Mahdi, Z., Zhu, Y., McKee, Z., Parris, D. S., Parikh, H. I., et al. (2015). Anti-cytomegalovirus Activity of the Anthraquinone Atanyl Blue PRL. *Antivir. Res.* 114, 86–95. doi:10.1016/j.antiviral.2014.12.003

Alves, D. S., Pérez-Fons, L., Estepa, A., and Micol, V. (2004). Membrane-related Effects Underlying the Biological Activity of the Anthraquinones Emodin and Barbaloin. *Biochem. Pharmacol.* 68 (3), 549–561. doi:10.1016/j.bcp.2004.04.012

Andersen, D. O., Weber, N. D., Wood, S. G., Hughes, B. G., Murray, B. K., and North, J. A. (1991). *In Vitro* virucidal Activity of Selected Anthraquinones and Anthraquinone Derivatives. *Antivir. Res.* 16 (2), 185–196. doi:10.1016/0166-3542(91)90024-l

Atkin-Smith, G. K., Duan, M., Chen, W., and Poon, I. K. H. (2018). The Induction and Consequences of Influenza A Virus-Induced Cell Death. *Cell Death Dis.* 9 (10), 1002. doi:10.1038/s41419-018-1035-6

Ban, E., Park, M., Jeong, S., Kwon, T., Kim, E. H., Jung, K., et al. (2017). Poloxamer-Based Thermoreversible Gel for Topical Delivery of Emodin: Influence of P407 and P188 on Solubility of Emodin and its Application in Cellular Activity Screening. *Molecules* 22 (2), 246. doi:10.3390/molecules22020246

Barnard, D. L., Huffman, J. H., Morris, J. L., Wood, S. G., Hughes, B. G., and Sidwell, R. W. (1992). Evaluation of the Antiviral Activity of Anthraquinones, Anthrones and Anthraquinone Derivatives against Human Cytomegalovirus. *Antivir. Res.* 17 (1), 63–77. doi:10.1016/0166-3542(92)90091-i

Basu, A., Sarkar, A., and Maulik, U. (2020). Molecular Docking Study of Potential Phytochemicals and Their Effects on the Complex of SARS-CoV2 Spike Protein and Human ACE2. *Sci. Rep.* 10 (1), 17699. doi:10.1038/s41598-020-74715-4

Batista, M. N., Braga, A. C. S., Campos, G. R. F., Souza, M. M., Matos, R. P. A., Lopes, T. Z., et al. (2019). Natural Products Isolated from Oriental Medicinal Herbs Inactivate Zika Virus. *Viruses* 11 (1), 49. doi:10.3390/v11010049

Batool, R., Aziz, E., Mahmood, T., Tan, B. K. H., and Chow, V. T. K. (2018). Inhibitory Activities of Extracts of Rumex Dentatus, Commelina Benghalensis, Ajuga Bracteosa, Ziziphus Mauritiana as Well as Their Compounds of Gallic Acid and Emodin against Dengue Virus. *Asian Pac J. Trop. Med.* 11 (4), 265–271. doi:10.4103/1995-7645.231466

Bei, Y., Tia, B., Li, Y., Guo, Y., Deng, S., Huang, R., et al. (2021). Anti-influenza A Virus Effects and Mechanisms of Emodin and its Analogs via Regulating PPARα/γ-AMPK-SIRT1 Pathway and Fatty Acid Metabolism. *Biomed. Res. Int.* 2021, 9066938. doi:10.1155/2021/9066938

Boozari, M., and Hosseinzadeh, H. (2021). Natural Products for COVID-19 Prevention and Treatment Regarding to Previous Coronavirus Infections and Novel Studies. *Phytother. Res.* 35 (2), 864–876. doi:10.1002/ptr.6873

Caruso, F., Rossi, M., Pedersen, J. Z., and Incerpi, S. (2020). Computational Studies Reveal Mechanism by Which Quinone Derivatives Can Inhibit SARS-CoV-2. Study of Embelin and Two Therapeutic Compounds of Interest, Methyl Prednisolone and Dexamethasone. *J. Infect. Public Health* 13 (12), 1868–1877. doi:10.1016/j.jiph.2020.09.015

Chai, Y., Kan, L., and Zhao, M. (2019). Enzymatic Extraction Optimization, Anti-HBV and Antioxidant Activities of Polysaccharides from Viscum Coloratum (Kom.) Nakai. *Int. J. Biol. Macromol.* 134, 588–594. doi:10.1016/j.ijbiomac.2019.04.173

Chien, R. N., and Liaw, Y. F. (2022). Current Trend in Antiviral Therapy for Chronic Hepatitis B. *Viruses* 14 (2). doi:10.3390/v14020434

Cohen, P. A., Hudson, J. B., and Towers, G. H. (1996). Antiviral Activities of Anthraquinones, Bianthrones and Hypericin Derivatives from Lichens. *Experientia* 52 (2), 180–183. doi:10.1007/bf01923366

Corey, L., Wald, A., Celum, C. L., and Quinn, T. C. (2004). The Effects of Herpes Simplex Virus-2 on HIV-1 Acquisition and Transmission: A Review of Two Overlapping Epidemics. *J. Acquir Immune Defic. Syndr.* 35 (5), 435–445. doi:10.1097/00126334-200404150-00001

Cui, X., and Snapper, C. M. (2021). Epstein Barr Virus: Development of Vaccines and Immune Cell Therapy for EBV-Associated Diseases. *Front. Immunol.* 12, 734471. doi:10.3389/fimmu.2021.734471

Dai, J. P., Wang, Q. W., Su, Y., Gu, L. M., Zhao, Y., Chen, X. X., et al. (2017). Emodin Inhibition of Influenza A Virus Replication and Influenza Viral Pneumonia via the Nrf2, TLR4, p38/JNK and NF-kappaB Pathways. *Molecules* 22 (10), 1754. doi:10.3390/molecules22101754

Dang, S. S., Jia, X. L., Song, P., Cheng, Y. A., Zhang, X., Sun, M. Z., et al. (2009). Inhibitory Effect of Emodin and Astragalus Polysaccharide on the Replication of HBV. *World J. Gastroenterol.* 15 (45), 5669–5673. doi:10.3748/wjg.15.5669

Dang, S. S., Zhang, Z. G., Chen, Y. R., Zhang, X., Wang, B. F., Yuan, L. C., et al. (2006). Inhibition of the Replication of Hepatitis B Virus *In Vitro* by Emodin. *Med. Sci. Monit.* 12 (9), BR302–BR306.

Deng, Y., Zhang, Y., Huang, Y., Zhang, M., and Lou, B. (2018). Preparation, Crystal Structures, and Oral Bioavailability of Two Cocrystals of Emodin with Berberine Chloride. *Cryst. Growth Des.* 18 (12), 7481–7488. doi:10.1021/acs.cgd.8b01257

Deng, Z. L., Dhingra, A., Fritz, A., Götting, J., Münch, P. C., Steinbrück, L., et al. (2021). Evaluating Assembly and Variant Calling Software for Strain-Resolved Analysis of Large DNA Viruses. *Brief. Bioinform* 22 (3), bbaa123. doi:10.1093/bib/bbaa123

Dhama, K., Khan, S., Tiwari, R., Sircar, S., Bhat, S., Malik, Y. S., et al. (2020). Coronavirus Disease 2019-COVID-19. *Clin. Microbiol. Rev.* 33 (4), e00028-20. doi:10.1128/CMR.00028-20

Di, X., Wang, X., Di, X., and Liu, Y. (2015). Effect of Piperine on the Bioavailability and Pharmacokinetics of Emodin in Rats. *J. Pharm. Biomed. Anal.* 115, 144–149. doi:10.1016/j.jpba.2015.06.027

Ding, Y., Xu, J., Cheng, L. B., Huang, Y. Q., Wang, Y. Q., Li, H., et al. (2020). Effect of Emodin on Coxsackievirus B3m-Mediated Encephalitis in Hand, Foot, and Mouth Disease by Inhibiting Toll-like Receptor 3 Pathway *In Vitro* and *In Vivo*. *J. Infect. Dis.* 222 (3), 443–455. doi:10.1093/infdis/jiaa093

Dolan, A., Jamieson, F. E., Cunningham, C., Barnett, B. C., and McGeoch, D. J. (1998). The Genome Sequence of Herpes Simplex Virus Type 2. *J. Virol.* 72 (3), 2010–2021. doi:10.1128/JVI.72.3.2010-2021.1998

Dong, X., Fu, J., Yin, X., Cao, S., Li, X., Lin, L., et al. (2016). Emodin: A Review of its Pharmacology, Toxicity and Pharmacokinetics. *Phytother. Res.* 30 (8), 1207–1218. doi:10.1002/ptr.5631

Dong, X., Fu, J., Yin, X., Yang, C., Zhang, X., Wang, W., et al. (2017). Cassiae Semen: A Review of its Phytochemistry and Pharmacology (Review). *Mol. Med. Rep.* 16 (3), 2331–2346. doi:10.3892/mmr.2017.6880

El Baba, R., and Herbein, G. (2021). Immune Landscape of CMV Infection in Cancer Patients: From "Canonical" Diseases toward Virus-Elicited Oncomodulation. *Front. Immunol.* 12, 730765. doi:10.3389/fimmu.2021.730765

Firdayani, F. F., Arsianti, A., Churiyah C, C., and Yanuar, A. (2018). Molecular Docking and Dynamic Simulation Studies of Benzoylated Emodin into HBV Core Protein. *Jyp* 10 (2), S20–S24. doi:10.5530/jyp.2018.2s.5

Goodrum, F., Caviness, K., and Zagallo, P. (2012). Human Cytomegalovirus Persistence. *Cell Microbiol.* 14 (5), 644–655. doi:10.1111/j.1462-5822.2012.01774.x

Gui, R., and Chen, Q. (2021). Molecular Events Involved in Influenza A Virus-Induced Cell Death. *Front. Microbiol.* 12, 797789. doi:10.3389/fmicb.2021.797789

Gupta, R., Warren, T., and Wald, A. (2007). Genital Herpes. *Lancet* 370 (9605), 2127–2137. doi:10.1016/S0140-6736(07)61908-4

Hamdi, A., Halouani, A., Aouf, I., Viaene, J., Marzouk, B., Kraiem, J., et al. (2021). Cytotoxicity and Antiviral Activities of Haplophyllum Tuberculatum Essential Oils, Pure Compounds, and Their Combinations against Coxsackievirus B3 and B4. *Planta Med.* 87 (10-11), 827–835. doi:10.1055/a-1538-5289

Ho, T. Y., Wu, S. L., Chen, J. C., Li, C. C., and Hsiang, C. Y. (2007). Emodin Blocks the SARS Coronavirus Spike Protein and Angiotensin-Converting Enzyme 2 Interaction. *Antivir. Res.* 74 (2), 92–101. doi:10.1016/j.antiviral.2006.04.014

Horvat, M., Avbelj, M., Durán-Alonso, M. B., Banjanac, M., Petković, H., and Iskra, J. (2021). Antiviral Activities of Halogenated Emodin Derivatives against Human Coronavirus NL63. *Molecules* 26 (22), 6825. doi:10.3390/molecules26226825

Hsiang, C. Y., and Ho, T. Y. (2008). Emodin Is a Novel Alkaline Nuclease Inhibitor that Suppresses Herpes Simplex Virus Type 1 Yields in Cell Cultures. *Br. J. Pharmacol.* 155 (2), 227–235. doi:10.1038/bjp.2008.242

Huang, Y., Li, X., Pan, C., Cheng, W., Wang, X., Yang, Z., et al. (2021). The Intervention Mechanism of Emodin on TLR3 Pathway in the Process of Central Nervous System Injury Caused by Herpes Virus Infection. *Neurol. Res.* 43 (4), 307–313. doi:10.1080/01616412.2020.1853989

Islam, R., Mamat, Y., Ismayil, I., Yan, M., Kadir, M., Abdugheny, A., et al. (2015). Toxicity of Anthraquinones: Differential Effects of Rumex Seed Extracts on Rat Organ Weights and Biochemical and Haematological Parameters. *Phytother. Res.* 29 (5), 777–784. doi:10.1002/ptr.5317

Jakhmola, S., Jonniya, N. A., Sk, M. F., Rani, A., Kar, P., and Jha, H. C. (2021). Identification of Potential Inhibitors against Epstein-Barr Virus Nuclear Antigen 1 (EBNA1): An Insight from Docking and Molecular Dynamic

Simulations. *ACS Chem. Neurosci.* 12 (16), 3060–3072. doi:10.1021/acschemneuro.1c00350

James, C., Harfouche, M., Welton, N. J., Turner, K. M., Abu-Raddad, L. J., Gottlieb, S. L., et al. (2020). Herpes Simplex Virus: Global Infection Prevalence and Incidence Estimates, 2016. *Bull. World Health Organ* 98 (5), 315–329. doi:10.2471/BLT.19.237149

Ksiazek, T. G., Erdman, D., Goldsmith, C. S., Zaki, S. R., Peret, T., Emery, S., et al. (2003). A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. *N. Engl. J. Med.* 348 (20), 1953–1966. doi:10.1056/NEJMoa030781

Li, L., Song, X., Yin, Z., Jia, R., Li, Z., Zhou, X., et al. (2016). The Antibacterial Activity and Action Mechanism of Emodin from Polygonum Cuspidatum against Haemophilus Parasuis *In Vitro*. *Microbiol. Res.* 186-187, 139–145. doi:10.1016/j.micres.2016.03.008

Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., et al. (2003). Angiotensin-converting Enzyme 2 Is a Functional Receptor for the SARS Coronavirus. *Nature* 426 (6965), 450–454. doi:10.1038/nature02145

Li, Y., Luan, Y., Qi, X., Li, M., Gong, L., Xue, X., et al. (2010). Emodin Triggers DNA Double-Strand Breaks by Stabilizing Topoisomerase II-DNA Cleavage Complexes and by Inhibiting ATP Hydrolysis of Topoisomerase II. *Toxicol. Sci.* 118 (2), 435–443. doi:10.1093/toxsci/kfq282

Liaw, Y. F., and Chu, C. M. (2009). Hepatitis B Virus Infection. *Lancet* 373 (9663), 582–592. doi:10.1016/S0140-6736(09)60207-5

Lin, C. J., Lin, H. J., Chen, T. H., Hsu, Y. A., Liu, C. S., Hwang, G. Y., et al. (2015). Polygonum Cuspidatum and its Active Components Inhibit Replication of the Influenza Virus through Toll-like Receptor 9-Induced Interferon Beta Expression. *PLoS One* 10 (2), e0117602. doi:10.1371/journal.pone.0117602

Lin, J., Ma, C., and Lin, H. H. (2019). Emodin Alleviates Viral Myocarditis in BALB/c Mice and Underlying Mechanisms. *Lat. Am. J. Pharm.* 38 (10), 1979–1984.

Lin, L., Ni, B., Lin, H., Zhang, M., Li, X., Yin, X., et al. (2015). Traditional Usages, Botany, Phytochemistry, Pharmacology and Toxicology of Polygonum Multiflorum Thunb.: A Review. *J. Ethnopharmacol.* 159, 158–183. doi:10.1016/j.jep.2014.11.009

Liu, H., and Luo, H. (2021). Development of Group B Coxsackievirus as an Oncolytic Virus: Opportunities and Challenges. *Viruses* 13 (6), 1082. doi:10.3390/v13061082

Liu, X., Liu, Y., Qu, Y., Cheng, M., and Xiao, H. (2015). Metabolomic Profiling of Emodin-Induced Cytotoxicity in Human Liver Cells and Mechanistic Study. *Toxicol. Res.* 4 (4), 948–955. doi:10.1039/c4tx00246f

Liu, Z., Ma, N., Zhong, Y., and Yang, Z. Q. (2015). Antiviral Effect of Emodin from Rheum Palmatum against Coxsakievirus B5 and Human Respiratory Syncytial Virus *In Vitro*. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 35 (6), 916–922. doi:10.1007/s11596-015-1528-9

Liu, Z., Wei, F., Chen, L. J., Xiong, H. R., Liu, Y. Y., Luo, F., et al. (2013). *In Vitro* and *In Vivo* Studies of the Inhibitory Effects of Emodin Isolated from Polygonum Cuspidatum on Coxsakievirus B4. *Molecules* 18 (10), 11842–11858. doi:10.3390/molecules181011842

Luo, T., Li, N., He, Y. Q., Weng, S. Q., Wang, T., Zou, Q. X., et al. (2015). Emodin Inhibits Human Sperm Functions by Reducing Sperm [Ca(2+)]i and Tyrosine Phosphorylation. *Reprod. Toxicol.* 51, 14–21. doi:10.1016/j.reprotox.2014.11.007

Ma, F., Lf, D., Ei, T., and Pa, G. (2021). Herpes Simplex Virus Interference with Immunity: Focus on Dendritic Cells. *Virulence* 12 (1), 2583–2607. doi:10.1080/21505594.2021.1980990

Monisha, B. A., Kumar, N., and Tiku, A. B. (2016). Emodin and its Role in Chronic Diseases. *Adv. Exp. Med. Biol.* 928, 47–73. doi:10.1007/978-3-319-41334-1_3

Mueller, S. O., Schmitt, M., Dekant, W., Stopper, H., Schlatter, J., Schreier, P., et al. (1999). Occurrence of Emodin, Chrysophanol and Physcion in Vegetables, Herbs and Liquors. Genotoxicity and Anti-genotoxicity of the Anthraquinones and of the Whole Plants. *Food Chem. Toxicol.*, 37(5), 481–491. doi:Doi doi:10.1016/S0278-6915(99)00027-7

Nawrot-Hadzik, I., Zmudzinski, M., Matkowski, A., Preissner, R., Kęsik-Brodacka, M., Hadzik, J., et al. (2021). Reynoutria Rhizomes as a Natural Source of SARS-CoV-2 Mpro Inhibitors-Molecular Docking and *In Vitro* Study. *Pharm. (Basel)* 14 (8), 742. doi:10.3390/ph14080742

Oshida, K., Hirakata, M., Maeda, A., Miyoshi, T., and Miyamoto, Y. (2011). Toxicological Effect of Emodin in Mouse Testicular Gene Expression Profile. *J. Appl. Toxicol.* 31 (8), 790–800. doi:10.1002/jat.1637

Park, B., Yoon, W., Yun, J., Ban, E., Yun, H., and Kim, A. (2019). Emodin-nicotinamide (1:2) Cocrystal Identified by Thermal Screening to Improve Emodin Solubility. *Int. J. Pharm.* 557, 26–35. doi:10.1016/j.ijpharm.2018.12.027

Peng, W., Qin, R., Li, X., and Zhou, H. (2013). Botany, phytochemistry, pharmacology, and potential application of Polygonum cuspidatum Sieb.et Zucc.: A review. *J. Ethnopharmacol.* 148 (3), 729–745. doi:10.1016/j.jep.2013.05.007

Polaris Observatory Collaborators (2018). Global Prevalence, Treatment, and Prevention of Hepatitis B Virus Infection in 2016: A Modelling Study. *Lancet Gastroenterol. Hepatol.* 3 (6), 383–403. doi:10.1016/S2468-1253(18)30056-6

Rolta, R., Yadav, R., Salaria, D., Trivedi, S., Imran, M., Sourirajan, A., et al. (2021). In Silico screening of Hundred Phytocompounds of Ten Medicinal Plants as Potential Inhibitors of Nucleocapsid Phosphoprotein of COVID-19: An Approach to Prevent Virus Assembly. *J. Biomol. Struct. Dyn.* 39 (18), 7017–7034. doi:10.1080/07391102.2020.1804457

Schwarz, S., Wang, K., Yu, W., Sun, B., and Schwarz, W. (2011). Emodin Inhibits Current through SARS-Associated Coronavirus 3a Protein. *Antivir. Res.* 90 (1), 64–69. doi:10.1016/j.antiviral.2011.02.008

Shao, Q., Wu, F., Liu, T., Wang, W., Liu, T., Jin, X., et al. (2021). JieZe-1 Alleviates HSV-2 Infection-Induced Genital Herpes in Balb/c Mice by Inhibiting Cell Apoptosis via Inducing Autophagy. *Front. Pharmacol.* 12, 775521. doi:10.3389/fphar.2021.775521

Shia, C. S., Hou, Y. C., Tsai, S. Y., Huieh, P. H., Leu, Y. L., and Chao, P. D. (2010). Differences in Pharmacokinetics and *Ex Vivo* Antioxidant Activity Following Intravenous and Oral Administrations of Emodin to Rats. *J. Pharm. Sci.* 99 (4), 2185–2195. doi:10.1002/jps.21978

Shukla, S. D., and Valyi-Nagy, T. (2022). Host Molecules that Promote Pathophysiology of Ocular Herpes. *Front. Microbiol.* 13, 818658. doi:10.3389/fmicb.2022.818658

Simmonds, P., Gorbalenya, A. E., Harvala, H., Hovi, T., Knowles, N. J., Lindberg, A. M., et al. (2020). Recommendations for the Nomenclature of Enteroviruses and Rhinoviruses. *Arch. Virol.* 165 (3), 793–797. doi:10.1007/s00705-019-04520-6

Teng, Z., Yuan, C., Zhang, F., Huan, M., Cao, W., Li, K., et al. (2012). Intestinal Absorption and First-Pass Metabolism of Polyphenol Compounds in Rat and Their Transport Dynamics in Caco-2 Cells. *PLoS One* 7 (1), e29647. doi:10.1371/journal.pone.0029647

Wang, C., Dai, X., Liu, H., Yi, H., Zhou, D., Liu, C., et al. (2015). Involvement of PPARγ in Emodin-Induced HK-2 Cell Apoptosis. *Toxicol Vitro* 29 (1), 228–233. doi:10.1016/j.tiv.2014.10.021

Wu, C. C., Chen, M. S., Cheng, Y. J., Ko, Y. C., Lin, S. F., Chiu, I. M., et al. (2019). Emodin Inhibits EBV Reactivation and Represses NPC Tumorigenesis. *Cancers (Basel)* 11 (11). doi:10.3390/cancers11111795

Xiang, H., Zuo, J., Guo, F., and Dong, D. (2020). What We Already Know about Rhubarb: A Comprehensive Review. *Chin. Med.* 15 (1), 88. doi:10.1186/s13020-020-00370-6

Xiong, H. R., Luo, J., Hou, W., Xiao, H., and Yang, Z. Q. (2011). The Effect of Emodin, an Anthraquinone Derivative Extracted from the Roots of Rheum Tanguticum, against Herpes Simplex Virus *In Vitro* and *In Vivo*. *J. Ethnopharmacol.* 133 (2), 718–723. doi:10.1016/j.jep.2010.10.059

Yang, J., Zhu, A., Xiao, S., Zhang, T., Wang, L., Wang, Q., et al. (2019). Anthraquinones in the Aqueous Extract of Cassiae Semen Cause Liver Injury in Rats through Lipid Metabolism Disorder. *Phytomedicine* 64, 153059. doi:10.1016/j.phymed.2019.153059

Yiu, C. Y., Chen, S. Y., Yang, T. H., Chang, C. J., Yeh, D. B., Chen, Y. J., et al. (2014). Inhibition of Epstein-Barr Virus Lytic Cycle by an Ethyl Acetate Subfraction Separated from Polygonum Cuspidatum Root and its Major Component, Emodin. *Molecules* 19 (1), 1258–1272. doi:10.3390/molecules19011258

Yiu, C. Y., Chiu, Y. J., and Lin, T. P. (2021). The Ethyl Acetate Subfraction of Polygonum Cuspidatum Root Containing Emodin Affect EBV Gene Expression and Induce EBV-Positive Cells Apoptosis. *Biol. Pharm. Bull.* 44 (12), 1837–1842. doi:10.1248/bpb.b21-00508

Young, L. S., Yap, L. F., and Murray, P. G. (2016). Epstein-Barr Virus: More Than 50 Years Old and Still Providing Surprises. *Nat. Rev. Cancer* 16 (12), 789–802. doi:10.1038/nrc.2016.92

Yu, S., Zhu, Y., Xu, J., Yao, G., Zhang, P., Wang, M., et al. (2021). Glycyrrhizic Acid Exerts Inhibitory Activity against the Spike Protein of SARS-CoV-2. *Phytomedicine* 85, 153364. doi:10.1016/j.phymed.2020.153364

Zhang, H. M., Wang, F., Qiu, Y., Ye, X., Hanson, P., Shen, H., et al. (2016). Emodin Inhibits Coxsackievirus B3 Replication via Multiple Signalling Cascades Leading to Suppression of Translation. *Biochem. J.* 473 (4), 473–485. doi:10.1042/bj20150419

Zhang, Y. F., Lin, C., Yang, X. F., Wang, Y. B., Fang, Y. F., and Wang, F. L. (2016). Effect of Emodin on the Expression of TLR4 and P38MAPK in Mouse Cardiac Tissues with Viral Myocarditis. *Int. J. Clin. Exp. Pathol.* 9 (10), 10839–10845.

Zheng, Q., Li, S., Li, X., and Liu, R. (2021). Advances in the Study of Emodin: An Update on Pharmacological Properties and Mechanistic Basis. *Chin. Med.* 16 (1), 102. doi:10.1186/s13020-021-00509-z

Zheng, Y. F., Liu, C. F., Lai, W. F., Xiang, Q., Li, Z. F., Wang, H., et al. (2014). The Laxative Effect of Emodin Is Attributable to Increased Aquaporin 3 Expression in the Colon of Mice and HT-29 Cells. *Fitoterapia* 96, 25–32. doi:10.1016/j.fitote.2014.04.002

Zhi, H. J., Zhu, H. Y., Zhang, Y. Y., Lu, Y., Li, H., and Chen, D. F. (2019). *In Vivo* effect of Quantified Flavonoids-Enriched Extract of Scutellaria Baicalensis Root on Acute Lung Injury Induced by Influenza A Virus. *Phytomedicine* 57, 105–116. doi:10.1016/j.phymed.2018.12.009

Zhong, Q., Yang, Z., Liu, Y., Deng, H., Xiao, H., Shi, L., et al. (2009). Antiviral Activity of Arbidol against Coxsackie Virus B5 *In Vitro* and *In Vivo*. *Arch. Virol.* 154 (4), 601–607. doi:10.1007/s00705-009-0346-4

Zhong, T., Zhang, L. Y., Wang, Z. Y., Wang, Y., Song, F. M., Zhang, Y. H., et al. (2017). Rheum Emodin Inhibits Enterovirus 71 Viral Replication and Affects the Host Cell Cycle Environment. *Acta Pharmacol. Sin.* 38 (3), 392–401. doi:10.1038/aps.2016.110

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

REVIEW
published: 30 May 2022
doi: 10.3389/fphar.2022.891535

The Antimicrobial Potential of the Neem Tree *Azadirachta indica*

*Marina R. Wylie and D. Scott Merrell**

Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, United States

Azadirachta indica (A. Juss), also known as the neem tree, has been used for millennia as a traditional remedy for a multitude of human ailments. Also recognized around the world as a broad-spectrum pesticide and fertilizer, neem has applications in agriculture and beyond. Currently, the extensive antimicrobial activities of *A. indica* are being explored through research in the fields of dentistry, food safety, bacteriology, mycology, virology, and parasitology. Herein, some of the most recent studies that demonstrate the potential of neem as a previously untapped source of novel therapeutics are summarized as they relate to the aforementioned research topics. Additionally, the capacity of neem extracts and compounds to act against drug-resistant and biofilm-forming organisms, both of which represent large groups of pathogens for which there are limited treatment options, are highlighted. Updated information on the phytochemistry and safety of neem-derived products are discussed as well. Although there is a growing body of exciting evidence that supports the use of *A. indica* as an antimicrobial, additional studies are clearly needed to determine the specific mechanisms of action, clinical efficacy, and *in vivo* safety of neem as a treatment for human pathogens of interest. Moreover, the various ongoing studies and the diverse properties of neem discussed herein may serve as a guide for the discovery of new antimicrobials that may exist in other herbal panaceas across the globe.

Keywords: neem (*Azadirachta indica* A. Juss), antibacterial, antiviral, antifungal, antiparasitic, phytochemicals, natural products, antibiofilm

OPEN ACCESS

Edited by:
Alessandra Russo, University of Catania, Italy

Reviewed by:
Nagini S., Annamalai University, India
Yris Maria Fonseca-Bazzo, University of Brasilia, Brazil

****Correspondence:***
D. Scott Merrell
douglas.merrell@usuhs.edu

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 March 2022*
Accepted: *09 May 2022*
Published: *30 May 2022*

Citation:
Wylie MR and Merrell DS (2022) The Antimicrobial Potential of the Neem Tree Azadirachta indica. Front. Pharmacol. 13:891535. doi: 10.3389/fphar.2022.891535

MEDICINAL PLANTS AS SOURCES FOR NOVEL ANTIMICROBIAL AGENTS

The need to expand the available pharmaceutical repertoire is underlined by several recent reports, including the 2019 Antibiotic Resistance Threat Report by the Centers for Disease Control and Prevention; this document states that in the United States alone, more than 2.8 million antibiotic-resistant infections and more than 35,000 related deaths occur each year (CDC, 2019). These fatal infections are most frequently caused by the 18 species of bacteria and fungi listed as current urgent, serious, or concerning human health threats (CDCE, 2019). Additionally, on a global scale, infectious diseases cause approximately 20% of all deaths each year and are the leading cause of death of

Abbreviations: AIDS, acquired immunodeficiency syndrome; DENV, Dengue virus; EAF, ethyl acetate fraction; EC50, half maximal effective concentration; EPS, extracellular polymeric substance; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; SQDG, sulfonoquinovosyldiacylglyceride.

children under 5 years old (Martens and Demain, 2017). Many in the medical field agree that devastating statistics like these are a consequence of entering the "post-antibiotic era," a time in which the efficacies of antibiotics and other antimicrobials are unreliable (Wang et al., 2020; Streicher, 2021).

Despite the continuous popularity of herbal medicine across the globe, traditional antibiotics have previously overshadowed the exploration of plant-based products as therapeutics. However, due to the growing need for new antimicrobial agents, many scientists have now expanded their searches to include novel plant and other environmental sources. Indeed, mainstream medicine is increasingly receptive to the use of plant-derived drugs, especially those to which antimicrobial resistance is more difficult or unlikely to develop. Notably, 26% of all new approved drugs and 33% of all new small-molecule approved drugs between 1981 and 2014 were botanical drugs, unaltered natural products, or derivatives thereof (Newman and Cragg, 2016). This abundance underscores the vast, untapped potential of plants around the world to yield desperately needed novel drugs. In fact, only around 6% of the ~300,000 species of higher plants have been pharmacologically investigated (Cragg and Newman, 2013). However, recent reviews by Khameneh et al. (2019) and Chassagne et al. (2020) have highlighted the increasingly evident antibacterial properties of various plant species and phytochemicals. Furthermore, there is an increasing amount of evidence that suggests that phytochemicals may be used in conjunction with current antimicrobials to obtain synergistic effects (Aiyegoro and Okoh, 2009; Borges et al., 2016; Barbieri et al., 2017; Ayaz et al., 2019). For example, an early study by Ahmad and Aqil (2007) found that crude extracts of multiple plant species showed *in vitro* synergistic activity with existing antibiotics when used against two multidrug-resistant enteric bacterial species (Ahmad and Aqil, 2007). Thus, the combination of phytochemicals and antibiotics may help to combat resistance to conventional monotherapies for many diseases. As evidence for the use of natural products to treat human disease continues to accumulate, it will become increasingly important to perform in-depth safety studies on the identified extracts, compounds, and their derivatives. So far, there is a general consensus that natural products, as compared to synthetic drugs, have relatively low toxicity to mammals and have less harmful effects on nontarget beneficial organisms (Brahmachari, 2004), which is yet another appealing aspect of utilizing plant species for the identification of effective pharmaceuticals.

THE IMPORTANCE OF *AZADIRACHTA INDICA* (NEEM) AS A MEDICINAL PLANT

Azadirachta indica (A. Juss), commonly known as the neem tree, is a tropical evergreen tree that is native to the Indian subcontinent (Noorul Aneesa, 2016). For thousands of years, neem has been recognized for its wide array of beneficial properties, including those in agriculture for pest control and in traditional medicine for various common human ailments. *A. indica* originally provoked world-wide interest due to its capacity as a non-toxic infection-control agent for use in farming (Govindachari, 1992). Indeed, one of the most abundant compounds found within the neem plant, azadirachtin, is an increasingly common biopesticide (Chaudhary et al., 2017; Pasquoto-Stigliani et al., 2017; Kilani-Morakchi et al., 2021). However, various parts of the neem tree have been used for millennia in traditional Indian medicine for their claimed antipyretic, antacid, antiparasitic, antibacterial, antiviral, antidiabetic, contraceptive, antidermatitic, anticancer, anti-inflammatory, antioxidant, antifungal, dental, and other healing and protective properties (Govindachari, 1992; Alzohairy, 2016). Almost every part of *A. indica* (e.g., the stem, bark, roots, leaves, gum, seeds, fruits, flowers, etc.) have been used as house-hold remedies for human illnesses. Moreover, millions of people globally use neem twigs as a source of chewing sticks for dental hygiene (Brahmachari, 2004; Gupta et al., 2017). More recently, the neem tree has gained attention from modern medicine and infectious disease researchers as a potential source for new antimicrobials, in addition to the applications of *A. indica* in the fields of oncology, dentistry, dermatology, and endocrinology, among others; for reviews on some of these individual topics, see (Lakshmi et al., 2015; Patel et al., 2016; Aumeeruddy and Mahomoodally, 2021; Iman et al., 2021; Patil et al., 2021; Singh et al., 2021; Yarmohammadi et al., 2021).

Subsequent sections of this review seek to provide an overview of the most recent scientific findings that support the consideration of neem extracts and phytochemicals as antimicrobial agents. Specifically, the potential for neem and neem-related products to target pathogens that are resistant to first-line antibiotics, bacterial species that affect oral health and/or form difficult to eradicate biofilms, fungal infections that threaten food sources, and viral infections that have major impacts on human health are highlighted. Research in these fields is supported by a worldwide interest in neem products that stretches from ancient medicinal practices to an abundance of publications that highlight *A. indica* as a plant with modern pharmacological attributes. Apart from the activities highlighted herein, we would like to point the reader to the expert review by Saleem et al. (2018) that covers the most recent evidence supporting neem as a treatment for specific human ailments and known mechanisms of action of neem components; therein, the specific protective qualities of *A. indica* and related clinical trials are thoroughly reported.

PHYTOCHEMISTRY OF *AZADIRACHTA INDICA*

Although nearly every part of neem has been used for traditional medicinal purposes in India, the most widely available *A. indica* product on the market today is neem oil (Sir and Chopra, 1994). The country of India alone produces hundreds of thousands of tons of neem oil annually (National Research Council, 1992) and a byproduct of neem oil production includes neem cake, which is abundantly used in agriculture around the world at ~600 pounds per acre of farmland (Reddy, 2020). Neem oil is considered a vegetable oil that is cold pressed from the fruits and seeds of neem

(Noorul Aneesa, 2016). However, neem oil can be further processed into various types of extracts, via different solvents, that are then used for subsequent preclinical and clinical studies.

Although various solvents can be implemented to extract different active components from plant products, most of the compounds that are thought to be responsible for the biological activities of neem can be found in the extracts that are typically used in laboratories (e.g., water, ethanol, methanol, chloroform, and ether) (Cowan, 1999). In recently published literature, methanol and ethanol extracts are those that are most commonly used for antimicrobial testing. The general biological activities of the tested neem oil extracts have been attributed to the presence of many secondary plant metabolites, which include classes of compounds such as isoprenoids (e.g., terpenoids containing limonoid structures) and non-isoprenoids (e.g., tannins) (Saleem et al., 2018).

The neem tree contains hundreds of compounds (i.e., phytochemicals), many of which have been found to be bioactive and to have diverse utility on their own. Out of the more than 300 unique compounds have been identified within the neem tree, some of the more abundant phytochemicals (e.g., azadirachtin, gedunin, and nimbolide) have already been defined as potential drugs with a wide range of biological activities (Saleem et al., 2018; Braga et al., 2020; Nagini et al., 2021). The compounds that have been most thoroughly investigated for their individual antimicrobial properties thus far are limonoids, which are compounds that typically consist of four, six-membered rings and one five-membered aromatic ring (i.e., a furanolactone core) and make up one-third of the phytochemicals derived from the neem tree (Roy and Saraf, 2006; Gupta et al., 2017). This class of compounds has also been explored for its abundance of antioxidant activities [reviewed in (Tundis et al., 2014; Gualdani et al., 2016; Sarkar et al., 2021)] and includes nimbolide, nimbin, and nimbidin (triterpenoids), azadirachtin, and gedunin. Previously established *in vitro* activities of these compounds and others isolated from neem seed oil have been reviewed and range from anti-inflammatory and antiulcer to spermicidal and anti-psoriasis (Brahmachari, 2004). Two recent comprehensive reviews by Saleem et al. (2018) and Gupta et al. (2017) have expertly covered the extensive phytochemistry of neem.

ANTIMICROBIAL TESTING AND SAFETY OF *AZADIRACHTA INDICA*

To test the antimicrobial activities of neem oil extracts and phytochemicals, *in vitro* methods such as broth dilution, disc or agar diffusion, and agar overlay assays are commonly used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each treatment. A few *in vivo* models have been implemented to more accurately reflect human infection and disease testing; these models include intraperitoneal or intravenous injection, or oral or gastric administration of neem oil-related drugs in mice, rats, guinea pigs, and rabbits. These published animal studies indicate that the acute toxicity level of neem greatly depends on the plant component and solvent used to make the extract, as well as on the treatment route and species used in the model; for a comprehensive review on this topic, see (Braga et al., 2021). As an example, oral administration of an ethanolic neem leaf extract less than 2000 mg/kg body weight did not cause mortality in mice (Kanagasanthosh et al., 2015). Conversely, the ethanolic extract of neem stem bark given to rats at 50–200 mg/kg altered the biochemical markers of toxicity and may have consequential effects on organ function (Ashafa et al., 2012). In contrast, one small human study showed that the lyophilized powder of an aqueous neem bark extract given at doses of 30–60 mg twice daily for 10 weeks had therapeutic potential in adults for controlling gastric hypersecretion and gastroesophageal and gastroduodenal ulcers; there were no obvious effects on blood parameters indicative of organ toxicity (Bandyopadhyay et al., 2004). Also, after 1 year of external exposure to 1% neem oil, 156 adults and 110 children did not experience any major adverse effects (Brahmachari, 2004). Of note, Arsene et al. (2021) showed that the *Galleria mellonella* (wax moth larva) model is a reliable method to assess the acute *in vivo* toxicity of medicinal plants, in which ethanolic extracts of neem leaves and seeds had higher levels of toxicity than aqueous extracts of the same materials. Although the available *in vivo* data will need to be further developed before neem oil extracts and phytochemicals are applied in a clinical setting, the United States Environmental Protection Agency has stated that cold-pressed neem oil should have "no unreasonable adverse effects to the US population and the environment" (Agency U.S E.P, 2012). For neem-derived products, non-aqueous extracts are generally the most toxic, while unprocessed materials and pure phytochemicals from the neem tree have relatively low toxicities (Boeke et al., 2004). Given the currently available information summarized here, an important goal of future antimicrobial testing of neem oil and its products should be the standardization of extracts and administration methods. Additional more consistent studies on this topic may allow researchers to draw more detailed conclusions about the potential use of *A. indica* in a clinical setting.

ANTIBACTERIAL EVIDENCE

The rising rates of antibiotic resistance for bacterial pathogens has led to the need for novel therapeutics; thus, much of the recent work on the antimicrobial potential of neem has focused on the antibacterial properties of the plant. This area of research is supported by the traditional use of neem products for dental hygiene and the successful applications of neem in the food industry. In addition to standard antibiotic resistance, the ability of pathogenic bacterial species to form biofilms has led to an increased interest in describing how these communities contribute to heightened tolerance to antibacterial substances. Although the importance of biofilm-associated infections to human disease is well-recognized, few novel solutions that effectively eliminate biofilms have been developed thus far. However, encouraging data suggest that neem is consistently

more effective at prohibiting bacterial growth and at targeting biofilm-grown cells than many other herbal extracts and is, therefore, worth pursuing as a source for drug discovery (Noor, 2011). For a more comprehensive list of the antibacterial properties of the neem tree described in the following sections, see **Supplementary Table S1**.

Azadirachta indica and Dentistry

As mentioned above, the use of neem twigs as dental cleaning sticks is commonplace in many countries to which *A. indica* is a native species (Gupta et al., 2017). This traditional use has translated to a growing number of studies that have tested neem products for their ability to improve dental hygiene and to prevent or treat oral diseases. Typically, these studies aim to identify an application for neem extracts as a mouthwash alternative, root canal irrigant, toothpaste, etc. Previously, the antimicrobial activity of *A. indica* against common endodontic pathogens, such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans*, has been well-established (Mistry et al., 2014; Barua et al., 2017; Singh et al., 2020). For example, a decade ago, it was shown that at 7.5%, aqueous neem leaf extract was able to inhibit the growth of *E. faecalis*, *S. mutans*, and *C. albicans* and that the MIC of an ethanolic neem leaf extract was 1.88%, 7.5%, and 3.75% against these three important dental pathogens, respectively (Nayak Aarati et al., 2011). More recently, another group was able to determine that a methanolic extract of *A. indica* showed considerable antimicrobial activity against a three-week-old polymicrobial dental biofilm grown on extracted human teeth consisting of *S. mutans, E. faecalis, S. aureus* and *C. albicans* (Mistry et al., 2015). The results from two small human studies indicate that a neem-based toothpaste or gel can reduce the levels of *S. mutans* in the mouth and that the gel formulation can reduce plaque and gingivitis to the same degree as a chlorhexidine gel control (Nimbulkar et al., 2020; Selvaraj et al., 2020). Additional biologically relevant studies may be able to provide even more evidence for the use of neem in dentistry in combination with, or as an alternative to, antimicrobials already used in the field. Of note, several studies have already suggested that neem extracts have similar levels of activity as chlorhexidine or hypochlorite (typical components of oral washes) against plaque, gingivitis, and pain *in vivo* (Jalaluddin et al., 2017; Hosny et al., 2021) and against biofilm-forming bacteria (e.g., *Streptococcus viridans, Porphyromonas gingivalis,* and *S. aureus*) and *C. albicans in vitro* or *ex vivo* (Joy Sinha et al., 2015; Anand et al., 2016; Kankariya et al., 2016; Heyman et al., 2017; Andonissamy et al., 2019; Bansal et al., 2019; Tasanarong et al., 2021). Another human pathogen that causes plaque and other biofilm-related diseases in the body, *E. faecalis*, is also just as susceptible to various neem extracts as it is to chlorhexidine *in vitro* (Chandrappa et al., 2015; Mustafa, 2016; Bhardwaj et al., 2017; Joy Sinha et al., 2017).

The relevance of neem as an antimicrobial in dentistry is, so far, the most researched area and has led to many conclusions about *A. indica* extracts as compared to those from other plants used in traditional medicine. In fact, some studies suggest that neem has greater antibacterial activity than *Commiphora myrrha* (myrrh), *Acacia* tree (e.g., catechu), *Cinnamomum verum* (cinnamon), *Salvadora persica* (miswak), *Syzygium aromaticum* (clove), *Zingiber officinale* (ginger), *Allium sativum* (garlic) and *Curcuma longa* (tumeric) extracts against some species of bacteria and cultured dental caries (Kanth et al., 2016; Jagannathan et al., 2020; Arora et al., 2021). This being said, it is important to note that certain bacterial species (e.g., *S. mutans* and *E. faecalis*) appear to be more susceptible to extracts from other plants in some studies (Jain et al., 2015; Dedhia et al., 2018; Kalita et al., 2019; Panchal et al., 2020). While this does not diminish the antimicrobial potential of *A. indica*, it does underline the importance of thoroughly taking advantage of the wide variety of antimicrobial plants and compounds that are at the disposal of modern medicine.

The Use of *Azadirachta indica* in the Food Industry

Originally introduced around the world as a potent pesticide and fertilizer for use in agriculture (Govindachari, 1992; Nicoletti et al., 2012; Chaudhary et al., 2017), neem has been recognized in more recent years as a safe and effective broad-spectrum antimicrobial with uses throughout the food industry that range from food production and storage to packaging and human consumption. During meat production, the presence of several species of bacteria can affect the quality and safety of the product, including *Campylobacter*, *Lactobacillus*, and *Carnobacterium* spp. Neem cake extract, which is a waste product from neem seed oil production, has antibacterial activities against all of these potentially pathogenic species (Del Serrone et al., 2015a). Additionally, Ravva and Korn (2015) found that neem leaf and bark supplements were able to successfully eliminate *Escherichia coli* O157:H7 from cultured cow manure; because this *E. coli* strain was isolated from an apple juice outbreak of O157:H7, these results may have broad applications on farms where crops and orchards frequently exist in close proximity to cattle (Ravva and Korn, 2015). In the production of another source of protein for human consumption, specifically in shrimp aquaculture, antibiotic-resistant *Vibrio parahaemolyticus* can compromise both shrimp and human health. A thorough study on the potential use of neem in this industry included *in vitro* and *in vivo* assays that showed that aqueous neem extract had a MIC against *V. parahaemolyticus* of 62.5 mg/ml and was able to significantly increase survival of shrimp by 76% as compared to the untreated control (Morales-Covarrubias et al., 2016).

One of the next steps in the production of pathogen-free human food is storage and/or packaging. In the last couple of years, several groups have shown that food preservation films that are made from polyethylene or sustainable materials such as seaweed can be manufactured to incorporate neem leaf extracts, neem oil, and other plant-based products (e.g., turmeric and curcumin) (Ahmed et al., 2022). The resulting composite films are shelf-stable, block ultraviolet light, and have increased antifungal and antibacterial activities against *C. albicans* and a wide range of Gram-negative and Gram-positive organisms, including *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, and

Bacillus subtilis (Sunthar et al., 2020; Uthaya Kumar et al., 2020; Oyekanmi et al., 2021; Subbuvel and Kavan, 2021). Furthermore, the ability of *A. indica* to prevent activity of food-spoiling fungi is evident by several recent reports that outline the following: 1) the ability of neem oil to prevent the growth of the grape product-spoiling species *Aspergillus carbonarius* and to inhibit the production of mycotoxin by strains of this fungus (Rodrigues et al., 2019), 2) the ability of neem leaves to prevent the production of aflatoxins by *Aspergillus parasiticus* during long-term storage of rice, wheat, and maize (Sultana et al., 2015), 3) the ability of neem seed methanol and ethanol extracts to inhibit *Aspergillus flavus* and *A. parasiticus* by 10% in the context of maize storage (An et al., 2019), 4) the ability of multiple neem seed, bark, and leaf extracts to inhibit the growth of three major potato-spoiling fungi, *Aspergillus niger*, *Fusarium oxyporium*, and *Pythium* spp., by 72–100% (Ezeonu et al., 2019), and 5) the ability of aqueous neem leaf extract to inhibit growth of *A. niger* and *A. parasiticus*, as well as to detoxify aflatoxin B1 and ochratoxin A *in vivo* (Hamad et al., 2021). These diverse antifungal properties of neem are highlighted in **Supplementary Table S1**. Altogether, these food-related studies should encourage further investigation of the utility of *A. indica*-derived products throughout the food industry; these products may serve as a sustainable antimicrobial alternative that can potentially improve food security via improved stable long-term storage, as well as improve human health through the elimination of foodborne pathogens.

Additional Antibacterial Activities of Neem

In several other areas of antibacterial discovery, *A. indica* has been shown to be effective against many important human pathogens. Overall, a large number of studies have been published in the last decade on this topic, especially as they relate to the ever-growing number of antibiotic-resistant organisms. In this vein, several bacterial species that cause wound infections are found on the long list of antibiotic resistant threats, including *S. aureus* and *P. aeruginosa* (CDC, 2019). Several groups have tested the antibacterial activity of neem against these species. For example, Garg et al. (2015) demonstrated that methanol and chloroform neem extracts performed better than other plant extracts and better than several different antibiotics against both *S. aureus* and *P. aeruginosa* (Garg et al., 2015). More specifically, it has also been determined that the MIC of limonoid compounds that were isolated from neem seeds are between 32 μg/ml and 128 μg/ml against *P. aeruginosa* and another opportunistic skin pathogen, *Staphylococcus epidermidis* (Lu et al., 2019). As a translational approach to this area of research, several studies have demonstrated that an aqueous neem leaf extract that was used to make alginate fibers for wound dressings (Hussain et al., 2017), a nanofibrous mat embedded with neem leaf extract (Ali et al., 2019), and a topical gel containing a methanolic neem extract all inhibit *S. aureus* growth (Raju and Jose, 2019). In the case of a polyesteramide synthesized from neem oil to produce a nanofibrous mat, the incorporation of *A. indica* into this wound treatment method resulted in increased tissue regeneration in rats, as compared to the control commercial cream (Killi et al., 2019). These results potentially have broad applications for many areas of medicine in which the risk of antibiotic resistant skin/wound infections is high.

Another large group of infectious bacteria that cause morbidity and mortality all over the world are the gastrointestinal pathogens, which include foodborne and diarrhea-causing organisms. Relatedly, some of the traditional uses of neem are antidiarrheal, antacid, and antiulcer; this has led to a large body of research that has investigated the antibacterial properties of neem products against pathogens such as *Salmonella* spp., *Shigella* spp., *E. coli*, *Listeria monocytogenes*, and *Bacillus cereus*, as shown in **Figure 1**. To summarize, the *Salmonella* spp. and *Shigella* spp. that have been tested, which includes more than a dozen multidrug-resistant isolates from patients suffering from typhoid fever complications, are susceptible to seed, bark, and leaf extracts of neem from either ethanol, methanol, or acetone extraction; in some cases, the activity of neem extract was also found to be greater than that of gentamycin, erythromycin, and other plants used in traditional medicine (Mahfuzul Hoque et al., 2007; Susmitha et al., 2013; Tesso et al., 2015; Melese et al., 2016; Al Akeel et al., 2017; Panchal et al., 2020; Essuman et al., 2021). Similarly, dried leaf, seed, and bark neem extracts in any of the three previously mentioned solvents have significant antibacterial activity against *E. coli*, with the methanolic extract of neem seeds demonstrating the greatest level of activity (Susmitha et al., 2013; Sharma and Nupur, 2014; Melese et al., 2016). Additionally, neem oil was as effective as ciprofloxacin against 48 tested isolates of *E. coli*, 14 of which were diarrheagenic strains (Del Serrone et al., 2015b). Given that there are more than a quarter of a million cases of *E. coli* each year in the United States alone, associated world-wide morbidity is a huge issue for this pathogen (CDCE, 2019). Indeed, resistant *E. coli* infections were found to be responsible for nearly a quarter of all disability-adjusted life years caused by resistant bacterial infections in the European Union in 2015 (Cassini et al., 2019). On the chronic disease spectrum of gastrointestinal illnesses, *Helicobacter pylori* is a pathogen that colonizes approximately 50% of the human population and causes ulcers in millions of people each year; it also causes stomach cancer in ~1% of people who are infected (Kuipers, 1999; Moodley et al., 2012; Bray et al., 2018). Recently, two studies found that neem oil extract and a neem-associated phytochemical, nimbolide, have potent *in vitro* bactericidal activity against *H. pylori* in liquid cultures and in biofilms (Blum et al., 2019; Wylie et al., 2021). Similarly, another group determined that an ethanolic neem leaf extract has activity against this species as well (Saxena et al., 2021). Overall, studies like those described above provide strong evidence that novel plant-derived treatments, like neem oil extracts and/or the phytochemicals contained therein, may be able to reduce the burden of pervasive organisms like *E. coli* and *H. pylori*.

Finally, there have been many studies published on the antibacterial properties of neem against a variety of diverse human pathogens. The overwhelming conclusion from the majority of these investigations is that many elements of *A. indica* (e.g., seeds, bark, leaves, etc.) produce extracts that have moderate to significant levels of antimicrobial activity against several pathogens: *S. aureus*, *E. coli*, *E. faecalis*, *P. aeruginosa*, *Salmonella typhi*, *Streptococcus agalactiae*, *Shigella boydii*, *B.*

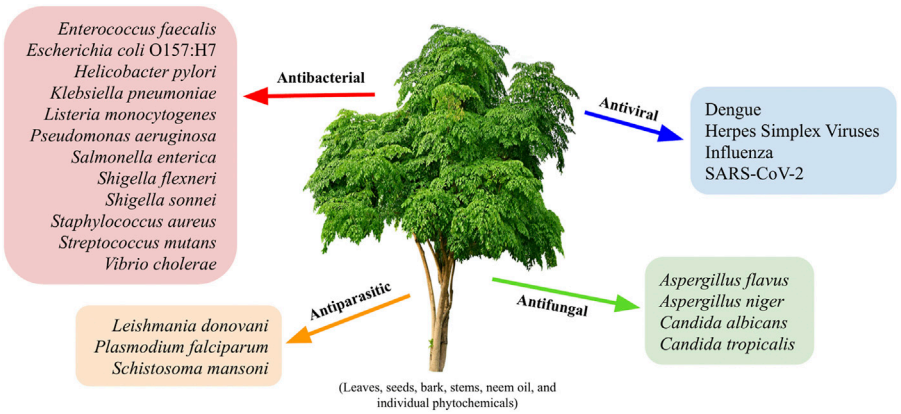


FIGURE 1 | Representative antimicrobial targets of the neem tree, *Azadirachta indic*a. Virtually all parts of the neem tree (leaves, seeds, bark, and stems), neem oil, and individual neem-associated phytochemicals have been shown to possess antibacterial, antiviral, antiparasitic, and/or antifungal activities. Some of the pathogens that have been studied and shown to be susceptible to *A. indica*-associated compounds are listed in their respective categories; the length of the provided list indicates the relative amount of published information on each of the four topics. A more exhaustive list of the pathogens that are susceptible to neem-derived products is available in **Supplementary Table S1**.

subtilis, *Klebsiella pneumoniae*, and *Candida tropicalis*, as listed in **Figure 1** (Dahiya and Purkayastha, 2012; Melese et al., 2016; Al Saiqali et al., 2018; Ibrahim and Kebede, 2020). Moreover, in side-by-side comparisons *A. indica*-based components often show superiority to other plants; a few exceptions include aqueous garlic extract and ethanolic green tea extract that were each shown to work better than neem extracts to kill *Bacillus anthracis* or *S. aureus* and *E. coli*, respectively (Zihadi et al., 2019; Kaur et al., 2021). Overall, studies support the notion that *A. indica* is an omnipotent plant that possesses antimicrobial activity against many bacterial pathogens. Future studies should include more standardized research approaches to test the potential of neem-derived products and individual compounds against other microbes both *in vitro* and *in vivo*; a priority should also be placed on determining the mechanisms of action of these products in order to fully understand the ideal clinical significance of *A. indica*.

Neem and Biofilm-Forming Pathogens

Biofilms, or communities of bacteria composed of biofilm-associated cells and extracellular polymeric substance (EPS) components (e.g., proteins, polysaccharides, and extracellular DNA), are notably recalcitrant to outside stressors, including those from the immune system and from therapeutics (Bae and Jeon, 2013; Yonezawa et al., 2019; de Vor et al., 2020). Depending on the species of biofilm-associated bacteria, mechanisms of tolerance include slow diffusion/limited penetration of the drug, metabolic heterogeneity and decreased growth rates among the cells within the biofilm, and the formation of persister cells (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Attaran et al., 2017). Backed by thorough research, plants such as *A. indica* may provide the tools needed to treat pervasive biofilm infections. To this end, there is evidence that natural products may be an ideal source of quorum sensing inhibitors, efflux pump inhibitors, and metal chelators, which are all potentially powerful antibiofilm agents (Borges et al., 2016). Additionally, synergistic interactions between the many constituents found within plant extracts may provide a benefit over a single isolated ingredient; this may explain the efficacy of lower doses of herbal products like neem oil as compared to individual compounds (Aiyegoro and Okoh, 2009).

Significant biofilm-associated human infections are caused by species such as *S. aureus*, *E. faecalis*, and *P. aeruginosa* (Vestby et al., 2020). There is already evidence that *A. indica* has activity against biofilm-forming strains of some of these pathogens. For example, a neem leaf ethanolic extract was found to inhibit *S. aureus* and methicillin-resistant *S. aureus* (MRSA) biofilm adherence at 62.5 and 125 μg/ml, respectively (Quelemes et al., 2015). More recently, another group found that a petroleum ether neem extract had a MIC and MBC of 125 and 500 μg/ml, respectively, against a strain of MRSA (S et al., 2020). In the same *in vitro* study, the addition of 1 mg/ml of the neem extract resulted in a 68.9% reduction in MRSA biofilm; 2 mg/ml of the same extract resulted in a 83.8% reduction (S et al., 2020). Additionally, Guchhait *et al.* found that ripe neem seed extracts had antibiofilm activity against *S. aureus* and *Vibrio cholerae*. The minimum biofilm inhibitory concentrations (MBIC) and minimum biofilm eradication concentrations (MBEC) for this extract were 100 and 300 μg/ml, respectively, against *S. aureus* and 300 and 500 μg/ml, respectively, against *V. cholerae* (Guchhait et al., 2022). Furthermore, using a mouse model of *V. cholerae* infection, Thakurta *et al.* showed that administration of methanolic neem leaf extract at 100–1800 mg/kg body weight reduced intestinal fluid secretion by 27.7%–77.9% and doses ≥300 mg/kg inhibited *Vibrio*-induced hemorrhage in the murine intestine without signs of toxicity (Thakurta et al., 2007). Of note, individual neem-associated phytochemicals, such as catechin, may have greater potential for biofilm eradication, persister cell damage, disruption of EPS structural components, and prevention of quorum sensing (Lahiri et al., 2021). In addition, a methanolic neem oil extract and nimbolide both killed cells within *in vitro* biofilms produced

by the Gram-negative carcinogenic pathogen, *H. pylori* (Wylie et al., 2021). Overall, evidence indicates that neem has great potential to be used as a therapeutic for resistant bacterial infections. However, future research that utilizes animal models will be crucial to determine whether neem-derived products fit in with established antibiotic regimens and/or work alone to eradicate biofilms *in vivo*.

ANTIVIRAL EVIDENCE

Although most recent studies have investigated the antibacterial and antifungal potential of *A. indica*, some work has also explored the antiviral activities of neem; this topic has previously been reviewed in (Dhawan, 2012). To date, most publications have centered around human immunodeficiency virus (HIV), as well as the herpes, Dengue, and influenza viruses; however, recent reports have also included SARS-CoV-2, which is responsible for the COVID-19 pandemic. Moreover, a few groups have successfully experimented with the use of neem products against other viruses, such as Japanese encephalitis virus (Dwivedi et al., 2021b), hepatitis C (Ashfaq et al., 2016), and coxsackie virus (Younus et al., 2016). The antiviral studies have primarily focused on the ability of individual neem-associated phytochemicals to block critical processes of the viral life cycle, including cell entry and replication. Intriguingly, this means that the obtained results may describe a mechanism of action as well as identify a distinct drug candidate that can be directly modified for pharmaceutical development. Finally and importantly, as an alternative method to control specific classes of viral or parasitic diseases, many researchers have demonstrated the ability of *A. indica* derivatives to deter and/or to negatively affect the many species of insect vectors that transmit these pathogens [reviewed in (Benelli et al., 2017a)] (Soni and Prakash, 2014; Abiy et al., 2015; Benelli et al., 2015; Maheswaran and Ignacimuthu, 2015; Poopathi et al., 2015; Chandramohan et al., 2016; Murugan et al., 2016; Yerbanga et al., 2016; Benelli et al., 2017b; Kaura et al., 2019; Paramo et al., 2020; Rasool et al., 2020; Ejeta et al., 2021; Zatelli et al., 2022).

Neem and HIV

Human immunodeficiency virus, or HIV, is arguably one of the most devastating modern human pathogens. Since its discovery in the early 1980s, there have been over one million new HIV infections each year; hundreds of thousands of people still die from the subsequent acquired immunodeficiency syndrome (AIDS) annually (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984; UNAIDS, 2022). Although antiretroviral therapy (ART) is well-established and is successful at diminishing viral load and preventing disease progression, ART drugs are expensive, and require life-long treatment that is not without side effects (CDC, 2022). To this end, natural products, such as those obtained from *A. indica*, that are traditionally used for HIV-associated infections (Nagata et al., 2011; Anywar et al., 2020), have been explored for their abilities to protect the $CD4^+$ T cell population that is vulnerable during HIV infection, to reduce persistent immune activation during ART, and to decrease the toxicity of ART drugs. For instance, small trials have concluded that neem leaf extract given daily is safe and effective at improving $CD4^+$ T cell counts in HIV patients (Udeinya et al., 2004; Mbah et al., 2007). Furthermore, when *A. indica* and *Senna siamea* leaf extracts were given in combination with ART, this HIV patient group had improved T cell numbers and fewer markers of hepatic and renal toxicity than the group that was given ART alone (Goni Hamad et al., 2021). To address the possibility of T cell exhaustion in HIV-infected patients, Olwenyi et al. (2021) performed an *in vitro* study with peripheral blood cells isolated from infected and uninfected individuals; following exposure to enterotoxin, the lymphocytic response indicated that *A. indica* extract, but not the extracts from two other plants, was able to down-regulate $CD4^+$ T cell activation in a concentration-dependent manner without affecting general T cell-specific functions. Overall, these results support the idea that neem has immunomodulatory abilities that can be exploited to increase the efficacies of certain treatments and to improve the condition of chronically infected patients, such as those with HIV.

Herpes and Sulfonoquinovosyldiacylglyceride From *Azadirachta indica*

Herpes simplex viruses (HSV) most commonly cause oral and genital infections, with an estimated half a billion people living with HSV type 2 and nearly four billion people with HSV type 1 in any given year (James et al., 2020). Because there is no cure for HSV infections and they can recur many times throughout a person's life, broad searches for novel antiviral medications are warranted in order to identify agents that may reduce the morbidity associated with these infections; these searches include phytochemicals isolated from medicinal plant such as *A. indica*. For example, the glycolipid sulfonoquinovosyldiacylglyceride (SQDG) that has been isolated from neem leaves was shown to have potent antiviral activity against HSV-1 and -2; the half maximal effective concentrations (EC_{50}) were 9.1 and 8.5 μg/ml, respectively. The same study also found that HSV-infected, SQDG-treated macrophages produced significantly less proinflammatory cytokines than untreated controls (Bharitkar et al., 2014). It has been suggested that the antiviral and anti-inflammatory properties of SQDG may indicate that *A. indica* contains other phytochemicals with therapeutic potential against viruses (Shanmugam et al., 2020). Concurrently, it was found that two polysaccharides isolated from neem leaves, along with their sulfated derivates, are able to inhibit HSV-1 nucleic acid synthesis at concentrations that were not cytotoxic (Faccin-Galhardi et al., 2019). Additionally, an aqueous neem bark extract was able to block HSV-1 glycoprotein binding to and virus entry into target cells *in vitro* (Tiwari et al., 2010). The preliminary data reported here indicate that products derived from *A. indica* can act at several steps of the viral life cycle to prevent herpes infections.

Azadirachta indica Components Against Dengue Proteins

Several groups have demonstrated that computational screening methods can be successfully used to identify novel inhibitors of viruses that infect hundreds of millions of people globally each year, including vector-borne diseases such as Dengue (CDC, 2021). In the context of Dengue virus (DENV), 49 different bioflavonoids that are present in the neem tree were virtually screened for binding to the DENV serine protease, NS2B-NS3pro. Subsequent *in vitro* assays with promising candidates revealed that kaempferol 3-O-β-rutinoside and epicatechin were able to inhibit DENV-2 infectivity by 77.7% and 66.2%, respectively, without significant cell toxicity (Dwivedi et al., 2021a). Similarly, it was previously shown that three members of another important class of neem phytochemicals, the terpenoids, were able to bind to NS2B-NS3pro with high affinity *in silico*; this binding ability was subsequently confirmed *in vitro* (Dwivedi et al., 2016). Nimbin, one of the more common triterpenoids isolated from neem leaf extracts, was shown to be effective against the envelope protein of all four types of DENV *in silico* (Lavanya et al., 2015). Overall, the ability of neem-derived phytochemicals to block the activities of both the protease and envelope proteins of DENV, and potentially other viruses, further suggest that *A. indica* may be a novel source for antiviral drugs (Shanmugam et al., 2020).

Influenza and Neem Phytochemicals

Flu leads to an estimated 290,000-650,000 deaths annually (WHO, 2020). Due to these consistently high levels of associated morbidity and mortality around the world, influenza is one of the most intensely researched viruses. Though new vaccines for influenza are constantly in development, the burden of flu could be additionally reduced by the introduction of an antiviral that is effective at all stages of disease. Recent evidence suggests that *A. indica* may represent a robust source of novel drugs against viruses such as influenza. To summarize, molecular docking experiments identified a total of four neem phytochemicals that interact with conserved residues of either the nucleoprotein or the non-structural (NS1) protein of influenza. Though requiring further testing, this may indicate the ability to act as a universal drug against the flu virus (Ahmad et al., 2015; Ahmad et al., 2016).

Azadirachta indica and SARS-CoV-2

Taking the lives of more than five million people over the last 2 years, SARS-CoV-2 and the associated disease, COVID-19, continue to be significant threats to public health for which there are few treatment options (WHO, 2021b). Consequently, many researchers have successfully screened chemical libraries for viral inhibitors that act against SARS-CoV-2 (Garg et al., 2020; Vardhan and Sahoo, 2020; Baildya et al., 2021; Borkotoky and Banerjee, 2021; Gogoi et al., 2021; Nallusamy et al., 2021; Navabshan et al., 2021; Senapati et al., 2021; Ogidigo et al., 2022; Vardhan and Sahoo, 2022); detailed reviews on the potential for medicinal plants to be used against SARS-CoV-2 are available (Thota et al., 2020; Adithya et al., 2021). Phytochemicals and plant-derived products like those from *A. indica* are sometimes included in these chemical libraries. For example, *in silico* binding simulations by Parida et al. (2020) identified nimolicinol as a compound with strong affinity for both the main protease and the spike protein of SARS-CoV-2; similar docking studies found that nimbocinol binds to the papain-like protease of SARS-CoV-2 with higher affinity than remdesivir, indicating its potential to hinder viral replication (Balkrishna et al., 2021). Although these types of studies are high throughput and can detect candidates for further investigation, virtual studies must typically be followed by *in vitro* experiments before an antiviral can have clinical relevance. Despite this, given the devastation associated with the ongoing pandemic, several clinical trials are in progress to test neem as a component of mouthwashes, nasal sprays, and capsules for suspected, confirmed, or hospitalized COVID-19 patients, or for prophylaxis (Khan et al., 2020; Farhan Raza Khan, 2021; Nesari et al., 2021). Additionally, one animal study has been completed in which coronavirus-infected mice were treated orally or intranasally with neem bark extract; overall, the therapy was able to prevent systemic injury and pathologic effects of the virus both *in vivo* and *in vitro* using multiple model systems (Sarkar et al., 2022). Advocating for additional trials of neem against SARS-CoV-2, Eze et al. (2022) made connections between some of the known mechanisms of *A. indica* products against other illnesses and the potential efficacy of neem against the virus that causes COVID-19. For additional information about the pharmacological implications for the use of medicinal plants against respiratory infections, see the recent review by Timalsina et al. (2021). All in all, the antiviral action of *A. indica* against a variety of viruses that cause human disease is evident (**Supplementary Table S1**). Moreover, outside of HIV, HSV, DENV, influenza, and SARS-CoV-2, there is a strong indication that the aforementioned phytochemical screening methods may be used to identify novel inhibitors of other viral life cycles.

ANTIPARASITIC EVIDENCE

Although the area of research regarding *A. indica* as a potential therapeutic against parasites is relatively underdeveloped, most of the investigations that have been published so far have focused on *Plasmodium*, the human pathogen of global importance that causes malaria; more than half a million deaths attributed to malaria are estimated to occur each year (WHO, 2021c). While there are effective prophylactics and therapeutics approved for this disease, these resources are not readily available in all parts of the world. In these instances, there is the possibility that natural products may be more easily acquired, distributed, and accepted; neem products could be part of the solution. Recently, a comprehensive review on the antiplasmodial activities of African medicinal plants was published and found that neem extracts consistently performed well across experiments (Tajbakhsh et al., 2021). Studies using both *in vitro* and *in vivo* models indicate that aqueous, methanolic, or ethanolic extracts of neem stems, leaves, or bark all have significant antimalarial activity against a variety of *Plasmodium*

falciparum and *Plasmodium berghei* strains (Benoit et al., 1996; MacKinnon et al., 1997; El-Tahir et al., 1999; Gathirwa et al., 2011; BC et al., 2013; Tepongning et al., 2018). These publications suggest that *A. indica* and other medicinal plants are active against malaria; however, only one clinical trial with an African plant species has been conducted so far (Benoit-Vical et al., 2003). Despite the lack of controlled human studies, the malaria mouse model has been used to demonstrate that neem bark and seed extracts are active against *Plasmodium* infection both alone and as part of a polyherbal mixture; in the case of the bark extract, this was indicated by parasite suppression or decreased erythrocyte infection (Habluetzel et al., 2019; Alaribe et al., 2021). The gestational malaria mouse model has also been used to show that administration of *A. indica* leaves improves the overall health of *P. falciparum*-infected dams, including reduced parasitemia, increased platelet counts, lower levels of preeclampsia biomarkers, and increased birth weight of pups (Amadi et al., 2021). Additionally, the limonoid deacetylnimbin, which is found within neem seed extracts, is able to interfere with the early sporogony stages of *P. bergehi*; this finding suggests that certain neem-associated phytochemicals may have the ability to limit *Plasmodium* transmissibility, warranting further investigation in other models and clinical trials (Tapanelli et al., 2016). Another commonly studied phytochemical of *A. indica*, azadirachtin, was shown to bind to Gephyrin E almost as well as artesunate during *in silico* analyses, which indicates that, upon further study, azadirachtin may be an effective treatment for cerebral malaria (Okoh et al., 2021). Providing additional convincing data for the investigation of neem products as therapeutics, Somsak et al. (2015) used the mouse model of *Plasmodium* infection to show that an aqueous neem leaf extract was able to reduce the blood markers of malaria-induced renal injury to normal levels without being toxic to the animals. Taken together, the available *in vitro* and *in vivo* data suggest that plants used in traditional medicine (e.g., neem) should be further explored in clinical trials for their antimalarial capacity.

Throughout tropical and subtropical areas around the world, the neglected tropical protozoan leishmania causes an estimated 700,000 to 1 million infections each year and is highly fatal if left untreated; visceral leishmaniasis is the most severe form of associated disease (WHO, 2021a). Leishmaniasis can be treated, but there is not currently a drug available that eliminates the leishmania parasite from the body; thus, the patient is susceptible to relapse if immunosuppression occurs (WHO, 2021a). Although studies on this topic are limited, there is some evidence that plant extracts can kill leishmania parasites, including an ethyl acetate fraction (EAF) of neem leaf extract (Dayakar et al., 2015). When treated with this EAF, promastigotes underwent an apoptosis-like death and intracellular amastigotes were also killed *in vitro* and *in vivo* (Dayakar et al., 2015). With this evidence, more *in vivo* and translational research on the antileishmanial activity of neem is merited.

Finally, the last antimicrobial activity of *A. indica* that has been recently explored is that against parasitic worms (**Figure 1**). As the most widespread neglected tropical diseases globally, the burden of schistosomiasis and soil-transmitted helminth infections could undoubtedly be reduced with the addition of new and accessible plant-derived treatments or prophylactics to the already available drug regimens (Molyneux et al., 2017). Although still a minor area of research, the antischistosomal and anthelminthic properties of neem suggest that natural products can be potent inhibitors of larger eukaryotic pathogens as well. Indeed, the same neem leaf extract that was effective against *S. aureus* and MRSA biofilms also caused severe tegument morphology changes, significant reduction of motor activity, or death of *Schistosoma mansoni* worms *in vitro* (Quelemes et al., 2015). For helminth related studies, an *in vivo* experiment showed that neem leaf powder used at 500 mg/kg body weight worked as well as 5 mg/kg fenbendazole to treat cows with bovine *Strongyloides* infections (Jamra et al., 2015); *Strongyloides* is a major hurdle to profitable farming in tropical and subtropical regions and can have a substantial economic impact (Jamra et al., 2015). Similarly, problematic helminths for the poultry industry are *Raillietina* spp., which are parasitic tapeworms. These organisms were found to be severely paralyzed, damaged, or killed by short exposures to SQDG, a glycolipid from neem extracts that also has antiviral activity (Ash et al., 2017). Although not yet tested on human helminth infections, neem-derived products appear to have strong potential against animal pathogens, thus supporting the need for a deeper investigation of the overall antiparasitic activity of *A. indica*.

THE FUTURE OF NEEM AS AN ANTIMICROBIAL

As summarized in this review, a variety of extracts and phytochemicals from the Indian evergreen tree, *A. indica*, have significant antimicrobial activity against a multitude of pathogens that affect human health. The dental application of neem appears to be one of the most researched areas, with the general antibacterial properties of neem not far behind. Intriguingly, there are several recent studies that suggest that neem may have broad applications in the food industry, aside from its traditional uses in agriculture for pest-control and fertilizer. Some of the least-developed areas, however, are the antiviral and antiparasitic activities of neem-derived products. Overall, there is sufficient evidence to warrant further investigation into these properties of *A. indica*; undoubtedly, *in vivo* models will be crucial to understanding the clinical relevance of neem against all of the microorganisms mentioned here and those that have yet to be tested.

Of note in recent years, there are several groups who have incorporated neem into novel materials and technologies that have broad implications for human health. Specifically, green-synthesized copper or silver nanoparticles and hydrogels, nanocellulose films, chitosan-copper oxide biopolymers, and hydroxyapatite have all been constructed to include neem extracts and have substantial antimicrobial activity, including against multidrug-resistant bacterial species (Nagaraj and Samiappan, 2019; Revathi and Thambidurai, 2019; Algebaly et al., 2020; Asghar and Asghar, 2020; Lakkim et al., 2020;

Sharma and Bhardwaj, 2020; Ulaeto et al., 2020; Chinnasamy et al., 2021; Ghazali et al., 2022; Lan Chi et al., 2022). Both *in vitro* and *in vivo* data suggest that these composite materials represent a growing industry of creative antimicrobial technologies that have the potential to revolutionize infectious disease treatments and biomedical science as a whole.

In addition to the many recently published manuscripts that indicate that *A. indica*-derived substances have broad-spectrum antimicrobial properties, it is worth noting that dozens of patents are filed each year that mention neem-based products. Indeed, a simple query of the United States Patent and Trademark Office's online Patent Public Search tool (USPTO, 2022) using the terms "neem and antimicrobial" yields over 400 results since 2015. This available list of patents helps to demonstrate the limitless applications of the antimicrobial properties of neem. While too many exist to cover in detail here, some notable examples include very diverse applications. For example, medical gloves (Wong et al., 2022) and a polymeric yarn for use in hygienic textiles (Mandawewala and Mandawewala, 2021) have both been enriched with neem derivates and shown to have beneficial antimicrobial properties. Furthermore, in line with the reported antimicrobial uses of neem in the fields of dentistry, dermatology, and agriculture, many of the patented neem-containing products fall into these categories as well; patents have been awarded for neem-containing dental rinses and composites (Tatch, 2021; Ramana, 2022), for neem and/or other plant extract-containing topical treatments for mild skin disorders (Balaraman, 2015), and for neem-based pest control formulas (Mazariegos, 2016). Clearly, the investigation of neem as an antimicrobial is an area of research that is constantly expanding and is generating valuable products that may improve human health.

In order to develop realistic *A. indica*-based treatment regimens that could be used in humans, there are clearly many intriguing areas for future investigation. Undoubtably, future experiments will need to elucidate the mechanisms of action of neem and the associated phytochemicals. Given the available data summarized in this review, some of the most promising areas of investigation moving forward appear to be 1) the application of individual neem phytochemicals and derivatives thereof as antimicrobial agents alone or used in combination with existing treatments, 2) additional pre-clinical and clinical studies to determine the toxicity and effective *in vivo* dosing of specific phytochemicals as compared to parent neem extracts, and 3) the inclusion of hundreds of available medicinal plant products, extracts, and phytochemicals in screens for potential inhibitors of emerging and resistant infectious diseases. It is important to note that to gain maximal utility from these areas of research, close attention should be paid to the types of extracts (including both the particular part of the plant and the solvent) that have already been tested against which organisms. Some level of standardization should be considered so that comparisons can be made and patterns can be recognized across multiple studies. This may become easier when the antimicrobial activities of more individual phytochemicals are determined. *En masse*, *A. indica* represents a novel source of antimicrobials that may be used to combat drug resistance and emerging threats to human health. Furthermore, the research that has been done on the neem tree can be used as a guide to encourage the investigation of other traditionally used natural products for their utility as modern pharmaceuticals.

AUTHOR CONTRIBUTIONS

MW and DM both contributed to conceptualization of the review. MW performed the investigation and wrote the original draft. MW and DM contributed to manuscript revision and both authors read and approved the final version.

FUNDING

Research in the Merrell lab is supported by funding from the NIH and the United States DoD.

ACKNOWLEDGMENTS

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the Uniformed Services University or the Department of Defense (DoD). We would like to acknowledge Jeannette Whitmire for critical review of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.891535/full#supplementary-material

REFERENCES

Abiy, E., Gebre-Michael, T., Balkew, M., and Medhin, G. (2015). Repellent Efficacy of DEET, MyggA, Neem (*Azedirachta indica*) Oil and Chinaberry (*Melia azedarach*) Oil against *Anopheles arabiensis*, the Principal Malaria Vector in Ethiopia. *Malar. J.* 14, 187. doi:10.1186/s12936-015-0705-4

Adithya, J., Nair, B., Aishwarya, T. S., and Nath, L. R. (2021). The Plausible Role of Indian Traditional Medicine in Combating Corona Virus (SARS-CoV 2): A Mini-Review. *Curr. Pharm. Biotechnol.* 22 (7), 906–919. doi:10.2174/1389201021666200807111359

Ahmad, A., Ahad, A., Rao, A. Q., and Husnain, T. (2015). Molecular Docking Based Screening of Neem-Derived Compounds with the NS1 Protein of Influenza Virus. *Bioinformation* 11 (7), 359–365. doi:10.6026/97320630011359

Ahmad, A., Javed, M. R., Rao, A. Q., and Husnain, T. (2016). Designing and Screening of Universal Drug from Neem (*Azadirachta indica*) and Standard Drug Chemicals against Influenza Virus Nucleoprotein. *BMC Complement. Altern. Med.* 16 (1), 519. doi:10.1186/s12906-016-1469-2

Ahmad, I., and Aqil, F. (2007). *In Vitro* efficacy of Bioactive Extracts of 15 Medicinal Plants against ESbetaL-Producing Multidrug-Resistant Enteric Bacteria. *Microbiol. Res.* 162 (3), 264–275. doi:10.1016/j.micres.2006.06.010

Ahmed, W., Azmat, R., Khojah, E., Ahmed, R., Qayyum, A., Shah, A. N., et al. (2022). The Development of a Green Innovative Bioactive Film for Industrial Application as a New Emerging Technology to Protect the Quality of Fruits. *Molecules* 27 (2), 486. doi:10.3390/molecules27020486

Al Akeel, R., Mateen, A., Janardhan, K., and Gupta, V. C. (2017). Analysis of Antibacterial and Anti Oxidative Activity of *Azadirachta indica* Bark Using Various Solvents Extracts. *Saudi J. Biol. Sci.* 24 (1), 11–14. doi:10.1016/j.sjbs.2015.08.006

Al Saiqali, M., Tangutur, A. D., Banoth, C., and Bhukya, B. (2018). Antimicrobial and Anticancer Potential of Low Molecular Weight Polypeptides Extracted and Characterized from Leaves of *Azadirachta indica*. *Int. J. Biol. Macromol.* 114, 906–921. doi:10.1016/j.ijbiomac.2018.03.169

Aiyegoro, A., and Okoh, A. I. (2009). Use of Bioactive Plant Products in Combination with Standard Antibiotics: Implications in Antimicrobial Chemotherapy. *J. Med. Plants Res.* 3 (13), 1147–1152.

Alaribe, S. C., Oladipupo, A. R., Uche, G. C., Onumba, M. U., Ota, D., Awodele, O., et al. (2021). Suppressive, Curative, and Prophylactic Potentials of an Antimalarial Polyherbal Mixture and its Individual Components in *Plasmodium Berghei*-Infected Mice. *J. Ethnopharmacol.* 277, 114105. doi:10.1016/j.jep.2021.114105

Algebaly, A. S., Mohammed, A. E., Abutaha, N., and Elobeid, M. M. (2020). Biogenic Synthesis of Silver Nanoparticles: Antibacterial and Cytotoxic Potential. *Saudi J. Biol. Sci.* 27 (5), 1340–1351. doi:10.1016/j.sjbs.2019.12.014

Ali, A., Shahid, M. A., Hossain, M. D., and Islam, M. N. (2019). Antibacterial Bilayered Polyvinyl Alcohol (PVA)-chitosan Blend Nanofibrous Mat Loaded with *Azadirachta indica* (Neem) Extract. *Int. J. Biol. Macromol.* 138, 13–20. doi:10.1016/j.ijbiomac.2019.07.015

Alzohairy, M. A. (2016). Therapeutics Role of *Azadirachta indica* (Neem) and Their Active Constituents in Diseases Prevention and Treatment. *Evid. Based Complement. Altern. Med.* 2016, 7382506. doi:10.1155/2016/7382506

Amadi, P. U., Agomuo, E. N., Ukaga, C. N., Njoku, U. C., Amadi, J. A., and Nwaekpe, C. G. (2021). Preclinical Trial of Traditional Plant Remedies for the Treatment of Complications of Gestational Malaria. *Med. (Basel)* 8 (12). doi:10.3390/medicines8120079

An, A., Je, A., Cb, U., and Mn, I. (2019). Identification and Control of Specific Aflatoxin-Producing Fungi in Stored Maize Seeds in Awka Using *Azadirachta indica* (Neem) and *garcinia Kola* Seeds. *Pak J. Pharm. Sci.* 32 (4), 1679–1686.

Anand, P. J., Athira, S., Chandramohan, S., Ranjith, K., Raj, V. V., and Manjula, V. D. (2016). Comparison of Efficacy of Herbal Disinfectants with Chlorhexidine Mouthwash on Decontamination of Toothbrushes: An Experimental Trial. *J. Int. Soc. Prev. Community Dent.* 6 (1), 22–27. doi:10.4103/2231-0762.175406

Andonissamy, L., Karthigeyan, S., Ali, S. A., and Felix, J. W. (2019). Effect of Chemical Denture Disinfectants and Tree Extracts on Biofilm-Forming *Staphylococcus aureus* and *Viridans Streptococcus* Species Isolated from Complete Denture. *J. Contemp. Dent. Pract.* 20 (11), 1307–1314.

Anywar, G., Kakudidi, E., Byamukama, R., Mukonzo, J., Schubert, A., and Oryem-Origa, H. (2020). Indigenous Traditional Knowledge of Medicinal Plants Used by Herbalists in Treating Opportunistic Infections Among People Living with HIV/AIDS in Uganda. *J. Ethnopharmacol.* 246, 112205. doi:10.1016/j.jep.2019.112205

Arora, S., Saquib, S. A., Algarni, Y. A., Kader, M. A., Ahmad, I., Alshahrani, M. Y., et al. (2021). Synergistic Effect of Plant Extracts on Endodontic Pathogens Isolated from Teeth with Root Canal Treatment Failure: An *In Vitro* Study. *Antibiot. (Basel)* 10 (5). doi:10.3390/antibiotics10050552

Arsene, M. M. J., Viktorovna, P. I., and Davares, A. K. L. (2021). *Galleria mellonella* (Greater Wax Moth) as an Eco-Friendly *In Vivo* Approach for the Assessment of the Acute Toxicity of Medicinal Plants: Application to Some Plants from Cameroon. *Open Vet. J.* 11 (4), 651–661. doi:10.5455/OVJ.2021.v11.i4.15

Asghar, M. A., and Asghar, M. A. (2020). Green Synthesized and Characterized Copper Nanoparticles Using Various New Plants Extracts Aggravate Microbial Cell Membrane Damage after Interaction with Lipopolysaccharide. *Int. J. Biol. Macromol.* 160, 1168–1176. doi:10.1016/j.ijbiomac.2020.05.198

Ash, A., Bharitkar, Y. P., Murmu, S., Hazra, A., Ravichandiran, V., Kar, P. K., et al. (2017). Ultrastructural Changes in *Raillietina* (Platyhelminthes: Cestoda), Exposed to Sulfonoquinovosyldiacylglyceride (SQDG), Isolated from Neem (*Azadirachta indica*). *Nat. Prod. Res.* 31 (20), 2445–2449. doi:10.1080/14786419.2017.1305383

Ashafa, A. O., Orekoya, L. O., and Yakubu, M. T. (2012). Toxicity Profile of Ethanolic Extract of *Azadirachta indica* Stem Bark in Male Wistar Rats. *Asian Pac J. Trop. Biomed.* 2 (10), 811–817. doi:10.1016/S2221-1691(12)60234-2

Ashfaq, U. A., Jalil, A., and Ul Qamar, M. T. (2016). Antiviral Phytochemicals Identification from *Azadirachta indica* Leaves against HCV NS3 Protease: an In Silico Approach. *Nat. Prod. Res.* 30 (16), 1866–1869. doi:10.1080/14786419.2015.1075527

Attaran, B., Falsafi, T., and Ghorbanmehr, N. (2017). Effect of Biofilm Formation by Clinical Isolates of *Helicobacter pylori* on the Efflux-Mediated Resistance to Commonly Used Antibiotics. *World J. Gastroenterol.* 23 (7), 1163–1170. doi:10.3748/wjg.v23.i7.1163

Aumeeruddy, M. Z., and Mahomoodally, M. F. (2021). Ethnomedicinal Plants for the Management of Diabetes Worldwide: A Systematic Review. *Curr. Med. Chem.* 28 (23), 4670–4693. doi:10.2174/0929867328666210121123037

Ayaz, M., Ullah, F., Sadiq, A., Ullah, F., Ovais, M., Ahmed, J., et al. (2019). Synergistic Interactions of Phytochemicals with Antimicrobial Agents: Potential Strategy to Counteract Drug Resistance. *Chem. Biol. Interact.* 308, 294–303. doi:10.1016/j.cbi.2019.05.050

Agency, U.S.E.P. (2012). in *Cold Pressed Neem Oil PC Code 025006*. Editor P. P. D. B.A (NW Washington DC: Office of Pesticide Programs).

Bae, J., and Jeon, B. (2013). Increased Emergence of Fluoroquinolone-Resistant *Campylobacter jejuni* in Biofilm. *Antimicrob. Agents Chemother.* 57 (10), 5195–5196. doi:10.1128/AAC.00995-13

Baildya, N., Khan, A. A., Ghosh, N. N., Dutta, T., and Chattopadhyay, A. P. (2021). Screening of Potential Drug from *Azadiractha indica* (Neem) Extracts for SARS-CoV-2: An Insight from Molecular Docking and MD-simulation Studies. *J. Mol. Struct.* 1227, 129390. doi:10.1016/j.molstruc.2020.129390

Balaraman, B. (2015). *Compositions and Methods for Their Dermatological Use. 14/646049*.

Balkrishna, A., Mittal, R., and Arya, V. (2021). Computational Evidences of Phytochemical Mediated Disruption of PLpro Driven Replication of SARS-CoV-2: A Therapeutic Approach against COVID-19. *Curr. Pharm. Biotechnol.* 22 (10), 1350–1359. doi:10.2174/1389201021999201110204116

Bandyopadhyay, U., Biswas, K., Sengupta, A., Moitra, P., Dutta, P., Sarkar, D., et al. (2004). Clinical Studies on the Effect of Neem (*Azadirachta indica*) Bark Extract on Gastric Secretion and Gastroduodenal Ulcer. *Life Sci.* 75 (24), 2867–2878. doi:10.1016/j.lfs.2004.04.050

Bansal, V., Gupta, M., Bhaduri, T., Shaikh, S. A., Sayed, F. R., Bansal, V., et al. (2019). Assessment of Antimicrobial Effectiveness of Neem and Clove Extract against *Streptococcus Mutans* and *Candida Albicans*: An *In Vitro* Study. *Niger. Med. J.* 60 (6), 285–289. doi:10.4103/nmj.NMJ_20_19

Barbieri, R., Coppo, E., Marchese, A., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S. F., et al. (2017). Phytochemicals for Human Disease: An Update on Plant-Derived Compounds Antibacterial Activity. *Microbiol. Res.* 196, 44–68. doi:10.1016/j.micres.2016.12.003

Barré-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., et al. (1983). Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). *Science* 220 (4599), 868–871. doi:10.1126/science.6189183

Barua, D. R., Basavanna, J. M., and Varghese, R. K. (2017). Efficacy of Neem Extract and Three Antimicrobial Agents Incorporated into Tissue Conditioner in Inhibiting the Growth of *C. albicans* and *S. mutans*. *J. Clin. Diagn Res.* 11 (5), ZC97–ZC101. doi:10.7860/JCDR/2017/23784.9950

Bc, A.-O., Aj, N., Salisu, I., Hajia Mairo, I., E, O., E, A., et al. (2013). Antimalarial Effect of Neem Leaf and Neem Stem Bark Extracts on *Plasmodium berghei* Infected in the Pathology and Treatment of Malaria. *Int. J. Res. Biochem. Biophysics* 3 (1), 7–14.

Benelli, G., Bedini, S., Cosci, F., Toniolo, C., Conti, B., and Nicoletti, M. (2015). Larvicidal and Ovideterrent Properties of Neem Oil and Fractions against the Filariasis Vector *Aedes albopictus* (Diptera: Culicidae): a Bioactivity Survey across Production Sites. *Parasitol. Res.* 114 (1), 227–236. doi:10.1007/s00436-014-4183-3

Benelli, G., Canale, A., Toniolo, C., Higuchi, A., Murugan, K., Pavela, R., et al. (2017a). Neem (*Azadirachta indica*): towards the Ideal Insecticide? *Nat. Prod. Res.* 31 (4), 369–386. doi:10.1080/14786419.2016.1214834

Benelli, G., Chandramohan, B., Murugan, K., Madhiyazhagan, P., Kovendan, K., Panneerselvam, C., et al. (2017b). Neem Cake as a Promising Larvicide and

Adulticide against the Rural Malaria Vector *Anopheles culicifacies* (Diptera: Culicidae): a HPTLC Fingerprinting Approach. *Nat. Prod. Res.* 31 (10), 1185–1190. doi:10.1080/14786419.2016.1222390

Benoit, F., Valentin, A., Pelissier, Y., Diafouka, F., Marion, C., Kone-Bamba, D., et al. (1996). *In Vitro* antimalarial Activity of Vegetal Extracts Used in West African Traditional Medicine. *Am. J. Trop. Med. Hyg.* 54 (1), 67–71. doi:10.4269/ajtmh.1996.54.67

Benoit-Vical, F., Valentin, A., Da, B., Dakuyo, Z., Descamps, L., and Mallié, M. (2003). N'Dribala (*Cochlospermum Planchonii*) versus Chloroquine for Treatment of Uncomplicated *Plasmodium Falciparum* Malaria. *J. Ethnopharmacol.* 89 (1), 111–114. doi:10.1016/s0378-8741(03)00277-0

Bhardwaj, A., Srivastava, N., Rana, V., Adlakha, V. K., and Asthana, A. K. (2017). How Efficacious Are Neem, Tulsi, Guduchi Extracts and Chlorhexidine as Intracanal Disinfectants? A Comparative *Ex Vivo* Study. *Ayu* 38 (1-2), 70–75. doi:10.4103/ayu.AYU_72_16

Bharitkar, Y. P., Bathini, S., Ojha, D., Ghosh, S., Mukherjee, H., Kuotsu, K., et al. (2014). Antibacterial and Antiviral Evaluation of Sulfonoquinovosyldiacylglyceride: a Glycolipid Isolated from *Azadirachta indica* Leaves. *Lett. Appl. Microbiol.* 58 (2), 184–189. doi:10.1111/lam.12174

Blum, F. C., Singh, J., and Merrell, D. S. (2019). *In Vitro* activity of Neem (*Azadirachta indica*) Oil Extract against *Helicobacter pylori*. *J. Ethnopharmacol.* 232, 236–243. doi:10.1016/j.jep.2018.12.025

Boeke, S. J., Boersma, M. G., Alink, G. M., van Loon, J. J., van Huis, A., Dicke, M., et al. (2004). Safety Evaluation of Neem (*Azadirachta indica*) Derived Pesticides. *J. Ethnopharmacol.* 94 (1), 25–41. doi:10.1016/j.jep.2004.05.011

Borges, A., Abreu, A. C., Dias, C., Saavedra, M. J., Borges, F., and Simões, M. (2016). New Perspectives on the Use of Phytochemicals as an Emergent Strategy to Control Bacterial Infections Including Biofilms. *Molecules* 21 (7). doi:10.3390/molecules21070877

Borkotoky, S., and Banerjee, M. (2021). A Computational Prediction of SARS-CoV-2 Structural Protein Inhibitors from *Azadirachta indica* (Neem). *J. Biomol. Struct. Dyn.* 39 (11), 4111–4121. doi:10.1080/07391102.2020.1774419

Braga, T. M., Rocha, L., Chung, T. Y., Oliveira, R. F., Pinho, C., Oliveira, A. I., et al. (2021). *Azadirachta indica* A. Juss. *In Vivo* Toxicity-An Updated Review. *Molecules* 26 (2). doi:10.3390/molecules26020252

Braga, T. M., Rocha, L., Chung, T. Y., Oliveira, R. F., Pinho, C., Oliveira, A. I., et al. (2020). Biological Activities of Gedunin-A Limonoid from the Meliaceae Family. *Molecules* 25 (3). doi:10.3390/molecules25030493

Brahmachari, G. (2004). Neem--an Omnipotent Plant: a Retrospection. *Chembiochem* 5 (4), 408–421. doi:10.1002/cbic.200300749

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* 68 (6), 394–424. doi:10.3322/caac.21492

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., et al. (2019). Attributable Deaths and Disability-Adjusted Life-Years Caused by Infections with Antibiotic-Resistant Bacteria in the EU and the European Economic Area in 2015: a Population-Level Modelling Analysis. *Lancet Infect. Dis.* 19 (1), 56–66. doi:10.1016/S1473-3099(18)30605-4

CDC (2021). *About Dengue: What You Need to Know cdc.Gov: Centers for Disease Control and Prevention*. Available: cdc.gov/dengue/about/index.html

CDC (2019). *Antibiotic Resistance Threats in the United States, 2019*. Available: www.cdc.gov/DrugResistance/Biggest-Threats.html.

CDC E (2019). *E. coli (Escherichia coli) Questions & Answers [Online]. U.S. Department of Health & Human Services*. Available: https://www.cdc.gov/ecoli/general/index.html#:~:text=How%20common%20are%20STEC%20infections,O157%20STEC%20cause%20the%20rest.

CDC (2022). *HIV Treatment cdc.Gov: Centers for Disease Control and Prevention*. Available: cdc.gov/hiv/basics/livingwithhiv/treatment.html

Chandramohan, B., Murugan, K., Panneerselvam, C., Madhiyazhagan, P., Chandirasekar, R., Dinesh, D., et al. (2016). Characterization and Mosquitocidal Potential of Neem Cake-Synthesized Silver Nanoparticles: Genotoxicity and Impact on Predation Efficiency of Mosquito Natural Enemies. *Parasitol. Res.* 115 (3), 1015–1025. doi:10.1007/s00436-015-4829-9

Chandrappa, P. M., Dupper, A., Tripathi, P., Arroju, R., Sharma, P., and Sulochana, K. (2015). Antimicrobial Activity of Herbal Medicines (Tulsi Extract, Neem Extract) and Chlorhexidine against *Enterococcus faecalis* in Endodontics: An *In Vitro* Study. *J. Int. Soc. Prev. Community Dent.* 5 (Suppl. 2), S89–S92. doi:10.4103/2231-0762.172952

Chassagne, F., Samarakoon, T., Porras, G., Lyles, J. T., Dettweiler, M., Marquez, L., et al. (2020). A Systematic Review of Plants with Antibacterial Activities: A Taxonomic and Phylogenetic Perspective. *Front. Pharmacol.* 11, 586548. doi:10.3389/fphar.2020.586548

Chaudhary, S., Kanwar, R. K., Sehgal, A., Cahill, D. M., Barrow, C. J., Sehgal, R., et al. (2017). Progress on *Azadirachta indica* Based Biopesticides in Replacing Synthetic Toxic Pesticides. *Front. Plant Sci.* 8, 610. doi:10.3389/fpls.2017.00610

Chinnasamy, G., Chandrasekharan, S., Koh, T. W., and Bhatnagar, S. (2021). Synthesis, Characterization, Antibacterial and Wound Healing Efficacy of Silver Nanoparticles from *Azadirachta indica*. *Front. Microbiol.* 12, 611560. doi:10.3389/fmicb.2021.611560

Cowan, M. M. (1999). Plant Products as Antimicrobial Agents. *Clin. Microbiol. Rev.* 12 (4), 564–582. doi:10.1128/CMR.12.4.564

Cragg, G. M., and Newman, D. J. (2013). Natural Products: a Continuing Source of Novel Drug Leads. *Biochim. Biophys. Acta* 1830 (6), 3670–3695. doi:10.1016/j.bbagen.2013.02.008

Dahiya, P., Dahiya, P., and Purkayastha, S. (2012). Phytochemical Screening and Antimicrobial Activity of Some Medicinal Plants against Multi-Drug Resistant Bacteria from Clinical Isolates. *Indian J. Pharm. Sci.* 74 (5), 443–450. doi:10.4103/0250-474X.108420

Dayakar, A., Chandrasekaran, S., Veronica, J., Sundar, S., and Maurya, R. (2015). *In Vitro* and *In Vivo* Evaluation of Anti-leishmanial and Immunomodulatory Activity of Neem Leaf Extract in *Leishmania Donovani* Infection. *Exp. Parasitol.* 153, 45–54. doi:10.1016/j.exppara.2015.02.011

de Vor, L., Rooijakkers, S. H. M., and van Strijp, J. A. G. (2020). *Staphylococci* Evade the Innate Immune Response by Disarming Neutrophils and Forming Biofilms. *FEBS Lett.* 594 (16), 2556–2569. doi:10.1002/1873-3468.13767

Dedhia, J., Mukharjee, E., Luke, A. M., Mathew, S., and Pawar, A. M. (2018). Efficacy of *Andrographis Paniculata* Compared to *Azadirachta indica, Curcuma longa*, and Sodium Hypochlorite when Used as Root Canal Irrigants against *Candida Albicans* and *Staphylococcus aureus*: An *In Vitro* Antimicrobial Study. *J. Conserv. Dent.* 21 (6), 642–645. doi:10.4103/JCD.JCD_118_18

Del Serrone, P., Failla, S., and Nicoletti, M. (2015a). Natural Control of Bacteria Affecting Meat Quality by a Neem (*Azadirachta indica* A. Juss) Cake Extract. *Nat. Prod. Res.* 29 (10), 985–987. doi:10.1080/14786419.2014.964708

Del Serrone, P., Toniolo, C., and Nicoletti, M. (2015b). Neem (*Azadirachta indica* A. Juss) Oil to Tackle Enteropathogenic *Escherichia coli*. *Biomed. Res. Int.* 2015, 343610. doi:10.1155/2015/343610

Dhawan, B. N. (2012). Anti-Viral Activity of Indian Plants. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 82 (1), 209–224. doi:10.1007/s40011-011-0016-7

Dwivedi, V. D., Bharadwaj, S., Afroz, S., Khan, N., Ansari, M. A., Yadava, U., et al. (2021a). Anti-dengue Infectivity Evaluation of Bioflavonoid from *Azadirachta indica* by Dengue Virus Serine Protease Inhibition. *J. Biomol. Struct. Dyn.* 39 (4), 1417–1430. doi:10.1080/07391102.2020.1734485

Dwivedi, V. D., Singh, A., El-Kafraway, S. A., Alandijany, T. A., Faizo, A. A., Bajrai, L. H., et al. (2021b). Mechanistic Insights into the Japanese Encephalitis Virus RNA Dependent RNA Polymerase Protein Inhibition by Bioflavonoids from *Azadirachta indica*. *Sci. Rep.* 11 (1), 18125. doi:10.1038/s41598-021-96917-0

Dwivedi, V. D., Tripathi, I. P., and Mishra, S. K. (2016). In Silico evaluation of Inhibitory Potential of Triterpenoids from *Azadirachta indica* against Therapeutic Target of Dengue Virus, NS2B-NS3 Protease. *J. Vector Borne Dis.* 53 (2), 156–161.

Ejeta, D., Asme, A., and Asefa, A. (2021). Insecticidal Effect of Ethnobotanical Plant Extracts against *Anopheles arabiensis* under Laboratory Conditions. *Malar. J.* 20 (1), 466. doi:10.1186/s12936-021-04004-6

El-Tahir, A., Satti, G. M., and Khalid, S. A. (1999). Antiplasmodial Activity of Selected Sudanese Medicinal Plants with Emphasis on *Acacia Nilotica*. *Phytother. Res.* 13 (6), 474–478. doi:10.1002/(sici)1099-1573(199909)13:6<474:aid-ptr482>3.0.co;2-6

Essuman, E. K., Boakye, A. A., Tettey, C. O., Hunkpe, G., Kortei, N. K., Kwansa-Bentum, H., et al. (2021). Evaluation of the Antidiarrheal and Antioxidant Effects of Some Chewing Sticks Commonly Used for Oral Hygiene in Ghana. *Evid. Based Complement. Altern. Med.* 2021, 7270250. doi:10.1155/2021/7270250

Eze, M. O., Ejike, C. E. C. C., Ifeonu, P., Udeinya, I. J., Udenigwe, C. C., and Uzoegwu, P. N. (2022). Anti-COVID-19 Potential of *Azadirachta indica* (Neem) Leaf Extract. *Sci. Afr.* 16, e01184. doi:10.1016/j.sciaf.2022.e01184

Ezeonu, C. S., Tatah, V. S., Imo, C., Mamma, E., Mayel, M. H., Kukoyi, A. J., et al. (2019). Inhibitory Effect of Aqueous and Ethanolic Extracts of Neem Parts on Fungal Rot Disease of *Solanum tuberosum*. *Pak J. Biol. Sci.* 22 (5), 206–213. doi:10.3923/pjbs.2019.206.213

Faccin-Galhardi, L. C., Ray, S., Lopes, N., Ali, I., Espada, S. F., Dos Santos, J. P., et al. (2019). Assessment of Antiherpetic Activity of Nonsulfated and Sulfated Polysaccharides from *Azadirachta indica*. *Int. J. Biol. Macromol.* 137, 54–61. doi:10.1016/j.ijbiomac.2019.06.129

Farhan Raza Khan, B. (2021). *A Double Blind, Randomized Controlled Pilot Trial of Gargling Agents in Reducing Intraoral Viral Load Among Laboratory Confirmed COVID-19 Patients: GARGLES STUDY [Online]*. MS, FCPS, Aga Khan University (Identifier NCT04341688). Available: https://clinicaltrials.gov/ct2/show/NCT04341688.

Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., et al. (1984). Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS. *Science* 224 (4648), 500–503. doi:10.1126/science.6200936

Garg, R., Perveen, S., Gupta, B., and Bajpai, V. K. (2015). *Evaluation of Antibacterial Activity of* Annona Squamosa, Psidium Guajava *and* Azadirachta indica *against Pathogenic Bacterial Cultures*.

Garg, S., Anand, A., Lamba, Y., and Roy, A. (2020). Molecular Docking Analysis of Selected Phytochemicals against SARS-CoV-2 Mpro Receptor. *Vegetos* 33 (4), 1–16. doi:10.1007/s42535-020-00162-1

Gathirwa, J. W., Rukunga, G. M., Mwitari, P. G., Mwikwabe, N. M., Kimani, C. W., Muthaura, C. N., et al. (2011). Traditional Herbal Antimalarial Therapy in Kilifi District, Kenya. *J. Ethnopharmacol.* 134 (2), 434–442. doi:10.1016/j.jep.2010.12.043

Ghazali, S. Z., Mohamed Noor, N. R., and Mustaffa, K. M. F. (2022). Anti-plasmodial Activity of Aqueous Neem Leaf Extract Mediated Green Synthesis-Based Silver Nitrate Nanoparticles. *Prep. Biochem. Biotechnol.* 52 (1), 99–107. doi:10.1080/10826068.2021.1913602

Gogoi, B., Chowdhury, P., Goswami, N., Gogoi, N., Naiya, T., Chetia, P., et al. (2021). Identification of Potential Plant-Based Inhibitor against Viral Proteases of SARS-CoV-2 through Molecular Docking, MM-PBSA Binding Energy Calculations and Molecular Dynamics Simulation. *Mol. Divers* 25 (3), 1963–1977. doi:10.1007/s11030-021-10211-9

Goni Hamadama, O., Leonel Javeres, M. N., Nyemb, N., Mba Fabrice, M., and Manuela Elsa, P. T. (2021). Effect of *Azadirachta indica* and *Senna siamea* Decoction on CD4+ and CD8+ Level, Toxicological, and Antioxidant Profile in HIV/AIDS Positive Persons. *J. Toxicol.* 2021, 5594505. doi:10.1155/2021/5594505

Govindachari, T. R. (1992). Chemical and Biological Investigations on *Azadirachta indica* (The Neem Tree). *Curr. Sci.* 63 (3), 117–122.

Gualdani, R., Cavalluzzi, M. M., Lentini, G., and Habtemariam, S. (2016). The Chemistry and Pharmacology of Citrus Limonoids. *Molecules* 21 (11). doi:10.3390/molecules21111530

Guchhait, K. C., Manna, T., Barai, M., Karmakar, M., Nandi, S. K., Jana, D., et al. (2022). Antibiofilm and Anticancer Activities of Unripe and Ripe *Azadirachta indica* (Neem) Seed Extracts. *BMC Complement. Med. Ther.* 22 (1), 42. doi:10.1186/s12906-022-03513-4

Gupta, S. C., Prasad, S., Tyagi, A. K., Kunnumakkara, A. B., and Aggarwal, B. B. (2017). Neem (*Azadirachta indica*): An Indian Traditional Panacea with Modern Molecular Basis. *Phytomedicine* 34, 14–20. doi:10.1016/j.phymed.2017.07.001

Habluetzel, A., Pinto, B., Tapanelli, S., Nkouangang, J., Saviozzi, M., Chianese, G., et al. (2019). Effects of *Azadirachta indica* Seed Kernel Extracts on Early Erythrocytic Schizogony of *Plasmodium Berghei* and Pro-inflammatory Response in Inbred Mice. *Malar. J.* 18 (1), 35. doi:10.1186/s12936-019-2671-8

Hamad, G. M., Mohdaly, A. A. A., El-Nogoumy, B. A., Ramadan, M. F., Hassan, S. A., and Zeitoun, A. M. (2021). Detoxification of Aflatoxin B1 and Ochratoxin A Using *Salvia farinacea* and *Azadirachta indica* Water Extract and Application in Meat Products. *Appl. Biochem. Biotechnol.* 193 (10), 3098–3120. doi:10.1007/s12010-021-03581-1

Heyman, L., Houri-Haddad, Y., Heyman, S. N., Ginsburg, I., Gleitman, Y., and Feuerstein, O. (2017). Combined Antioxidant Effects of Neem Extract, Bacteria, Red Blood Cells and Lysozyme: Possible Relation to Periodontal Disease. *BMC Complement. Altern. Med.* 17 (1), 399. doi:10.1186/s12906-017-1900-3

Hosny, N. S., El Khodary, S. A., El Boghdadi, R. M., and Shaker, O. G. (2021). Effect of Neem (*Azadirachta indica*) versus 2.5% Sodium Hypochlorite as Root Canal Irrigants on the Intensity of Post-operative Pain and the Amount of Endotoxins in Mandibular Molars with Necrotic Pulps: a Randomized Controlled Trial. *Int. Endod. J.* 54 (9), 1434–1447. doi:10.1111/iej.13532

Hussain, F., Khurshid, M. F., Masood, R., and Ibrahim, W. (2017). Developing Antimicrobial Calcium Alginate Fibres from Neem and Papaya Leaves Extract. *J. Wound Care* 26 (12), 778–783. doi:10.12968/jowc.2017.26.12.778

Ibrahim, N., and Kebede, A. (2020). *In Vitro* antibacterial Activities of Methanol and Aqueous Leave Extracts of Selected Medicinal Plants against Human Pathogenic Bacteria. *Saudi J. Biol. Sci.* 27 (9), 2261–2268. doi:10.1016/j.sjbs.2020.06.047

Iman, M., Taheri, M., and Bahari, Z. (2021). The Anti-cancer Properties of Neem (*Azadirachta indica*) through its Antioxidant Activity in the Liver; its Pharmaceutics and Toxic Dosage Forms; a Literature Review. *J. Complement. Integr. Med.* doi:10.1515/jcim-2021-0009

Jagannathan, J., Nagar, P., Kaniappan, A. S., Raveendran, A., and Shekhar, S. (2020). Comparison of Antimicrobial Efficacy of Natural Extracts as a Disinfectant for Removable Orthodontic Appliances: An *Ex Vivo* Study. *Int. J. Clin. Pediatr. Dent.* 13 (6), 640–643. doi:10.5005/jp-journals-10005-1850

Jain, I., Jain, P., Bisht, D., Sharma, A., Srivastava, B., and Gupta, N. (2015). Use of Traditional Indian Plants in the Inhibition of Caries-Causing Bacteria--*Streptococcus Mutans*. *Braz Dent. J.* 26 (2), 110–115. doi:10.1590/0103-6440201300102

Jalaluddin, M., Rajasekaran, U. B., Paul, S., Dhanya, R. S., Sudeep, C. B., and Adarsh, V. J. (2017). Comparative Evaluation of Neem Mouthwash on Plaque and Gingivitis: A Double-Blind Crossover Study. *J. Contemp. Dent. Pract.* 18 (7), 567–571. doi:10.5005/jp-journals-10024-2085

James, C., Harfouche, M., Welton, N. J., Turner, K. M., Abu-Raddad, L. J., Gottlieb, S. L., et al. (20202016). Herpes Simplex Virus: Global Infection Prevalence and Incidence Estimates, 2016. *Bull. World Health Organ* 98 (5), 315–329. doi:10.2471/BLT.19.237149

Jamra, N., Das, G., Singh, P., and Haque, M. (2015). Anthelmintic Efficacy of Crude Neem (*Azadirachta indica*) Leaf Powder against Bovine Strongylosis. *J. Parasit. Dis.* 39 (4), 786–788. doi:10.1007/s12639-014-0423-9

Joy Sinha, D., D S Nandha, K., Jaiswal, N., Vasudeva, A., Prabha Tyagi, S., and Pratap Singh, U. (2017). Antibacterial Effect of *Azadirachta indica* (Neem) or *Curcuma Longa* (Turmeric) against *Enterococcus faecalis* Compared with that of 5% Sodium Hypochlorite or 2% Chlorhexidine *In Vitro*. *Bull. Tokyo Dent. Coll.* 58 (2), 103–109. doi:10.2209/tdcpublication.2015-0029

Joy Sinha, D., Garg, P., Verma, A., Malik, V., Maccune, E. R., and Vasudeva, A. (2015). Dentinal Tubule Disinfection with Propolis & Two Extracts of *Azadirachta indica* against *Candida albicans* Biofilm Formed on Tooth Substrate. *Open Dent. J.* 9, 369–374. doi:10.2174/1874210601509010369

Kalita, C., Raja, D., Saikia, A., and Saikia, A. K. (2019). Antibacterial Property of *Azadirachta indica*, *Ocimum sanctum*, and *Vitex negundo* against Oral Microbes. *J. Conserv. Dent.* 22 (6), 602–606. doi:10.4103/JCD.JCD_268_19

Kanagasanthosh, K., Shanmugapriyan, S., and Kavirajan, V. (2015). Evaluation of Acute Toxicity, Anti-inflammatory Activity and Phytochemical Screening of Ethanolic Extract of *Azadirachta indica* Leaves. *Int. J. Res. Dev. Pharm. Life Sci.* 4 (5), 1737–1742.

Kankariya, A. R., Patel, A. R., and Kunte, S. S. (2016). The Effect of Different Concentrations of Water Soluble Azadirachtin (Neem Metabolite) on *Streptococcus Mutans* Compared with Chlorhexidine. *J. Indian Soc. Pedod. Prev. Dent.* 34 (2), 105–110. doi:10.4103/0970-4388.180394

Kanth, M. R., Prakash, A. R., Sreenath, G., Reddy, V. S., and Huldah, S. (2016). Efficacy of Specific Plant Products on Microorganisms Causing Dental Caries. *J. Clin. Diagn Res.* 10 (12), ZM01–ZM03. doi:10.7860/JCDR/2016/19772.9025

Kaur, R., Tiwari, A., Manish, M., Maurya, I. K., Bhatnagar, R., and Singh, S. (2021). Common Garlic (*Allium Sativum* L.) Has Potent Anti-*Bacillus Anthracis* Activity. *J. Ethnopharmacol.* 264, 113230. doi:10.1016/j.jep.2020.113230

Kaura, T., Mewara, A., Zaman, K., Sharma, A., Agrawal, S. K., Thakur, V., et al. (2019). Utilizing Larvicidal and Pupicidal Efficacy of Eucalyptus and Neem Oil against *Aedes* Mosquito: An Approach for Mosquito Control. *Trop. Parasitol.* 9 (1), 12–17. doi:10.4103/tp.TP_35_18

Khameneh, B., Iranshahy, M., Soheili, V., and Fazly Bazzaz, B. S. (2019). Review on Plant Antimicrobials: a Mechanistic Viewpoint. *Antimicrob. Resist Infect. Control* 8, 118. doi:10.1186/s13756-019-0559-6

Khan, F. R., Kazmi, S. M. R., Iqbal, N. T., Iqbal, J., Ali, S. T., and Abbas, S. A. (2020). A Quadruple Blind, Randomised Controlled Trial of Gargling Agents in Reducing Intraoral Viral Load Among Hospitalised COVID-19 Patients: A Structured Summary of a Study Protocol for a Randomised Controlled Trial. *Trials* 21 (1), 785. doi:10.1186/s13063-020-04634-2

Kilani-Morakchi, S., Morakchi-Goudjil, H., and Sifi, K. (2021). Azadirachtin-Based Insecticide: Overview, Risk Assessments, and Future Directions. *Front. Agron.* 3. doi:10.3389/fagro.2021.676208

Killi, N., Pawar, A. T., and Gundloori, R. V. (2019). Polyesteramide of Neem Oil and its Blends as an Active Nanomaterial for Tissue Regeneration. *ACS Appl. Bio Mater* 2 (8), 3341–3351. doi:10.1021/acsabm.9b00354

Kuipers, E. J. (1999). Review Article: Exploring the Link between *Helicobacter pylori* and Gastric Cancer. *Aliment. Pharmacol. Ther.* 13 (Suppl. 1), 3–11. doi:10.1046/j.1365-2036.1999.00002.x

Lahiri, D., Nag, M., Dutta, B., Mukherjee, I., Ghosh, S., Dey, A., et al. (2021). Catechin as the Most Efficient Bioactive Compound from *Azadirachta indica* with Antibiofilm and Anti-Quorum Sensing Activities against Dental Biofilm: an *In Vitro* and In Silico Study. *Appl. Biochem. Biotechnol.* 193 (6), 1617–1630. doi:10.1007/s12010-021-03511-1

Lakkim, V., Reddy, M. C., Pallavali, R. R., Reddy, K. R., Reddy, C. V., Inamuddin, et al. (2020). Green Synthesis of Silver Nanoparticles and Evaluation of Their Antibacterial Activity against Multidrug-Resistant Bacteria and Wound Healing Efficacy Using a Murine Model. *Antibiot. (Basel)* 9 (12). doi:10.3390/antibiotics9120902

Lakshmi, T., Krishnan, V., Rajendran, R., and Madhusudhanan, N. (2015). *Azadirachta indica*: A Herbal Panacea in Dentistry - an Update. *Pharmacogn. Rev.* 9 (17), 41–44. doi:10.4103/0973-7847.156337

Lan Chi, N. T., Narayanan, M., Chinnathambi, A., Govindasamy, C., Subramani, B., Brindhadevi, K., et al. (2022). Fabrication, Characterization, Anti-inflammatory, and Anti-diabetic Activity of Silver Nanoparticles Synthesized from *Azadirachta indica* Kernel Aqueous Extract. *Environ. Res.* 208, 112684. doi:10.1016/j.envres.2022.112684

Lavanya, P., Ramaiah, S., and Anbarasu, A. (2015). Computational Analysis Reveal Inhibitory Action of Nimbin against Dengue Viral Envelope Protein. *Virusdisease* 26 (4), 243–254. doi:10.1007/s13337-015-0280-x

Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M., and Oshiro, L. S. (1984). Isolation of Lymphocytopathic Retroviruses from San Francisco Patients with AIDS. *Science* 225 (4664), 840–842. doi:10.1126/science.6206563

Lu, X. F., Lin, P. C., Zi, J. C., and Fan, X. N. (2019). Limonoids from Seeds of *Azadirachta indica* and Their Antibacterial Activity. *Zhongguo Zhong Yao Za Zhi* 44 (22), 4864–4873. doi:10.19540/j.cnki.cjcmm.20190813.202

MacKinnon, S., Durst, T., Arnason, J. T., Angerhofer, C., Pezzuto, J., Sanchez-Vindas, P. E., et al. (1997). Antimalarial Activity of Tropical *Meliaceae* Extracts and Gedunin Derivatives. *J. Nat. Prod.* 60 (4), 336–341. doi:10.1021/np9605394

Mah, T. F., and O'Toole, G. A. (2001). Mechanisms of Biofilm Resistance to Antimicrobial Agents. *Trends Microbiol.* 9 (1), 34–39. doi:10.1016/s0966-842x(00)01913-2

Maheswaran, R., and Ignacimuthu, S. (2015). A Novel Biopesticide PONNEEM to Control Human Vector Mosquitoes *Anopheles stephensi* L. And *Culex quinquefasciatus* Say. *Environ. Sci. Pollut. Res. Int.* 22 (17), 13153–13166. doi:10.1007/s11356-015-4586-4

Mahfuzul Hoque, M. D., Bari, M. L., Inatsu, Y., Juneja, V. K., and Kawamoto, S. (2007). Antibacterial Activity of Guava (*Psidium Guajava* L.) and Neem (*Azadirachta indica* A. Juss.) Extracts against Foodborne Pathogens and Spoilage Bacteria. *Foodborne Pathog. Dis.* 4 (4), 481–488. doi:10.1089/fpd.2007.0040

Mandawewala, A. R., and Mandawewala, K. A. (2021). POLYMERIC YARN, COMPOSITION AND METHOD. *17/148618*.

Martens, E., and Demain, A. L. (2017). The Antibiotic Resistance Crisis, with a Focus on the United States. *J. Antibiot. (Tokyo)* 70 (5), 520–526. doi:10.1038/ja.2017.30

Mazariegos, L. A. (2016). *Pest Control Formulation of Neem and Beauveria Bassiana and Methods of Making and Using Same*. 14/494199.

Mbah, A. U., Udeinya, I. J., Shu, E. N., Chijioke, C. P., Nubila, T., Udeinya, F., et al. (2007). Fractionated Neem Leaf Extract Is Safe and Increases CD4+ Cell Levels in HIV/AIDS Patients. *Am. J. Ther.* 14 (4), 369–374. doi:10.1097/MJT.0b013e3180a72199

Melese, S., Kumar, S., Nair, P., and Sharmila, B. (2016). Preliminary Phytochemical Analysis and *In Vitro* Antibacterial Activity of Bark and Seeds of Ethiopian Neem (*Azadirachta indica* A. Juss). *WORLD J. Pharm. Pharm. Sci.* 5 (4), 1714–1723.

Mistry, K. S., Sanghvi, Z., Parmar, G., Shah, S., and Pushpalatha, K. (2015). Antibacterial Efficacy of *Azadirachta indica, Mimusops elengi* and 2% CHX on Multispecies Dentinal Biofilm. *J. Conserv. Dent.* 18 (6), 461–466. doi:10.4103/0972-0707.168810

Mistry, K. S., Sanghvi, Z., Parmar, G., and Shah, S. (2014). The Antimicrobial Activity of *Azadirachta indica, Mimusops elengi, Tinospora cardifolia, Ocimum sanctum* and 2% Chlorhexidine Gluconate on Common Endodontic Pathogens: An *In Vitro* Study. *Eur. J. Dent.* 8 (2), 172–177. doi:10.4103/1305-7456.130591

Molyneux, D. H., Savioli, L., and Engels, D. (2017). Neglected Tropical Diseases: Progress towards Addressing the Chronic Pandemic. *Lancet* 389 (10066), 312–325. doi:10.1016/S0140-6736(16)30171-4

Moodley, Y., Linz, B., Bond, R. P., Nieuwoudt, M., Soodyall, H., Schlebusch, C. M., et al. (2012). Age of the Association between *Helicobacter pylori* and Man. *PLoS Pathog.* 8 (5), e1002693. doi:10.1371/journal.ppat.1002693

Morales-Covarrubias, M. S., García-Aguilar, N., Bolan-Mejía, M. D., and Puello-Cruz, A. C. (2016). Evaluation of Medicinal Plants and Colloidal Silver Efficiency against *Vibrio Parahaemolyticus* Infection in *Litopenaeus Vannamei* Cultured at Low Salinity. *Dis. Aquat. Organ* 122 (1), 57–65. doi:10.3354/dao03060

Murugan, K., Panneerselvam, C., Samidoss, C. M., Madhiyazhagan, P., Suresh, U., Roni, M., et al. (2016). *In Vivo* and *In Vitro* Effectiveness of *Azadirachta indica*-Synthesized Silver Nanocrystals against Plasmodium Berghei and Plasmodium Falciparum, and Their Potential against Malaria Mosquitoes. *Res. Vet. Sci.* 106, 14–22. doi:10.1016/j.rvsc.2016.03.001

Mustafa, M. (2016). Antibacterial Efficacy of Neem (*Azadirachta indica*) Extract against *Enterococcus faecalis*: An *In Vitro* Study. *J. Contemp. Dent. Pract.* 17 (10), 791–794. doi:10.5005/jp-journals-10024-1932

Nagaraj, A., and Samiappan, S. (2019). Presentation of Antibacterial and Therapeutic Anti-inflammatory Potentials to Hydroxyapatite via Biomimetic with *Azadirachta indica*: An *In Vitro* Anti-inflammatory Assessment in Contradiction of LPS-Induced Stress in RAW 264.7 Cells. *Front. Microbiol.* 10, 1757. doi:10.3389/fmicb.2019.01757

Nagata, J. M., Jew, A. R., Kimeu, J. M., Salmen, C. R., Bukusi, E. A., and Cohen, C. R. (2011). Medical Pluralism on Mfangano Island: Use of Medicinal Plants Among Persons Living with HIV/AIDS in Suba District, Kenya. *J. Ethnopharmacol.* 135 (2), 501–509. doi:10.1016/j.jep.2011.03.051

Nagini, S., Nivetha, R., Palrasu, M., and Mishra, R. (2021). Nimbolide, a Neem Limonoid, Is a Promising Candidate for the Anticancer Drug Arsenal. *J. Med. Chem.* 64 (7), 3560–3577. doi:10.1021/acs.jmedchem.0c02239

Nallusamy, S., Mannu, J., Ravikumar, C., Angamuthu, K., Nathan, B., Nachimuthu, K., et al. (2021). Exploring Phytochemicals of Traditional Medicinal Plants Exhibiting Inhibitory Activity against Main Protease, Spike Glycoprotein, RNA-dependent RNA Polymerase and Non-structural Proteins of SARS-CoV-2 through Virtual Screening. *Front. Pharmacol.* 12, 667704. doi:10.3389/fphar.2021.667704

National Research Council (1992). *Neem: A Tree for Solving Global Problems*. Washington (DC): The National Academies Press. doi:10.17226/1924

Navabshan, I., Sakthivel, B., Pandiyan, R., Antoniraj, M. G., Dharmaraj, S., Ashokkumar, V., et al. (2021). Computational Lock and Key and Dynamic Trajectory Analysis of Natural Biophors against COVID-19 Spike Protein to Identify Effective Lead Molecules. *Mol. Biotechnol.* 63 (10), 898–908. doi:10.1007/s12033-021-00358-z

Nayak Aarati, N. R. N., Soumya, G. B., Kishore, B., and Mithun, K. (2011). Evaluation of Antibacterial and Anticandidial Efficacy of Aqueous and Alcoholic Extract of Neem (*Azadirachta indica*) an *In Vitro* Study. *Int. J. Res. Ayurveda Pharm.* 2 (1), 230–235.

Nesari, T. M., Bhardwaj, A., ShriKrishna, R., Ruknuddin, G., Ghildiyal, S., Das, A., et al. (2021). Neem (*Azadirachta indica* A. Juss) Capsules for Prophylaxis of COVID-19 Infection: A Pilot, Double-Blind, Randomized Controlled Trial. *Altern. Ther. Health Med.* 27 (S1), 196–203.

Newman, D. J., and Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* 79 (3), 629–661. doi:10.1021/acs.jnatprod.5b01055

Nicoletti, M., Maccioni, O., Coccioletti, T., Mariani, S., and Vitali, F. (2012). "Neem Tree (*Azadirachta indica* A. Juss) as Source of Bioinsectides," in Insecticides - Advances In Integrated Pest Management. *IntechOpen)*. doi:10.5772/28786

Nimbulkar, G., Garacha, V., Shetty, V., Bhor, K., Srivastava, K. C., Shrivastava, D., et al. (2020). Microbiological and Clinical Evaluation of Neem Gel and Chlorhexidine Gel on Dental Plaque and Gingivitis in 20-30 Years Old Adults: A Randomized Parallel-Armed, Double-Blinded Controlled Trial. *J. Pharm. Bioallied Sci.* 12 (Suppl. 1), S345–S351. doi:10.4103/jpbs.JPBS_101_20

Noor, A. A. (2011). Dose Response Curve of Plants Extracts against the Human Pathogens. *Gomal Univ. J. Res.* 27 (2).

Noorul Aneesa, G. (2016). Beneficial Effects of Neem Oil-An Updated Review. *J. Pharm. Sci. Res.* 8 (8), 756–758.

Ogidigo, J. O., Iwuchukwu, E. A., Ibeji, C. U., Okpalefe, O., and Soliman, M. E. S. (2022). Natural Phyto, Compounds as Possible Noncovalent Inhibitors against SARS-CoV2 Protease: Computational Approach. *J. Biomol. Struct. Dyn.* 40 (5), 2284–2301. doi:10.1080/07391102.2020.1837681

Okoh, M. P., Singla, R. K., Madu, C., Soremekun, O., Adejoh, J., Alli, L. A., et al. (2021). Phytomedicine in Disease Management: In-Silico Analysis of the Binding Affinity of Artesunate and Azadirachtin for Malaria Treatment. *Front. Pharmacol.* 12, 751032. doi:10.3389/fphar.2021.751032

Olwenyi, O. A., Asingura, B., Naluyima, P., Anywar, G. U., Nalunga, J., Nakabuye, M., et al. (2021). In-vitro Immunomodulatory Activity of *Azadirachta indica* A.Juss. Ethanol: Water Mixture against HIV Associated Chronic CD4+ T-Cell Activation/Exhaustion. *BMC Complement. Med. Ther.* 21 (1), 114. doi:10.1186/s12906-021-03288-0

Oyekanmi, A. A., Kumar, U. S. U., H. P. S., A. K., Olaiya, N. G., Amirul, A. A., Rahman, A. A., et al. (2021). Functional Properties of Antimicrobial Neem Leaves Extract Based Macroalgae Biofilms for Potential Use as Active Dry Packaging Applications. *Polymers* 13 (10), 1664. doi:10.3390/polym13101664

Panchal, V., Gurunathan, D., and Muralidharan, N. P. (2020). Comparison of Antibacterial Efficacy of Cinnamon Extract, Neem Extract as Irrigant and Sodium Hypochlorite against *Enterococcus Fecalis*: An *In Vitro* Study. *Indian J. Dent. Res.* 31 (1), 124–128. doi:10.4103/ijdr.IJDR_177_18

Páramo, M. E. R., Falvo, M., García, J., and Lastra, C. C. L. (2020). Compatibility between *Leptolegnia chapmanii* and Diflubenzuron and Neem Oil for the Control of *Aedes aegypti*. *Rev. Argent. Microbiol.* 52 (3), 240–244. doi:10.1016/j.ram.2019.10.001

Parida, P. K., Paul, D., and Chakravorty, D. (2020). The Natural Way Forward: Molecular Dynamics Simulation Analysis of Phytochemicals from Indian Medicinal Plants as Potential Inhibitors of SARS-CoV-2 Targets. *Phytother. Res.* 34 (12), 3420–3433. doi:10.1002/ptr.6868

Pasquoto-Stigliani, T., Campos, E. V. R., Oliveira, J. L., Silva, C. M. G., Bilesky-José, N., Guilger, M., et al. (2017). Nanocapsules Containing Neem (*Azadirachta indica*) Oil: Development, Characterization, and Toxicity Evaluation. *Sci. Rep.* 7 (1), 5929. doi:10.1038/s41598-017-06092-4

Patel, S. M., Nagulapalli Venkata, K. C., Bhattacharyya, P., Sethi, G., and Bishayee, A. (2016). Potential of Neem (*Azadirachta indica* L.) for Prevention and Treatment of Oncologic Diseases. *Semin. Cancer Biol.* 40-41, 100–115. doi:10.1016/j.semcancer.2016.03.002

Patil, S. M., Shirahatti, P. S., and Ramu, R. (2021). *Azadirachta indica* A. Juss (Neem) against Diabetes Mellitus: a Critical Review on its Phytochemistry, Pharmacology, and Toxicology. *J. Pharm. Pharmacol.* doi:10.1093/jpp/rgab098

Poopathi, S., De Britto, L. J., Praba, V. L., Mani, C., and Praveen, M. (2015). Synthesis of Silver Nanoparticles from *Azadirachta indica*-A Most Effective Method for Mosquito Control. *Environ. Sci. Pollut. Res. Int.* 22 (4), 2956–2963. doi:10.1007/s11356-014-3560-x

Quelemes, P. V., Perfeito, M. L., Guimarães, M. A., dos Santos, R. C., Lima, D. F., Nascimento, C., et al. (2015). Effect of Neem (*Azadirachta indica* A. Juss) Leaf Extract on Resistant *Staphylococcus aureus* Biofilm Formation and *Schistosoma Mansoni* Worms. *J. Ethnopharmacol.* 175, 287–294. doi:10.1016/j.jep.2015.09.026

Raju, D., and Jose, J. (2019). Development and Evaluation of Novel Topical Gel of Neem Extract for the Treatment of Bacterial Infections. *J. Cosmet. Dermatol* 18 (6), 1776–1783. doi:10.1111/jocd.12965

Ramana, V. (2022). *Dental Enamel Compositions with Anti-inflammatory Agents for Animals*, 17–523091.

Rasool, S., Raza, M. A., Manzoor, F., Kanwal, Z., Riaz, S., Iqbal, M. J., et al. (2020). Biosynthesis, Characterization and Anti-dengue Vector Activity of Silver Nanoparticles Prepared from *Azadirachta indica* and *Citrullus colocynthis*. *R. Soc. Open Sci.* 7 (9), 200540. doi:10.1098/rsos.200540

Ravva, S. V., and Korn, A. (2015). Effect of Neem (*Azadirachta indica*) on the Survival of *Escherichia coli* O157:H7 in Dairy Manure. *Int. J. Environ. Res. Public Health* 12 (7), 7794–7803. doi:10.3390/ijerph120707794

Reddy, J. (2020). Neem Cake Fertilizer, Uses, Application, Benefits [Online]. Agrifarming. Available: https://www.agrifarming.in/neem-cake-fertilizer-uses-application-benefits.

Revathi, T., and Thambidurai, S. (2019). Cytotoxic, Antioxidant and Antibacterial Activities of Copper Oxide Incorporated Chitosan-Neem Seed Biocomposites. *Int. J. Biol. Macromol.* 139, 867–878. doi:10.1016/j.ijbiomac.2019.07.214

Rodrigues, M. P., Astoreca, A. L., Oliveira, Á. A., Salvato, L. A., Biscoto, G. L., Keller, L. A. M., et al. (2019). *In Vitro* Activity of Neem (*Azadirachta indica*) Oil on Growth and Ochratoxin A Production by *Aspergillus carbonarius* Isolates. *Toxins (Basel)* 11 (10). doi:10.3390/toxins11100579

Roy, A., and Saraf, S. (2006). Limonoids: Overview of Significant Bioactive Triterpenes Distributed in Plants Kingdom. *Biol. Pharm. Bull.* 29 (2), 191–201. doi:10.1248/bpb.29.191

S, L. P., A, U., and S J, G. F. (2020). Investigation on the Biofilm Eradication Potential of Selected Medicinal Plants against Methicillin-Resistant *Staphylococcus aureus*. *Biotechnol. Rep. (Amst)* 28, e00523. doi:10.1016/j.btre.2020.e00523

Saleem, S., Muhammad, G., Hussain, M. A., and Bukhari, S. N. A. (2018). A Comprehensive Review of Phytochemical Profile, Bioactives for Pharmaceuticals, and Pharmacological Attributes of *Azadirachta indica*. *Phytother. Res.* 32 (7), 1241–1272. doi:10.1002/ptr.6076

Sarkar, L., Oko, L., Gupta, S., Bubak, A. N., Das, B., Gupta, P., et al. (2022). *Azadirachta indica* A. Juss Bark Extract and its Nimbin Isomers Restrict β-coronaviral Infection and Replication. *Virology* 569, 13–28. doi:10.1016/j.virol.2022.01.002

Sarkar, S., Singh, R. P., and Bhattacharya, G. (2021). Exploring the Role of *Azadirachta indica* (Neem) and its Active Compounds in the Regulation of Biological Pathways: an Update on Molecular Approach. *3 Biotech.* 11 (4), 178. doi:10.1007/s13205-021-02745-4

Saxena, A., Arivaradarajan, P., Arivaradarajan, P., Mukhopadhyay, A. K., and Nandi, S. P. (2021). Bactericidal Effect of Neem (*Azadirachta indica*) Leaf Extract on *Helicobacter pylori*. *Jeb* 42, 1591–1597. doi:10.22438/jeb/42/6/mrn-2070

Selvaraj, K., Bharath, N., Natarajan, R., Dinesh, S., Murugesan, S., and Selvaraj, S. (2020). Comparative Evaluation of Antimicrobial Efficacy of Toothpastes Containing Probiotic and Neem as Primary Ingredient on Salivary *Streptococcus mutans* in Melmaruvathur Population: An *In Vivo* Study. *J. Pharm. Bioallied Sci.* 12 (Suppl. 1), S595–S600. doi:10.4103/jpbs.JPBS_209_20

Senapati, S., Banerjee, P., Bhagavatula, S., Kushwaha, P. P., and Kumar, S. (2021). Contributions of Human ACE2 and TMPRSS2 in Determining Host-Pathogen Interaction of COVID-19. *J. Genet.* 100. doi:10.1007/s12041-021-01262-w

Shanmugam, A., Ramakrishnan, C., Velmurugan, D., and Gromiha, M. M. (2020). Identification of Potential Inhibitors for Targets Involved in Dengue Fever. *Curr. Top. Med. Chem.* 20 (19), 1742–1760. doi:10.2174/1568026620666200618123026

Sharma, C., and Bhardwaj, N. K. (2020). Fabrication of Natural-Origin Antibacterial Nanocellulose Films Using Bio-Extracts for Potential Use in Biomedical Industry. *Int. J. Biol. Macromol.* 145, 914–925. doi:10.1016/j.ijbiomac.2019.09.182

Sharma, D. D., and Nupur, S. S. (2014). Comparative Study of Different Parts of *Azadirachta indica* (Neem) Plant on the Basis of Antibacterial Activity, Phytochemical Screening and its Effect on Rat Pc-12 (Pheochromocytoma) Cell Line. *Int. J. Biotechnol. Humanit. Sports Allied Fields* 2 (7), 144–154.

Singh, M., Sharma, D., Kumar, D., Singh, G., Swami, G., and Rathore, M. S. (2020). Formulation, Development, and Evaluation of Herbal Effervescent Mouthwash Tablet Containing *Azadirachta indica* (Neem) and Curcumin for the Maintenance of Oral Hygiene. *Recent Pat. Drug Deliv. Formul.* 14 (2), 145–161. doi:10.2174/1872211314666200820142509

Singh, V., Roy, M., Garg, N., Kumar, A., Arora, S., and Malik, D. S. (2021). An Insight into the Dermatological Applications of Neem: A Review on Traditional and Modern Aspect. *Recent Adv. Antiinfect Drug Discov.* 16 (2), 94–121. doi:10.2174/2772434416666210604105251

Sir, C., and Chopra, R. N. (1994). *Indigenous Drugs of India*. Academic Publishers.

Somsak, V., Chachiyo, S., Jaihan, U., and Nakinchat, S. (2015). Protective Effect of Aqueous Crude Extract of Neem (*Azadirachta indica*) Leaves on *Plasmodium Berghei*-Induced Renal Damage in Mice. *J. Trop. Med.* 2015, 961205. doi:10.1155/2015/961205

Soni, N., and Prakash, S. (2014). Silver Nanoparticles: a Possibility for Malarial and Filarial Vector Control Technology. *Parasitol. Res.* 113 (11), 4015–4022. doi:10.1007/s00436-014-4069-4

Stewart, P. S., and Costerton, J. W. (2001). Antibiotic Resistance of Bacteria in Biofilms. *Lancet* 358 (9276), 135–138. doi:10.1016/s0140-6736(01)05321-1

Streicher, L. M. (2021). Exploring the Future of Infectious Disease Treatment in a Post-antibiotic Era: A Comparative Review of Alternative Therapeutics. *J. Glob. Antimicrob. Resist* 24, 285–295. doi:10.1016/j.jgar.2020.12.025

Subbuvel, M., and Kavan, P. (2022). Preparation and Characterization of Polylactic Acid/fenugreek Essential Oil/curcumin Composite Films for Food Packaging Applications. *Int. J. Biol. Macromol.* 194, 470–483. doi:10.1016/j.ijbiomac.2021.11.090

Sultana, B., Naseer, R., and Nigam, P. (2015). Utilization of Agro-Wastes to Inhibit Aflatoxins Synthesis by *Aspergillus parasiticus*: A Biotreatment of Three Cereals for Safe Long-Term Storage. *Bioresour. Technol.* 197, 443–450. doi:10.1016/j.biortech.2015.08.113

Sunthar, T. P. M., Marin, E., Boschetto, F., Zanocco, M., Sunahara, H., Ramful, R., et al. (2020). Antibacterial and Antifungal Properties of Composite Polyethylene Materials Reinforced with Neem and Turmeric. *Antibiot. (Basel)* 9 (12). doi:10.3390/antibiotics9120857

Susmitha, S., Vidyamol, K. K., Ranganayaki, P., and Vijayaragavan, R. (2013). Phytochemical Extraction and Antimicrobial Properties of *Azadirachta indica* (Neem). *Glob. J. Pharmacol.* 7 (3), 316–320.

Tajbakhsh, E., Kwenti, T. E., Kheyri, P., Nezaratizade, S., Lindsay, D. S., and Khamesipour, F. (2021). Antiplasmodial, Antimalarial Activities and Toxicity of African Medicinal Plants: a Systematic Review of Literature. *Malar. J.* 20 (1), 349. doi:10.1186/s12936-021-03866-0

Tapanelli, S., Chianese, G., Lucantoni, L., Yerbanga, R. S., Habluetzel, A., and Taglialatela-Scafati, O. (2016). Transmission Blocking Effects of Neem (*Azadirachta indica*) Seed Kernel Limonoids on *Plasmodium Berghei* Early Sporogonic Development. *Fitoterapia* 114, 122–126. doi:10.1016/j.fitote.2016.09.008

Tasanarong, T., Patntirapong, S., and Aupaphong, V. (2021). The Inhibitory Effect of a Novel Neem Paste against Cariogenic Bacteria. *J. Clin. Exp. Dent.* 13 (11), e1083–e1088. doi:10.4317/jced.58781

Tatch, W. (2021). Compositions and Methods for Promoting and Maintaining Oral Health. *U. S. Pat. Appl.* 17, 282430.

Tepongning, R. N., Mbah, J. N., Avoulou, F. L., Jerme, M. M., Ndanga, E. K., and Fekam, F. B. (2018). Hydroethanolic Extracts of *Erigeron Floribundus* and *Azadirachta indica* Reduced *Plasmodium Berghei* Parasitemia in Balb/c Mice. *Evid. Based Complement. Altern. Med.* 2018, 5156710. doi:10.1155/2018/5156710

Tesso, H., Nisha, A. K., and Kumsa, K. (2015). Antibacterial Activity and Phytochemical Screening of Some Important Medicinal Plants against Human Diarrheal Pathogens in Adama City, Ethiopia. *Int. J. Microbiol. Immunol. Res.* 3 (3), 029–035.

Thakurta, P., Bhowmik, P., Mukherjee, S., Hajra, T. K., Patra, A., and Bag, P. K. (2007). Antibacterial, Antisecretory and Antihemorrhagic Activity of *Azadirachta indica* Used to Treat Cholera and Diarrhea in India. *J. Ethnopharmacol.* 111 (3), 607–612. doi:10.1016/j.jep.2007.01.022

Thota, S. M., Balan, V., and Sivaramakrishnan, V. (2020). Natural Products as Home-Based Prophylactic and Symptom Management Agents in the Setting of COVID-19. *Phytother. Res.* 34 (12), 3148–3167. doi:10.1002/ptr.6794

Timalsina, D., Pokhrel, K. P., and Bhusal, D. (2021). Pharmacologic Activities of Plant-Derived Natural Products on Respiratory Diseases and Inflammations. *Biomed. Res. Int.* 2021, 1636816. doi:10.1155/2021/1636816

Tiwari, V., Darmani, N. A., Yue, B. Y., and Shukla, D. (2010). *In Vitro* antiviral Activity of Neem (*Azardirachta indica* L.) Bark Extract against Herpes Simplex Virus Type-1 Infection. *Phytother. Res.* 24 (8), 1132–1140. doi:10.1002/ptr.3085

Tundis, R., Loizzo, M. R., and Menichini, F. (2014). An Overview on Chemical Aspects and Potential Health Benefits of Limonoids and Their Derivatives. *Crit. Rev. Food Sci. Nutr.* 54 (2), 225–250. doi:10.1080/10408398.2011.581400

Udeinya, I. J., Mbah, A. U., Chijioke, C. P., and Shu, E. N. (2004). An Antimalarial Extract from Neem Leaves Is Antiretroviral. *Trans. R. Soc. Trop. Med. Hyg.* 98 (7), 435–437. doi:10.1016/j.trstmh.2003.10.016

Ulaeto, S. B., Mathew, G. M., Pancrecious, J. K., Nair, J. B., Rajan, T. P. D., Maiti, K. K., et al. (2020). Biogenic Ag Nanoparticles from Neem Extract: Their Structural Evaluation and Antimicrobial Effects against *Pseudomonas nitroreducens* and *Aspergillus unguis* (NII 08123). *ACS Biomater. Sci. Eng.* 6 (1), 235–245. doi:10.1021/acsbiomaterials.9b01257

UNAIDS (2022). Global HIV & AIDS Statistics - Fact Sheet [Online]. unaids.Org: UNAIDS. Available at: unaids.org/en/resources/fact-sheet.

USPTO (2022). Patent Public Search [Online]. United States Patent and Trademark Office. Available: https://ppubs.uspto.gov/pubwebapp/.

Uthaya Kumar, U. S., Abdulmadjid, S. N., Olaiya, N. G., Amirul, A. A., Rizal, S., Rahman, A. A., et al. (2020). Extracted Compounds from Neem Leaves as Antimicrobial Agent on the Physico-Chemical Properties of Seaweed-Based Biopolymer Films. *Polym. (Basel)* 12 (5). doi:10.3390/polym12051119

Vardhan, S., and Sahoo, S. K. (2020). In Silico ADMET and Molecular Docking Study on Searching Potential Inhibitors from Limonoids and Triterpenoids for COVID-19. *Comput. Biol. Med.* 124, 103936. doi:10.1016/j.compbiomed.2020.103936

Vardhan, S., and Sahoo, S. K. (2022). Virtual Screening by Targeting Proteolytic Sites of Furin and TMPRSS2 to Propose Potential Compounds Obstructing the Entry of SARS-CoV-2 Virus into Human Host Cells. *J. Tradit. Complement. Med.* 12 (1), 6–15. doi:10.1016/j.jtcme.2021.04.001

Vestby, L. K., Grønseth, T., Simm, R., and Nesse, L. L. (2020). Bacterial Biofilm and its Role in the Pathogenesis of Disease. *Antibiot. (Basel)* 9 (2). doi:10.3390/antibiotics9020059

Wang, C. H., Hsieh, Y. H., Powers, Z. M., and Kao, C. Y. (2020). Defeating Antibiotic-Resistant Bacteria: Exploring Alternative Therapies for a Post-Antibiotic Era. *Int. J. Mol. Sci.* 21 (3). doi:10.3390/ijms21031061

WHO (2020). Burden of Disease. Available: https://www.who.int/teams/global-influenza-programme/surveillance-and-monitoring/burden-of-disease.

Who (2021a). Leishmaniasis [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.

Who (2021b). WHO Coronavirus (COVID-19) Dashboard [Online]. World Health Organization. Available: https://covid19.who.int/.

Who (2021c). *World Malaria Report*. Geneva: Global Malaria Programme.

Wong, C. B., Mohamad, N., Chandrasakaran, P., Selvadurai, A., and Subramaniam, R. (2022). *Antimicrobial Elastomeric Article*, 17–483140.

Wylie, M. R., Windham, I. H., Blum, F. C., Wu, H., and Merrell, D. S. (2022). *In Vitro* antibacterial Activity of Nimbolide against *Helicobacter pylori*. *J. Ethnopharmacol.* 285, 114828. doi:10.1016/j.jep.2021.114828

Yarmohammadi, F., Mehri, S., Najafi, N., Salar Amoli, S., and Hosseinzadeh, H. (2021). The Protective Effect of *Azadirachta indica* (Neem) against Metabolic Syndrome: A Review. *Iran. J. Basic Med. Sci.* 24 (3), 280–292. doi:10.22038/ijbms.2021.48965.11218

Yerbanga, R. S., Rayaisse, J. B., Vantaux, A., Salou, E., Mouline, K., Hien, F., et al. (2016). Neemazal ® as a Possible Alternative Control Tool for Malaria and African Trypanosomiasis? *Parasit. Vectors* 9, 263. doi:10.1186/s13071-016-1538-x

Yonezawa, H., Osaki, T., Hojo, F., and Kamiya, S. (2019). Effect of *Helicobacter pylori* Biofilm Formation on Susceptibility to Amoxicillin, Metronidazole and Clarithromycin. *Microb. Pathog.* 132, 100–108. doi:10.1016/j.micpath.2019.04.030

Younus, I., Siddiq, A., Ishaq, H., Anwer, L., Badar, S., and Ashraf, M. (2016). Evaluation of Antiviral Activity of Plant Extracts against Foot and Mouth Disease Virus *In Vitro*. *Pak J. Pharm. Sci.* 29 (4), 1263–1268.

Zatelli, A., Fondati, A., Maroli, M., and Canine Leishmaniosis Working, G. (2022). The Knowns and Unknowns of the Efficacy of Neem Oil (*Azadirachta indica*) Used as a Preventative Measure against *Leishmania* Sand Fly Vectors (*Phlebotomus* Genus). *Prev. Vet. Med.* 202, 105618. doi:10.1016/j.prevetmed.2022.105618

Zihadi, M. A. H., Rahman, M., Talukder, S., Hasan, M. M., Nahar, S., and Sikder, M. H. (2019). Antibacterial Efficacy of Ethanolic Extract of *Camellia Sinensis* and *Azadirachta indica* Leaves on Methicillin-Resistant *Staphylococcus aureus* and Shiga-Toxigenic *Escherichia coli*. *J. Adv. Vet. Anim. Res.* 6 (2), 247–252. doi:10.5455/javar.2019.f340

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

REVIEW
published: 02 June 2022
doi: 10.3389/fphar.2022.837907

Effects of Traditional Chinese Medicine and its Active Ingredients on Drug-Resistant Bacteria

Jimin Li[1,2], *Shanshan Feng*[1], *Xin Liu*[4], *Xu Jia*[2,3], *Fengling Qiao*[1]*, *Jinlin Guo*[1,5]* and *Shanshan Deng*[2,3]*

[1]Chongqing Key Laboratory of Sichuan-Chongqing Co-construction for Diagnosis and Treatment of Infectious Diseases Integrated Traditional Chinese and Western Medicine, College of Medical Technology, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2]Non-Coding RNA and Drug Discovery Key Laboratory of Sichuan Province, Chengdu Medical College, Chengdu, China, [3]School of Basic Medical Sciences, Chengdu Medical College, Chengdu, China, [4]School of Public Health, Chengdu Medical College, Chengdu, China, [5]Key Laboratory of Systematic Research of Distinctive Chinese Medicine Resources in Southwest China, Chengdu University of Traditional Chinese Medicine, Chengdu, China

OPEN ACCESS

Edited by:
Joan Villena García,
Universidad de Valparaíso, Chile

Reviewed by:
Susan Semple,
University of South Australia, Australia
Javier Alberto Garza Cervantes,
Autonomous University of Nuevo León, Mexico
Ali Parsaeimehr,
Delaware State University, United States

***Correspondence:**
Fengling Qiao
qiaozhaoyi@cdutcm.edu.cn
Jinlin Guo
guo596@cdutcm.edu.cn
Shanshan Deng
jzlxddss@163.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: 17 December 2021
Accepted: 25 April 2022
Published: 02 June 2022

Citation:
Li J, Feng S, Liu X, Jia X, Qiao F, Guo J and Deng S (2022) Effects of Traditional Chinese Medicine and its Active Ingredients on Drug-Resistant Bacteria. Front. Pharmacol. 13:837907. doi: 10.3389/fphar.2022.837907

The increasing and widespread application of antibacterial drugs makes antibiotic resistance a prominent and growing concern in clinical practice. The emergence of multidrug-resistant bacteria presents a global threat. However, the development and use of novel antibacterial agents involves time-consuming and costly challenges that may lead to yet further drug resistance. More recently, researchers have turned to traditional Chinese medicine to stem the rise of antibiotic resistance in pathogens. Many studies have shown traditional Chinese medicines to have significant bacteriostatic and bactericidal effects, with the advantage of low drug resistance. Some of which when combined with antibiotics, have also demonstrated antibacterial activity by synergistic effect. Traditional Chinese medicine has a variety of active components, including flavonoids, alkaloids, phenols, and quinones, which can inhibit the growth of drug-resistant bacteria and be used in combination with a variety of antibiotics to treat various drug-resistant bacterial infections. We reviewed the interaction between the active ingredients of traditional Chinese medicines and antibiotic-resistant bacteria. At present, flavonoids and alkaloids are the active ingredients that have been most widely studied, with significant synergistic activity demonstrated when used in combination with antibiotics against drug-resistant bacteria. The reviewed studies show that traditional Chinese medicine and its active ingredients have antimicrobial activity on antibiotic-resistant bacteria, which may enhance the susceptibility of antibiotic-resistant bacteria, potentially reduce the required dosage of antibacterial agents and the rate of drug resistance. Our results provide direction for finding and developing alternative methods to counteract drug-resistant bacteria, offering a new therapeutic strategy for tackling antibiotic resistance.

Keywords: traditional Chinese medicine, active ingredient, combined, antibiotic, drug-resistant bacterial

INTRODUCTION

In the late 1950s, most *Staphylococcus aureus* strains became resistant to penicillin (Paul D Stapleton, 2002). Researchers then developed new drugs, such as methicillin and vancomycin, to treat penicillin-resistant bacteria. Unfortunately, the existence of methicillin-resistant *S. aureus* (MRSA) was first reported in 1961 (Barber, 1961). Antibiotic resistance is a global problem. Although it is a natural process for bacteria to develop antibiotic resistance, antibiotic resistance is accelerated by the misuse and abuse of antibiotics, which makes it more difficult to prevent and control bacterial infections (Piddock, 2012). Currently, more and more infections become complicated to treat or even untreatable, as overuse of antibiotics reduces their effectiveness. Thus far, there is no antibiotic capable of solving the problem of resistant strains, where it is predicted that antibiotic resistance will re-emerge even with the most vigorous research and development of new drugs (Barriere, 2014). Antibiotic resistance leads to higher hospital costs, delayed discharge times and higher mortality rates, where at least 700,000 people die worldwide each year as a result. The report on the review of Antimicrobial Resistance chaired by Jim O'Neill warns that if bacterial drug resistance remains to increase at the rate of today's levels, 10 million people per year may die of antibiotic resistance by 2050.

In recent years, the exploration of methods to control drug-resistant strains has attracted extensive attention from scholars hoping to find a promising alternative solution. Traditional Chinese medicine (TCM) has attracted the greatest interest among all methods. TCM has a long history and rich experience in treating infectious diseases. The antibacterial action of TCM and its compounds has a complex multi-link, multi-target, and multi-site process. Compared with antibiotics, TCM is characterised with more resources, easier access, lower drug resistance, more active ingredients (Yang et al., 2010; Wu et al., 2019) fewer adverse reactions, and more targets (Messier and Grenier, 2011; Eumkeb et al., 2012a). Many studies have shown that TCM has significant bacteriostatic or bactericidal effects. These effects occur mainly through inhibition of biofilm formation of drug-resistant bacteria, efflux pump system, enzyme activity, and changes in the permeability of bacteria and other drug-resistant mechanisms (Su et al., 2020). *Polygonum cuspidatum* (*Polygonum cuspidatum* Sieb. et Zucc.) extracts can exert antibacterial and bactericidal effects by destroying bacterial cell membranes and walls (Su et al., 2015). Extracts from *Hypericum perforatum* (*Hypericum perforatum* L.) and *Sophora moorcroftiana* (*Sophora moorcroftiana* (Benth.Baker)) also have antibacterial effects, as the extracts can inhibit the growth of drug-resistant bacteria by suppressing the efflux pump system (Wang et al., 2014; Dogan et al., 2019). Resveratrol can inhibit biofilm formation of avian pathogenic *Escherichia coli* to achieve a bacteriostatic effect (Ruan et al., 2021).

Studies have demonstrated that some TCM can directly inhibit drug-resistant bacteria. However, for TCM with no individually attributed antibacterial activity, if combined with antibacterial drugs, the synergistic effect of TCM can make these TCM play an important role in bacterial infection treatment. The synergistic effect by TCM can also enhance the susceptibility of drug-resistant bacteria to antibiotics and even reverse drug resistance. Studies on the antibacterial effects of pterostilbene and gentamicin alone and in combination showed no significant difference in antibacterial effects. However, when they were combined they completely inhibited the growth of bacteria and had synergistic antibacterial effects (Lee et al., 2017). The synergistic application of TCM and antibiotics in drug-resistant bacteria has stronger antibacterial activity, which is a recognised antibacterial treatment measure (Wagner and Ulrich-Merzenich, 2009). Several alternative antibiotic treatments for bacteria, such as bacteriocins (Cotter et al., 2013), essential oils (Esmael et al., 2020; Puvaca et al., 2021), antibodies (Berghman et al., 2005), and phage therapy (Chang et al., 2018), have been evaluated in studies and confirmed *in vitro* and with the use of animal models. However, these still present with many issues to consider, including cost, side effects, and safety, where most of them are still far from clinical use. As TCM has already been used clinically with a long history, combining antibiotics and TCM is a promising alternative therapy to resolve antibiotic resistance. As extracts from TCM may contain hundreds of chemical components, the isolation of active compounds under the guidance of bioassays is crucial to study their synergistic effects in detail. This review summarises the effects of flavonoids, alkaloids, phenols, and quinones (chemical structures of key compounds in these classes are shown in **Figure 1**) combined with antibiotics on bacterial and drug-resistant bacterial infections. It provides the basis for an alternative approach, involving TCM to treat bacterial and drug-resistant bacterial infections in the future, by applying a relatively new and promising option in antibiotic resistant treatment.

METHODOLOGY

Search strategy and research criteria: English articles published from September 2001 to May 2021 were searched in the PubMed database, and related keywords such as: "Traditional Chinese medicine," "Chinese herbal medicine," "antibiotics," "drug-resistant bacteria," "flavonoids," "alkaloids," "phenols," and "quinones" were used to search the database. The study included published data but excluded TCM treatments for other diseases, such as cancer. 180 English language articles published mainly since 2011 were located which related to the use of components from TCM against drug-resistant bacteria. According to our criteria, we reviewed the abstract and content of the articles, with 115 studies included as references, among which 86 were identified. Most of these papers focus on the synergistic antibacterial activity of the active ingredients of TCM combined with antibiotics against drug-resistant bacteria, and how some active ingredients of TCM can reverse drug resistance.

Synergy judgment criteria: In order to assess if a TCM component in combination with an antibiotic demonstrated a synergistic activity, we used the published definition of the fractional inhibitory concentration index (FICI), which is the sum of the FICs of each of the drugs, which were defined as the

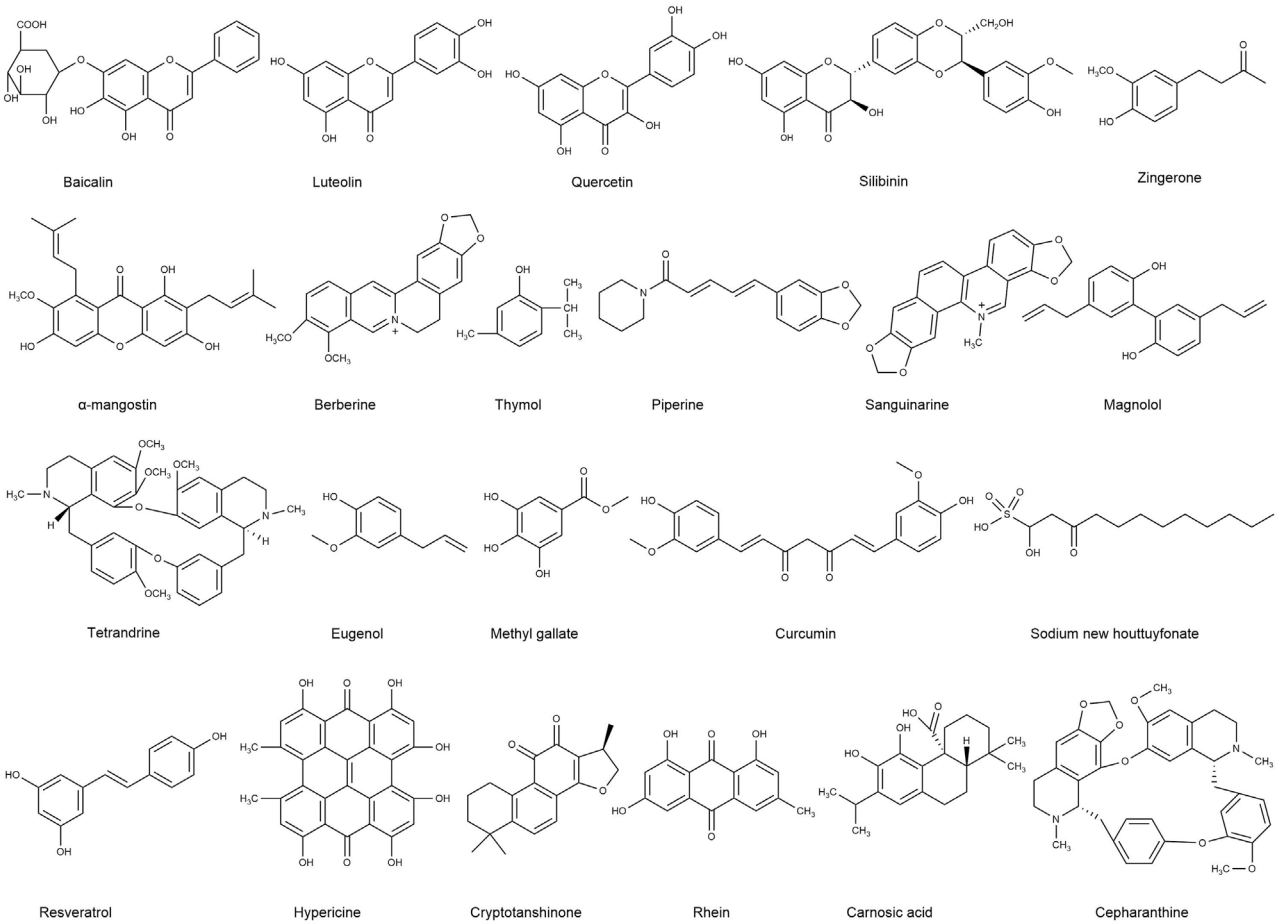


FIGURE 1 | List of key compounds studied for their synergy with antibiotics.

minimal inhibition concentration (MIC) of each drug when used in combination divided by the MIC of each drug when used alone, i.e., FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FICI were graded as: ≤ 0.5, synergy; > 0.5–≤ 1.0, additive; >1.0–≤ 2.0, indifference; and >2.0, antagonism (Kang et al., 2011).

REVIEW

Flavonoids Combined With Antibiotics for Antibacterial Effects

Flavonoids are compounds of some widely distributed plants and are found in photosynthetic cells, which exist broadly within the plant kingdom and in almost all parts of the plant (Havsteen, 1983). Baicalein and baicalin in the root of *Scutellaria baicalensis* Georgi, luteolin in the root and stem of *Reseda odorata* L., and quercetin in the flower and leaf of *Camellia sinensis* (L.) Kuntze are all flavonoids. For centuries, preparations containing flavonoids as the key physiologically active ingredients have been used by clinicians to treat human diseases. It is reported that flavonoids have anti-inflammatory and antibacterial effects, whilst potentially having antiviral, antioxidant and free radical scavenging abilities (Kumar and Pandey, 2013). Researchers have also actively investigated the antibacterial effects of flavonoids in combination with antibiotics.

Mai Fujita et al. (2005) demonstrated that the combination of baicalein with tetracycline and β-lactam antibiotics significantly reduced the MIC of MRSA such that it played an antibacterial role. When baicalein and ciprofloxacin were combined to treat MRSA infection, 12 of the 20 drug-resistant strains had FICI≤0.5, which mainly inhibited the efflux of ciprofloxacin by suppressing the efflux pump, thereby exerting a synergistic anti-MRSA effect (Chan et al., 2011). The main mechanism of the combination of active ingredients of TCM and antibiotics is shown in **Figure 2**. Qian et al. (2015) also found that the combined application of baicalein and penicillin can resist penicillinase-producing MRSA or *S. aureus* infection. When the concentration of baicalein increased from 8 μg/ml to 32 μg/ml, the MIC of penicillin decreased from 64 μg/ml to 4 μg/ml, significantly improving the resistant bacteria's susceptibility to penicillin. Recent studies have demonstrated that linezolid and baicalein can inhibit biofilm formation *in vivo* to play an anti-MRSA role (Liu T. et al., 2020). Baicalin has similar effects to baicalein, and if

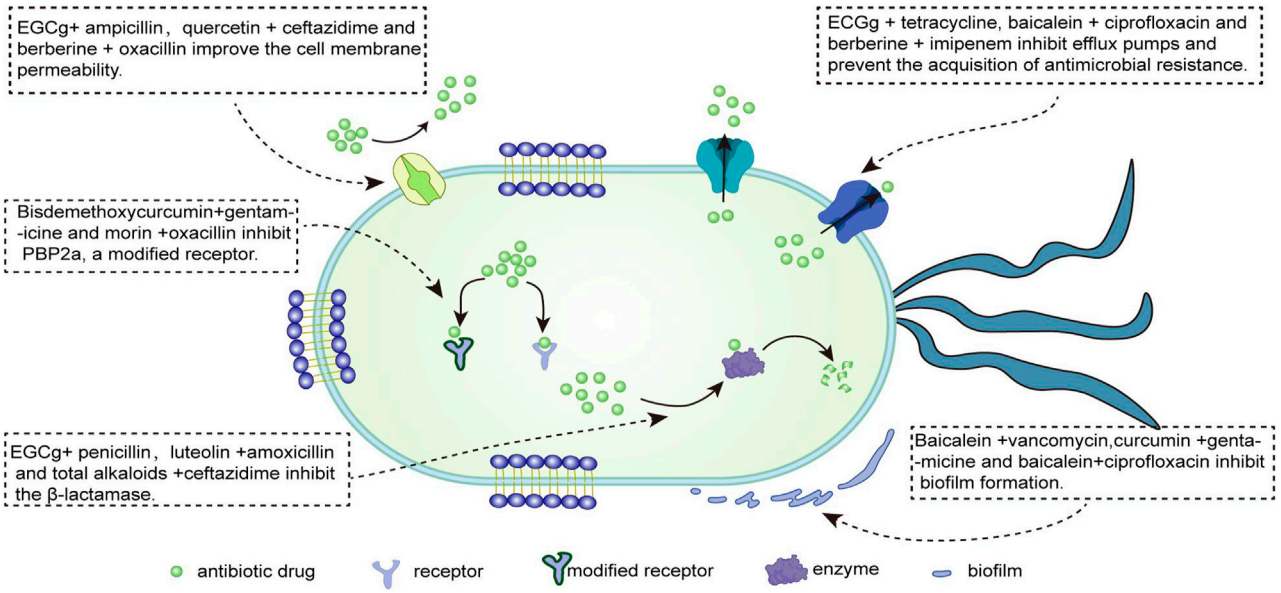


FIGURE 2 | The major mechanism and target of the antibacterial effect of antibiotics combined with active ingredients of traditional Chinese medicine.

Baicalin is used in combination with oxytetracycline and tetracycline, it can resist *S. aureus* infection, while in combination with β-lactam antibiotics, it yields anti-MRSA activity (Iain and Liu, 2000; Novy et al., 2011).

Usman Amin et al. (2016) demonstrated synergistic effects of luteolin and quercetin combined with ceftriaxone and imipenem against MRSA. In addition, luteolin combined with ampicillin, oxacillin, and gentamicin can synergically enhance the antibacterial action of aminoglycosides and β-lactam antibiotics against MRSA. The FICI of the combination of ½ MIC luteolin and ½ MIC antibiotics against MRSA ATCC 33591 for most strains was 0.125–0.562, and these combinations did not show additive or antagonistic effects (Joung et al., 2016). As well as inhibiting MRSA, luteolin can synergize with amoxicillin to reverse the resistance of amoxicillin-resistant *E. coli* and can fight *Streptococcus pyogenes* infection when combined with ceftazidime. Quercetin can also combat *S. pyogenes* combined with ceftazidime, where the FICIs of luteolin and quercetin paired with ceftazidime were 0.37 and 0.27, respectively (Eumkeb et al., 2012b; Siriwong et al., 2015). Siriwong et al. (2016) also demonstrated that quercetin with amoxicillin could reverse the resistance of amoxicillin-resistant *Staphylococcus epidermidis*. In addition, quercetin with ciprofloxacin, tetracycline, and erythromycin has an antibacterial effect on *S. aureus,* including MRSA. In the time-kill curves test, quercetin with tetracycline reduced the cell viability of resistant *E. coli* strains by more than eight times within 24 h compared with the drug group alone and had a FICI ≤0.5 (Abreu et al., 2016; Qu et al., 2019). Compared with other antibiotics, researchers found that ¼ MIC, ⅛ MIC quercetin combined with tobramycin and amikacin has potential systematic antibacterial activity against multidrug-resistant *Pseudomonas aeruginosa* (Vipin et al., 2020). Pal and Tripathi (Pal and Tripathi, 2019; 2020) reported that quercetin and meropenem had synergistic antibacterial effects on carbapenem-resistant *P. aeruginosa*, *A. baumannii*, *E. coli,* and *K. pneumoniae*, with FICI values of 0.18–0.50, 0.16–0.37, 0.187–0.375, and 0.093–0.500, respectively, which can not only significantly kill bacteria but also may reverse drug resistance.

It has been reported (Kang et al., 2011; Cai et al., 2018; Vivekanandan et al., 2018) that silibinin, an extract of *Silybum marianum* (L.) Gaertn., has anti-MRSA activity when combined with oxacillin or ampicillin. Another extract, silymarin, can improve the toxicity of linezolid and synergistic anti-MRSA infection, while a high concentration silibinin with kanamycin can inhibit the growth of *S. aureus*. Pimchan et al. (2017) demonstrated a synergistic effect between α-mangostin and ceftazidime in *A. baumannii*. The FICI of the combination of α-mangiferin and oxacillin against oxacillin-resistant *Staphylococcus saprophyticus* was 0.37. The number of bacterial colonies decreased by the combination of 2 μg/ml α-mangostin and 16 μg/ml oxacillin, and in the time-kill curves test ≥2 log10 cfu/ml also verified the synergy. When α-mangostin is combined with gentamicin and vancomycin hydrochloride, it can help inhibit vancomycin-resistant *Enterococci* (VRE) and MRSA infection, respectively (Sakagami et al., 2005; Phitaktim et al., 2016). **Table 1** lists the antibacterial effects of flavonoids combined with antibiotics.

Alkaloids Combined With Antibiotics for Antibacterial Effects

Alkaloids are components of botanical drugs and are widely distributed in nature. They are organic compounds with biological activity and are present within a wide range of plants, bacteria, and fungi (Qiu et al., 2014). Berberine is extracted from *Berberis vulgaris* L., total alkaloids from

TABLE 1 | Summary of flavonoids compounds in combination with antibiotics.

Species Name	Active ingredients	Drug resistant strains	Combination antibiotics	FICI	References
Thymus vulgaris L.	Baicalein	MRSA	Tetracycline, β -lactam antibiotics	—	Mai Fujita et al. (2005)
Scutellaria baicalensis Georgi	Baicalein	MRSA	Ciprofloxacin	≤0.5	Chan et al. (2011)
Scutellaria baicalensis Georgi	Baicalein	MRSA	Linezolid	—	Liu et al. (2020a)
Scutellaria baicalensis Georgi	Baicalein	*MRSA*,*Staphylococcus aureus*	penicillin	0.14–0.38	Qian et al. (2015)
Scutellaria baicalensis Georgi	Baicalin	*Staphylococcus* aureus	Oxytetracycline, Tetracycline	≤0.5	(Iain and Liu, 2000; Novy et al. (2011)
Scutellaria amoena C.H. Wright	Baicalin	MRSA	β -lactam antibiotics	≤0.5	-
Lonicera japonica Thunb., *Thymus vulgaris* L.	Luteolin	MRSA	Ceftriaxone, Imipenem	0.45–0.50	Usman Amin et al. (2016)
Thymus vulgaris L., *Daucus carota* L.	Luteolin	MRSA	Ampicillin, Oxacillin, Gentamicin	0.125–0.562	Joung et al. (2016)
Thymus vulgaris L., *Daucus carota* L.	Luteolin	*Escherichia coli*	Amoxicillin	≤0.5	Eumkeb et al. (2012b); Siriwong et al. (2015)
Daucus carota L., *Allium cepa* L.	Luteolin, Quercetin	*streptococcus pyogenes*	Ceftazidime	0.37、0.27	-
Allium cepa L., *Ginkgo biloba* L.	Quercetin	*Staphylococcus epidermidis*	Amoxicillin	0.5	Siriwong et al. (2016)
Allium cepa L., *Ginkgo biloba* L.	Quercetin	MRSA	Ciprofloxacin, Tetracycline and Erythromycin	—	(Abreu et al., 2016; Qu et al., 2019)
Allium cepa L., *Ginkgo biloba* L.	Quercetin	*Escherichia coli*	Tetracycline	≤0.5	-
Allium cepa L., *Ginkgo biloba L.*	Quercetin	*pseudomonas aeruginosa*	Tobramycin, Amikacin	0.25–0.5	Vipin et al. (2020)
Allium cepa L., *Berberis aristata* DC., *Camellia sinensis* (L.) Kuntze	Quercetin	*Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae*	Meropenem	0 .18-0.5、0.16-0 .37、0.187-0.375和 0.093-0.5	Pal and Tripathi, (2019); Pal and Tripathi, (2020)
Silybum marianum (L.) Gaertn.	Silibinin	MRSA	Oxacillin, Ampicillin	≤0.5	Kang et al. (2011); Cai et al. (2018); Vivekanandan et al. (2018)
Silybum marianum (L.) Gaertn.	Silibinin	*Staphylococcus aureus*	Kanamycin	—	-
Silybum marianum (L.) Gaertn.	Silymarin	MRSA	Linezolid	—	-
Garcinia mangostana L.	α-Mangostin	*Acinetobacter Baumannii*	Ceftazidime	<0.35	Pimchan et al. (2017)
Garcinia mangostana L.	α-Mangostin	*Staphylococcus saprophytic*	Oxacillin	0.37	Sakagami et al. (2005); Phitaktim et al. (2016).
Garcinia mangostana L.	α-Mangostin	*Enterococcus*, MRSA	Gentamicin, Vancomycin hydrochloride	≤0.5	-

Sophora alopecuroides L., and tetrandrine from *Stephania tetrandra* S. Moore are common alkaloids. Several clinical studies have reported that alkaloids have anti-inflammatory (Souza et al., 2020), antibacterial activities (Liu Y. et al., 2020) and antiviral (Gorpenchenko et al., 2019) pharmacological effects. Studies have shown that these alkaloid compounds are important in enhancing antibiotic effects for treating infections (Cushnie et al., 2014). In recent years, researchers have explored cooperative applications of alkaloids and antibiotics to fight against bacterial resistance.

Hyeon-Hee et al. (2005) showed the anti-MRSA effect of berberine. The FICI of berberine combined with ampicillin (0.625) had an additive effect, whereas if it joined with oxacillin (0.5) it had a synergistic effect. Some scholars have found that berberine combined with azithromycin has a synergistic antibacterial effect on MRSA and *P. aeruginosa*, and if it paired with levofloxacin, it could resist MRSA infection. The combination of ¼ MIC berberine and ⅛ MIC imipenem had a synergistic antibacterial effect on carbapenems resistant *P. aeruginosa* with a FICI of 0.375. In addition, berberine can increase the antibacterial activity of gentamicin and other aminoglycoside antibiotics against *P. aeruginosa* and reverse the resistance of antibacterial drugs. When berberine was combined with linezolid, cefoxitin, and erythromycin, the synergistic effect was significant in coagulase-negative *staphylococcus* (Zuo et al., 2012; Wojtyczka et al., 2014; Morita et al., 2016; Li et al., 2017; Su

TABLE 2 | Summary of alkaloids compounds in combination with antibiotics.

Species Name	Active ingredients	Drug resistant strains	Combination antibiotics	FICI	References
Coptis chinensis Franch., *Phellodendron amurense* Rupr.	Berberine	MRSA	Oxacillin	0.5	Hyeon-Hee Yu (2005)
Coptis chinensis Franch., *Phellodendron amurense* Rupr.	Berberine	MRSA	Azithromycin, Levofloxacin	0.188–0.5	Zuo et al. (2012); Wojtyczka et al. (2014); Morita et al. (2016); Li et al. (2017); Su and Wang, (2018)
Coptis chinensis Franch., *Phellodendron amurense* Rupr.	Berberine	*Pseudomonas aeruginosa*	Azithromycin	0.13–0.5	-
Coptis chinensis Franch., *Phellodendron amurense* Rupr.	Berberine	*Pseudomonas aeruginosa*	Gentamicin and other aminoglycoside antibiotics	<0.5	-
Coptis chinensis Franch., *Phellodendron amurense* Rupr.	Berberine	*Pseudomonas aeruginosa*	Imipenem	0.375	-
Coptis chinensis Franch., *Berberis vulgaris* L., *Berberis aristate* DC	Berberine	Coagulase negative *staphylococcus*	Linezolid, Cefoxitin and Erythromycin	—	-
Coptis chinensis Franch.	Berberine	*Salmonella, Klebsiella pneumoniae*	Ciprofloxacin	0.375–1	Zhou et al. (2016); Shi et al. (2018)
Coptis chinensis Franch.	Berberine	*Candida albicans, Candida tropicalis*	Fluconazole	0.03-0.27、0.13-1.0	Shi et al. (2017); Xu et al. (2017)
Coptis chinensis Franch., *Hydrastis canadensis* L., *Berberis vulgaris* L.	Berberine chloride	MRSA	Fusidic acid	0.19–0.5	Liang et al. (2014)
Coptis chinensis Franch., *Phellodendron amurense* Rupr., *Berberis aristate* DC.	Berberine hydrochloride	*Acinetobacter baumannii*	Tigecycline, Sulbactam, Meropenem and ciprofloxacin	<0.5	Li et al. (2021)
Coptis chinensis Franch., *Hydrastis canadensis* L., *Berberis vulgaris* L.	Berberine chloride	*Streptococcus orals*	Penicillin, Clindamycin and Erythromycin	—	Dziedzic et al. (2015); Wultanska et al. (2020); Yong et al. (2020)
Coptis chinensis Franch., *Hydrastis canadensis* L.	Berberine chloride	*Clostridium difficile*	Vancomycin	—	-
Coptis chinensis Franch.	Berberine hydrochloride	*Candida albicans*	Fluconazole	0.03–0.06	-
Piper nigrum L.	Piperine	MRSA	Gentamicin	0.5	Khameneh et al. (2015)
Sophora alopecuroides L.	Total alkaloid	*Escherichia coli*	Ciprofloxacin	0.131	Zhou et al. (2013); Pourahmad Jaktaji and Mohammadi, (2018)
Sophora alopecuroides L.	Total alkaloid	*Escherichia coli*	Cefotaxime, Ceftazidime	≤0.5	-
Stephania tetrandra S. Moore	Tetrandrine	*Candida albicans*	Ketoconazole	—	Zhang et al. (2010)
Stephania tetrandra S. Moore	Tetrandrine	MRSA	Cefazolin	0.188–0.625	Zuo et al. (2011)
Sanguinaria canadensis L.	Sanguinarine	MRSA	Ampicillin, Oxacillin, Norfloxacin, Ciprofloxacin	0.06–0.75	Obiang-Obounou et al. (2011)

and Wang, 2018). Although the FICI of berberine and ciprofloxacin against multidrug-resistant *Salmonella* and *K. pneumoniae* were between 0.375 and 1, the time-kill curves test confirmed the synergistic antibacterial effect of the combination (Zhou et al., 2016; Shi et al., 2018). Studies have shown that berberine and fluconazole can be combined to resist drug-resistant *Candida albicans* and fluconazole-resistant *Candida tropicalis*. Berberine can increase the biosynthesis of ergosterol, making it resistant to *C. albicans*. The effect of fluconazole on ergosterol can eliminate the resistance of berberine and synergise with berberine against drug-resistant *C. albicans*. Berberine and fluconazole also synergise against fluconazole-resistant *Candida tropicalis* by inhibiting efflux pumps (Shi et al., 2017; Xu et al., 2017). Liang et al. (2014) showed that an isoquinoline alkaloid may be extracted from *Berberis vulgaris* L. and other plants. The combination of berberine chloride and fusidic acid has shown a synergistic antibacterial effect on seven clinically isolated MRSA strains, with most significant inhibitions on two highly resistant strains, 4,806 and 7,155-1, and their FICIs were 0.19 and 0.38, respectively. Berberine chloride can increase the susceptibility of multidrug-resistant *A. baumannii* to tigecycline, sulbactam, meropenem, and ciprofloxacin to facilitate a more effective antibacterial role (Li et al., 2021). When berberine chloride combined with penicillin, clindamycin, and erythromycin, can also significantly inhibit the growth of *Streptococcus oralis* in a dose-dependent manner. Further, when combined with vancomycin, it can greatly inhibit the growth and motor capacity of *Clostridium difficile,* and can synergistically inhibit drug-resistant *C. albicans* when paired with fluconazole (Dziedzic et al., 2015; Wultanska et al., 2020; Yong et al., 2020).

Khameneh et al. (2015) demonstrated that the co-application of piperine and gentamicin nanoliposomes on MRSA had a significant synergistic antibacterial effect. Some researchers have shown that low-dose total alkaloids of *Sophora alopecuroides* L. and ciprofloxacin have synergistic antibacterial activity against

TABLE 3 | Summary of phenolic compounds in combination with antibiotics.

Species Name	Active ingredients	Drug resistant strains	Combination antibiotics	FICI	References
Camellia sinensis (L.) Kuntze	Epigallocatechin gallate	MRSA	Ampicillin, Sulbactam	0.19–0.56	(Hu et al., 2001; 2002)
Camellia sinensis (L.) Kuntze	Epigallocatechin gallate	MRSA	Imipenem, Panipenem	≤0.5	-
Camellia sinensis (L.) Kuntze	Epigallocatechin gallate	MRSA	Oxytetracycline	0.288–0.527	Novy et al. (2013)
Camellia sinensis (L.) Kuntze	Epigallocatechin gallate	*Staphylococcus aureus*	Penicillin, Ampicillin	≤0.5	Zhao et al. (2002)
Camellia sinensis (L.) Kuntze	Epigallocatechin gallate	*Staphylococcus aureus*	Tetracycline	—	Sudano Roccaro et al. (2004)
Magnolia officinalis Rehder & E.H.Wilson	Magnolol and Honokiol	MRSA	Oxacillin	≤0.5	Kim et al. (2015)
Magnolia officinalis Rehder & E.H.Wilson	Honokiol	*Candida albicans*	Fluconazole	0.125–0.5	Jin et al. (2010)
Thymus vulgaris L., *Origanum vulgare* L.	Thymol	*Staphylococcus aureus*	Tetracycline	—	Sousa Silveira et al. (2020)
Thymus vulgaris L., *Origanum vulgare* L.	Thymol	MRSA	Mupirocin	0.36–0.51	Kifer et al. (2016)
Eugenia cayophyllata Thunb., *Syzygium aromaticum* (L.) Merr. & L.M.Perry	Eugenol	Gram-negative bacilli	Vancomycin, Ampicillin, Oxacillin	—	Hemaiswarya and Doble, (2009)
Eugenia cayophyllata Thunb., *Syzygium aromaticum* (L.) Merr. & L.M.Perry	Eugenol	*Escherichia coli*	Colistin	0.375–0.5	Wang et al. (2018); Dhara and Tripathi, (2020)
Eugenia cayophyllata Thunb., *Syzygium aromaticum* (L.) Merr. & L.M.Perry, *Ocimum gratissimum* L.	Eugenol	*Enterobacter*	Cefotaxime, ciprofloxacin	0.08–0.5	-
Eugenia cayophyllata Thunb., *Syzygium aromaticum* (L.) Merr. & L.M.Perry	Eugenol	*Candida albicans*	Amphotericin B	0.27	Khan et al. (2019)
Rhus chinensis Mill.	Methyl gallate	Nalidixic acid resistant pathogens	Nalidixic acid	0.12–0.31	Choi et al. (2009)
Curcuma longa L.	Curcumin	*Pseudomonas aeruginosa*	Azithromycin, Gentamicin	0.25、0.37	Bahari et al. (2017)
Curcuma longa L.	Curcumin	*Pseudomonas aeruginosa*	Ceftazidime	0.26	Roudashti et al. (2017)
Curcuma longa L.	Curcumin	*Escherichia coli*	Ceftazidime	—	Kaur et al. (2018); Itzia Azucena et al. (2019); Sundaramoorthy et al. (2020)
Curcuma longa L.	Curcumin	*Escherichia coli, Klebsiella pneumoniae*	Colistin	0.03–0.5	-
Curcuma longa L.	Curcumin	*Acinetobacter baumannii*	Colistin	0.29	-
Curcuma longa L.	Bisdemethoxycurcumin	MRSA	Gentamicin, oxacillin	<0.1	Wang et al. (2020)
Rosmarinus officinalis L., *Salvia Rosmarinus* Spenn., *Punica granatum* L.	Phenols	*Pseudomonas aeruginosa*	Piperacillin, Ceftazidime, Imipenem, Gentamicin, Levofloxacin	≤0.5	Abu El-Wafa et al. (2020)
Salvia miltiorrhiza Bge.	Salvianolate	MRSA	Ampicillin	0.375	Liu et al. (2016)

multidrug-resistant *E. coli*. Total alkalids can enhance bacterial susceptibility to ciprofloxacin and cooperate with cefotaxime and ceftazidime against extended-spectrum β-lactamase (ESBL)-producing *E. coli* infection (Zhou et al., 2013; Pourahmad Jaktaji and Mohammadi, 2018). In time-kill curve tests, Zhang et al. (2010) showed that the combined application of 30 μg/ml tetrandrine and ketoconazole on drug-resistant *Candida* had synergistic antibacterial effects *in vitro* and *in vivo* but had no bactericidal effect. Tetrandrine and cefazolin in bisbenzylisoquinoline alkaloids presented a considerable synergistic effects against 90% of 10 clinically isolated MRSA strains, with the FICI between 0.188 and 0.625, while demethyltetrandrine and cefazolin had respective additive activities against 50% and 90% of tested MRSA strains, with the FICI ranging from 1.5 to 2.0 (Zuo et al., 2011). Another compound from TCM, called sanguinarine, can restore antibacterial activity of ampicillin, oxacillin, norfloxacin, and ciprofloxacin to treat MRSA by inhibiting the growth of drug-resistant bacteria (Obiang-Obounou et al., 2011). **Table 2** lists the antibacterial effects of the above alkaloids combined with antibiotics.

Phenolics Combined With Antibiotics for Antibacterial Effects

Phenolic compounds are some of the most diverse bioactive secondary metabolites in medicinal plants. They may also be a part of or the main component that contributes to a plants'

TABLE 4 | Summary of quinone compounds in combination with antibiotics.

Species Name	Active ingredients	Drug resistant strains	Combination antibiotics	FICI	References
Rheum palmatum L.	Rhein	MRSA	Ampicillin, Oxacillin	0.28-1、0.18-1.0	Joung et al. (2012)
Vitis vinifera L., *Morus alba* L.	Resveratrol	Gram-negative bacteria	Colistin	≤0.5	Cannatelli et al. (2018)
Morus alba L.	Oxyresveratrol	MRSA	Vancomycin, Ciprofloxacin	0.375	Joung et al. (2015)
Hypericum perforatum L.	Hypericin	MRSA	Oxacillin	0.1–0.16	Wang et al. (2019)
Salvia miltiorrhiza Bge.	Cryptotanshinone	*Staphylococcus aureus*	Ampicillin, Oxacillin, vancomycin	≤0.5	Cha et al. (2014)
Salvia miltiorrhiza Bge.	Cryptotanshinone	*Staphylococcus aureus*	Gentamicin, Streptomycin	0.25-0.5, 0.375-0.5	Teng et al. (2018); Ruan et al. (2020)
Salvia miltiorrhiza Bge.	Cryptotanshinone	*Staphylococcus aureus*	Fosfomycin	0.3125–0.375	-

bioactivity, with high antibacterial potential (Pinheiro et al., 2018). Phenolic compounds include: epigallocatechin gallate (EGCg), magnolol and honokiol, and eugenol, extracted from *Camellia sinensis* (L.) Kuntze, *Magnolia officinalis* Rehder & E.H.Wilson, and *Syzygium aromaticum* (L.) Merr. & L.M.Perry, respectively. Studies have found that they have anti-inflammatory, antibacteria and antioxidant effects (Daglia, 2012). These compounds may also be used to inhibit or kill pathogenic microorganisms (Marino et al., 2001). Researchers have also investigated the application of phenolic compounds with antibacterial drugs in the treatment of bacterial infections.

Hu et al. (Hu et al., 2001; 2002) demonstrated in 2001 that epigallocatechin gallate (EGCg) could be used together with β-lactam antibiotics, such as ampicillin or sulbactam for the treatment of MRSA infection. EGCg can also be combined with carbapenem antibiotics such as imipenem or panipenem in the treatment of MRSA infection, and reverse MRSA resistance. When EGCg is paired with oxytetracycline it has antibacterial effects on MRSA. EGCg at 4 μg/ml showed synergistic and additive effects on six and two clinically tested MRSA strains, respectively, with the FICI from 0.288 to 0.527 (Novy et al., 2013). A study showed that EGCg can further inhibit penicillinase to protect the antibacterial activity of penicillin and ampicillin against penicillinase-producing *S. aureus* (Zhao et al., 2002). It has been reported (Sudano Roccaro et al., 2004) that 50 μg/ml EGCg (½ MIC) joined with tetracycline can significantly reduce the MIC of tetracycline against *S. aureus* and exert an obvious antibacterial effect.

Kim et al. (2015) demonstrated that 10 μg/ml magnolol and 25 μg/ml honokiol combined with oxacillin has synergistic effects on MRSA. This application can increase the susceptibility of β-lactam antibiotics to MRSA. *In vivo* and *in vitro* experiments have demonstrated that the survival rate for honokiol combined with fluconazole in the treatment of fluconazole-resistant *C. albicans* infection reached 100%, compared with 20% for honokiol-treated or control group of mice over a period of 5 days (Jin et al., 2010). Sousa Silveira et al. (2020) found that thymol and tetracycline had an anti-*S. aureus* effect. In this study, the results of a fumigation bioassay showed that thymol had an obvious toxic effect on *Drosophila melanogaster* within 48 h of exposure with an EC_{50} (concentration for 50% of maximal effect) value of 17.96 μg/ml. Another study, showed the combination of mupirocin and thymol can enhance the antibacterial activity of mupirocin against MRSA (Kifer et al., 2016). Hemaiswarya and Doble (2009) found that eugenol combined with β-lactam antibiotics such as vancomycin, ampicillin, or oxacillin, had a synergistic antibacterial effect on Gram-negative bacilli. Some scholars (Wang et al., 2018; Dhara and Tripathi, 2020) showed that eugenol combined with colistin enhanced the antibacterial activity of the antibiotics against colistin-resistant *E. coli*, while the combination of eugenol with cefotaxime and ciprofloxacin could resist ESBL-producing quinolone-resistant pathogenic *Enterobacteria*, with FICI ≤0.5. Khan et al. (2019) demonstrated a synergistic effect of low doses (100 μg/ml) of eugenol together with amphotericin B (0.05 μg/ml) against *C. albicans*, with a FICI of 0.27. However, methyl gallate of *Galla Rhois* (*Rhus chinensis* Mill.), or carvacrol and nalidixic acid combination had a synergistic or partial synergistic effect (FICI = 0.31–0.75) on pathogens resistant to nalidixic acid, whereas methyl gallate or carvacrol restored the antibacterial activity of nalidixic acid (Choi et al., 2009).

Bahari et al. (2017) showed that sub-MIC of curcumin combined with azithromycin and gentamicin had a synergistic effect on *P. aeruginosa* PAO1. Moreover, the combination of sub-MIC curcumin and ceftazidime had a synergistic effect on *P. aeruginosa* PAO1 with a FICI of 0.26, and its combination with ciprofloxacin had a FICI of an additive effect (Roudashti et al., 2017). Several studies (Kaur et al., 2018; Itzia Azucena et al., 2019; Sundaramoorthy et al., 2020) showed that curcumin itself did not affect bacterial growth, but when combined with ceftazidime could resist enterotoxin *E. coli* infection. When combined with salicylate and colistin, curcumin could reduce the biological load of colisin-resistant *E. coli* U3790 and *K. pneumoniae* BC936. In addition, curcumin has a synergistic antibacterial effect on *A. baumannii* when paired with colistin. In another study, Wang et al. (Wang et al., 2020) demonstrated that the combination of ½ MIC bisdemethoxycurcumin and ½ MIC gentamicin had a significant synergistic effect on MRSA and a partial synergistic effect with oxacillin or a β-lactam antibiotic.

Abu El-Wafa et al. (2020) showed that the combination of phenolic extracts of pomegranate (*Punica granatum* L.) and rosemary (*Rosmarinus officinalis* L.) with piperacillin, ceftazidime, imipenem, gentamicin, and levofloxacin was effective in treating against *P. aeruginosa* PS-1 and exhibited a

TABLE 5 | Summary of other compounds in combination with antibiotics.

Species Name	Active ingredients	Drug resistant strains	Combination antibiotics	FICI	References
Houttuynia cordata Thumb.	Sodium new houttuyfonate.	MRSA	Cephalosporin, Meropenem, Oxacillin, Netilmicin	0.25–0.38	Lu et al. (2013)
Artemisia annua L.	Artesunate	MRSA	Oxacillin, Ampicillin	<0.37	Jiang et al. (2011); Li et al. (2011); Wei et al. (2020)
Artemisia annua L.	Artesunate	*Escherichia coli*	Ampicillin	≤0.5	-
Artemisia annua L.	Artesunate	*Escherichia coli*	Fluoroquinolone antibiotics	0.12–0.33	-
Caesalpinia sappan L.	3-Benzylchroman derivatives	MRSA	Aminoglycoside antibiotics	0.375–0.5	Zuo et al. (2014); Mun et al. (2015); Kuok et al. (2017); Wang et al. (2021)
Magnolia officinalis Rehder & E.H.Wilson, *Verbena officinalis* L., *Cinnamomum cassia* Presl	Morin, Tiliroside, Pinoresinol, Trans-Cinnamaldehyde	MRSA	Oxacillin	0.28–0.75	-
Pinus caribaea Morelet	Abietic acid	*Pseudo intermediate staphylococcus*	Oxacillin	0.375	-
Rosmarinus officinalis L.	Carnosic acid	MRSA	Gentamicin	0.5	Vazquez et al. (2016); Buommino et al. (2021)
Salvia chorassanica Bunge, *Artemisia khorassanica* Podlech, *Artemisia oliveriana* J.Gay ex Besser	Methanol extracts	*Acinetobacter baumannii*	Amikacin, Imipenem	0.185-0.625、0.18-0.37	-
Zingiber officinale Rosc.	Zingerone	*Pseudomonas aeruginosa*	Ciprofloxacin	—	Kumar et al. (2013); Yothin Teethaisong (2014)
Stephania suberosa Forman	Cepharanthine	MRSA	Ampicillin	<0.5	-

synergistic effect (FICI ≤0.5), which radically reduced the MIC of *P. aeruginosa*. Liu et al. (2016) found that the combination of salvianolic acid salt in *Salvia miltiorrhiza* (*Salvia miltiorrhiza* Bge.) and ampicillin applied to MRSA had the best antibacterial effects, which could also reverse MRSA resistance. **Table 3** lists the antibacterial effects of the above phenolic compounds combined with antibiotics.

Quinones Combined With Antibiotics for Antibacterial Effects

Quinone compounds in TCM can be divided into four types: benzoquinone, naphthoquinone, phenanthrene quinone, and anthraquinone. Anthraquinone and naphthoquinone are widely used in antibacterial treatment. Anthraquinone compounds from various plants were reported to have antibacterial activity (Novais et al., 2018) and anti-inflammatory, antifungal and antiviral effects (Li and Jiang, 2018). Naphthoquinone and naphthoquinone derivatives (Janeczko et al., 2016) were also reported to have antibacterial activity. Rhein extracted from *Rheum palmatum* L., resveratrol from the rhizome of *Polygonum cuspidatum* Sieb. et Zucc., and cryptotanshinone from *Salvia miltiorrhiza* Bge. are quinones. Quinone compounds in combination with antibiotics have been developed as a new measure for treating antibiotic resistance.

Joung et al. (2012) demonstrated that the FICI of rhein combined with ampicillin or oxacillin for all MRSA strains was 0.28–1 and 0.18–1, respectively and showed a synergistic or partial synergistic effect. Cannatelli et al. (2018) reported that resveratrol had no obvious intrinsic antibacterial activity but displayed synergistic effects with colistin on colistin-resistant Gram-negative bacilli of different species. Resveratrol oxide combined with vancomycin and ciprofloxacin had a synergistic effect on MRSA. It was partially additive or synergistic for the combination of resveratrol oxide with ampicillin, oxacillin, and norfloxacin. These combinations completely inhibited the growth of bacteria after 24 h (Joung et al., 2015). Studies have found that hypericin and β-lactam antibiotics such as oxacillin have anti-MRSA ability (Wang et al., 2019). Cha et al. (2014) demonstrated that cryptotanshinone combined with ampicillin, oxacillin, or vancomycin had synergistic effects on methicillin-resistant and vancomycin-resistant *S. aureus* and greatly inhibited the growth of bacteria. In addition, cryptotanshinone, together with gentamicin and streptomycin at safe doses (gentamicin ≤12 μg/ml and streptomycin ≤20 μg/ml) had a synergistic antibacterial effect on *S. aureus*. It reduced the resistance of aminoglycoside antibiotics to drug-resistant *S. aureus*, while the combination of cryptotanshinone with fosfomycin showed synergistic effect on fosfomycin-sensitive and fosfomycin-resistant *S. aureus* (FICI, 0.3125–0.375) (Teng et al., 2018; Ruan et al., 2020). **Table 4** lists the antibacterial effects of the above quinones in combination with antibiotics.

Other Compounds Combined With Antibiotics for Antibacterial Effects

Lu et al. (2013) demonstrated that sodium new houttuyfonate could be synergistic with cephalosporin, meropenem, oxacillin, and netilmicin against MRSA infection. The median FIC of the checkerboard method was 0.38, 0.38, 0.25, and 0.38, respectively. Several studies (Jiang et al., 2011; Li et al., 2011; Wei et al., 2020)

reported that artesunate combined with oxacillin and ampicillin had a synergistic antibacterial effect on MRSA. Combined with β-lactam antibiotics such as ampicillin, artesunate could also inhibit *E. coli* infection and enhance the antibacterial activity of fluoroquinolones against multidrug-resistant *E. coli*. The combination of 3-benzylchroman derivatives from the Chinese drug, *Caesalpinia sappan* L., with the aminoglycoside antibiotic can also be effective against MRSA. Morin, and trans-cinnamaldehyde combined with oxacillin has shown a synergistic effect against MRSA and potential for reversing the drug resistance of MRSA. M*agnolia officinalis* (*Magnolia officinalis* Rehder & E.H.Wilson) and *Verbena* (*Verbena officinalis* L.) extracts combined with oxacillin have otherwise showed a synergistic effect with partial efficacy against MRSA infection, where the colony number decreased by 3log10 cfu/mL (DPS-1 and DPS-3) after a treatment with a combination of ½ MIC morin and ½ MIC oxacillin for 24 h (Zuo et al., 2014; Mun et al., 2015; Kuok et al., 2017; Wang et al., 2021). Some scholars (Vazquez et al., 2016; Buommino et al., 2021) demonstrated that the pairing of rosin acid and oxacillin increased the susceptibility of methicillin-resistant *Staphylococcus pseudo intermediate* to oxacillin. Conversely, carnosic acid and gentamicin had obvious synergistic effects of bactericidal and bacteriostasis on clinical isolates of multidrug-resistant MRSA, while 4 μg/ml gentamicin combined with 4 μg/ml carnosic acid showed a 100% inhibition on bacterial growth. Fatemi et al. (2020) found that methanol extract of *Salvia chorassanica* (*Salvia chorassanica* Bunge) and *Artemisia khorassanica* (*Artemisia oliveriana* J. Gay ex Besser) synergically enhanced the susceptibility of multidrug-resistant *A. baumannii* with amikacin and imipenem. In addition, the combination of zingerone and ciprofloxacin significantly inhibited the formation of *P. aeruginosa* PAO1 biofilm and played an antibacterial role. *Stephania suberosa* Forman extract (2 mg/ml) in combination with ampicillin (0.15 μg/ml) had a significant effect on the treatment of MRSA infection and significantly reduced the dosage of ampicillin from >512 μg/ml (used alone) to 0.15 μg/ml (combined with the extract) (Kumar et al., 2013; Yothin Teethaisong 2014). **Table 5** lists the antibacterial effects of other active ingredients mentioned above in combination with antibiotics.

CONCLUSION

TCM has great antibacterial potential, with low toxicity, low drug resistance, and abundant resources. With further research on the mechanism of bacterial drug resistance and the continuous progress in the extraction technology of effective ingredients of TCM, the combined application of various active ingredients or compounds of TCM and antibiotics in the control of bacterial or drug-resistant bacteria infection has been widely studied. The active ingredients of TCM act as synergists by enhancing the antibacterial activity, improve the therapeutic effect and reduce the dosage of antibiotics and adverse reactions. At present, all studies on antibacterial or bacteriostatic effects from the combination of active ingredients of TCM and antibiotics have been conducted *in vitro*. There is insufficient evidence to prove the effectiveness, stability, selective toxicity, and targeted availability of these combinations in the human body. Therefore, further *in vivo* studies and animal models are needed. This paper summarises the interaction between different compounds of TCM, such as flavonoids, alkaloids, phenols and quinones, with antibiotics in the fight against drug-resistant bacteria. Using different active TCM ingredients with the same antibiotic, has a synergistic effect on drug-resistant bacteria. The same TCM ingredient can also have a synergistic antibacterial effect with different antibiotics. The above studies found that the combination of quercetin and berberine with antibiotics yielded good synergistic antibacterial effects and a broad antibacterial spectrum. Therefore, as the most researched active ingredients of TCM with strong antibacterial effects, flavonoids and alkaloids will be promising antibacterial choices when used in combination with antibiotics. This provides a new avenue to solve the problem of bacterial resistance through TCM and an important theoretical basis for finding alternative methods to counteract resistant bacteria. The combined use of TCM and antibiotics has become a new and alternative trend for antibacterial treatment. In the face of the current drug resistance crisis and the dilemma of new drug research and development, finding a more effective and safer alternative for the treatment of drug-resistant bacterial infection is crucial. The in-depth study of the synergistic antibacterial effect and synergistic mechanism of the combination of active components of TCM and antibiotics *in vivo,* may become an important research direction in the future.

AUTHOR CONTRIBUTIONS

JG, SD, and JL conceived and designed the work; XJ and FQ coordinated technical support and funding; JL wrote the manuscript and created the tables and figures; SF offered advice and explanation; XL checked the language of the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Natural Science Foundation of China (Grant Nos. 32170119 and 31870135).

REFERENCES

Abreu, A. C., Saavedra, M. J., Simões, L. C., and Simões, M. (2016). Combinatorial Approaches with Selected Phytochemicals to Increase Antibiotic Efficacy against *Staphylococcus aureus* Biofilms. *Biofouling* 32, 1103–1114. doi:10.1080/08927014.2016.1232402

Abu El-Wafa, W. M., Ahmed, R. H., and Ramadan, M. A. (2020). Synergistic Effects of Pomegranate and Rosemary Extracts in Combination with Antibiotics against Antibiotic Resistance and Biofilm Formation of

Pseudomonas aeruginosa. *Braz J. Microbiol.* 51, 1079–1092. doi:10.1007/s42770-020-00284-3

Bahari, S., Zeighami, H., Mirshahabi, H., Roudashti, S., and Haghi, F. (2017). Inhibition of *Pseudomonas aeruginosa* Quorum Sensing by Subinhibitory Concentrations of Curcumin with Gentamicin and Azithromycin. *J. Glob. Antimicrob. Resist* 10, 21–28. doi:10.1016/j.jgar.2017.03.006

Barber, M. (1961). Methicillin-resistant Staphylococci. *J. Clin. Pathol.* 14, 385–393. doi:10.1136/jcp.14.4.385

Barriere, S. L. (2014). Clinical, Economic and Societal Impact of Antibiotic Resistance. *Expert Opin. Pharmacother.* doi:10.1517/14656566.2015.983077

Berghman, L. R., Abi-Ghanem, D., and Ricke, S. C. (2005). Antibodies: An Alternative for Antibiotics? *Poult. Sci.*

Buommino, E., Vollaro, A., Nocera, F. P., Lembo, F., Dellagreca, M., De Martino, L., et al. (2021). Synergistic Effect of Abietic Acid with Oxacillin against Methicillin-Resistant Staphylococcus Pseudintermedius. *Antibiot. (Basel)* 10. doi:10.3390/antibiotics10010080

Cai, J. Y., Li, J., Hou, Y. N., Ma, K., Yao, G. D., Liu, W. W., et al. (2018). Concentration-dependent Dual Effects of Silibinin on Kanamycin-Induced Cells Death in *Staphylococcus aureus*. *Biomed. Pharmacother.* 102, 782–791. doi:10.1016/j.biopha.2018.03.133

Cannatelli, A., Principato, S., Colavecchio, O. L., Pallecchi, L., and Rossolini, G. M. (2018). Synergistic Activity of Colistin in Combination with Resveratrol against Colistin-Resistant Gram-Negative Pathogens. *Front. Microbiol.* 9, 1808. doi:10.3389/fmicb.2018.01808

Cha, J. D., Lee, J. H., Choi, K. M., Choi, S. M., and Park, J. H. (2014). Synergistic Effect between Cryptotanshinone and Antibiotics against Clinic Methicillin and Vancomycin-Resistant *Staphylococcus aureus*. *Evid. Based Complement. Altern. Med.* 2014, 450572. doi:10.1155/2014/450572

Chan, B. C., Ip, M., Lau, C. B., Lui, S. L., Jolivalt, C., Ganem-Elbaz, C., et al. (2011). Synergistic Effects of Baicalein with Ciprofloxacin against NorA Over-expressed Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Inhibition of MRSA Pyruvate Kinase. *J. Ethnopharmacol.* 137, 767–773. doi:10.1016/j.jep.2011.06.039

Chang, R. Y. K., Wallin, M., Lin, Y., Leung, S. S. Y., Wang, H., Morales, S., et al. (2018). Phage Therapy for Respiratory Infections. *Adv. Drug Deliv. Rev.* 133, 76–86. doi:10.1016/j.addr.2018.08.001

Choi, J. G., Kang, O. H., Lee, Y. S., Oh, Y. C., Chae, H. S., Jang, H. J., et al. (2009). Antibacterial Activity of Methyl Gallate Isolated from Galla Rhois or Carvacrol Combined with Nalidixic Acid against Nalidixic Acid Resistant Bacteria. *Molecules* 14, 1773–1780. doi:10.3390/molecules14051773

Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins - a Viable Alternative to Antibiotics? *Nat. Rev. Microbiol.* 11, 95–105. doi:10.1038/nrmicro2937

Cushnie, T. P., Cushnie, B., and Lamb, A. J. (2014). Alkaloids: an Overview of Their Antibacterial, Antibiotic-Enhancing and Antivirulence Activities. *Int. J. Antimicrob. Agents* 44, 377–386. doi:10.1016/j.ijantimicag.2014.06.001

da Costa Júnior, S. D., Da Silva, W. R. C., Da Silva, A. M. C. M., Maciel, M. A. V., and Cavalcanti, I. M. F. (2020). Synergistic Effect between Usnic Acid and Polymyxin B against Resistant Clinical Isolates of *Pseudomonas aeruginosa*. *Evid. Based Complement. Altern. Med.* 2020, 9852145. doi:10.1155/2020/9852145

Daglia, M. (2012). Polyphenols as Antimicrobial Agents. *Curr. Opin. Biotechnol.* 23, 174–181. doi:10.1016/j.copbio.2011.08.007

Dhara, L., and Tripathi, A. (2020). The Use of Eugenol in Combination with Cefotaxime and Ciprofloxacin to Combat ESBL-Producing Quinolone-Resistant Pathogenic Enterobacteriaceae. *J. Appl. Microbiol.* 129, 1566–1576. doi:10.1111/jam.14737

Dogan, S., Gokalsin, B., Senkardes, I., Dogan, A., and Sesal, N. C. (2019). Anti-Quorum Sensing and Anti-biofilm Activities of *Hypericum perforatum* Extracts against *Pseudomonas aeruginosa*. *J. Ethnopharmacol.* 235, 293–300.

Dziedzic, A., Wojtyczka, R. D., and Kubina, R. (2015). Inhibition of Oral Streptococci Growth Induced by the Complementary Action of Berberine Chloride and Antibacterial Compounds. *Molecules* 20, 13705–13724. doi:10.3390/molecules200813705

Esmael, A., Hassan, M. G., Amer, M. M., Abdelrahman, S., Hamed, A. M., Abd-Raboh, H. A., et al. (2020). Antimicrobial Activity of Certain Natural-Based Plant Oils against the Antibiotic-Resistant Acne Bacteria. *Saudi J. Biol. Sci.* 27, 448–455. doi:10.1016/j.sjbs.2019.11.006

Eumkeb, G., Siriwong, S., Phitaktim, S., Rojtinnakorn, N., and Sakdarat, S. (2012a). Synergistic Activity and Mode of Action of Flavonoids Isolated from Smaller Galangal and Amoxicillin Combinations against Amoxicillin-Resistant *Escherichia coli*. *J. Appl. Microbiol.* 112, 55–64. doi:10.1111/j.1365-2672.2011.05190.x

Eumkeb, G., Siriwong, S., and Thumanu, K. (2012b). Synergistic Activity of Luteolin and Amoxicillin Combination against Amoxicillin-Resistant *Escherichia coli* and Mode of Action. *J. Photochem Photobiol. B* 117, 247–253. doi:10.1016/j.jphotobiol.2012.10.006

Fatemi, N., Sharifmoghadam, M. R., Bahreini, M., Khameneh, B., and Shadifar, H. (2020). Antibacterial and Synergistic Effects of Herbal Extracts in Combination with Amikacin and Imipenem against Multidrug-Resistant Isolates of Acinetobacter. *Curr. Microbiol.* 77, 1959–1967. doi:10.1007/s00284-020-02105-0

Fujita, M., Shiota, S., Hatano, T., Kuroda, T., Hatano, T., Mizushima, T. T., et al. (2005). Remarkable Synergies between Baicalein and Tetracycline, and Baicalein and Beta-Lactams against Methicillin-Resistant *Staphylococcus aureus*. *Microbiol. Immunol.* 49, 391–396. doi:10.1111/j.1348-0421.2005.tb03732.x

Gorpenchenko, T. Y., Grigorchuk, V. P., Bulgakov, D. V., Tchernoded, G. K., and Bulgakov, V. P. (2019). Tempo-Spatial Pattern of Stepharine Accumulation in Stephania Glabra Morphogenic Tissues. *Int. J. Mol. Sci.* 20. doi:10.3390/ijms20040808

Havsteen, B. (1983). Flavonoids, a Class of Natural Products of High Pharmacological Potency. *Biochem. Pharmacol.* 32, 1141–1148. doi:10.1016/0006-2952(83)90262-9

Hemaiswarya, S., and Doble, M. (2009). Synergistic Interaction of Eugenol with Antibiotics against Gram Negative Bacteria. *Phytomedicine* 16, 997–1005. doi:10.1016/j.phymed.2009.04.006

Hu, Z. Q., Zhao, W. H., Asano, N., Yoda, Y., Hara, Y., and Shimamura, T. (2002). Epigallocatechin Gallate Synergistically Enhances the Activity of Carbapenems against Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 46, 558–560. doi:10.1128/aac.46.2.558-560.2002

Hu, Z. Q., Zhao, W. H., Hara, Y., Shimamura, T., Hara, Y., and Shimamura, T. (2001). Epigallocatechin Gallate Synergy with Ampicillin/sulbactam against 28 Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 48, 361–364. doi:10.1093/jac/48.3.361

Iain, X., and Liu, D. G. D. a. R. M. E. R. (2000). Baicalin Synergy with B-Lactam Antibiotics against Methicillinresistant *Staphylococcus aureus* and Other B-Lactam-Resistant Strains of *S. aureus*. *J. Pharm. Pharmacol.* 52, 361±366.

Itzia Azucena, R. C., José Roberto, C. L., Martin, Z. R., Rafael, C. Z., Leonardo, H. H., Gabriela, T. P., et al. (2019). Drug Susceptibility Testing and Synergistic Antibacterial Activity of Curcumin with Antibiotics against Enterotoxigenic *Escherichia coli*. *Antibiot. (Basel)* 8. doi:10.3390/antibiotics8020043

Janeczko, M., Demchuk, O. M., Strzelecka, D., Kubiński, K., and Masłyk, M. (2016). New Family of Antimicrobial Agents Derived from 1,4-naphthoquinone. *Eur. J. Med. Chem.* 124, 1019–1025. doi:10.1016/j.ejmech.2016.10.034

Jiang, W., Li, B., Zheng, X., Liu, X., Cen, Y., Li, J., et al. (2011). Artesunate in Combination with Oxacillin Protect Sepsis Model Mice Challenged with Lethal Live Methicillin-Resistant *Staphylococcus aureus* (MRSA) via its Inhibition on Proinflammatory Cytokines Release and Enhancement on Antibacterial Activity of Oxacillin. *Int. Immunopharmacol.* 11, 1065–1073. doi:10.1016/j.intimp.2011.02.028

Jin, J., Guo, N., Zhang, J., Ding, Y., Tang, X., Liang, J., et al. (2010). The Synergy of Honokiol and Fluconazole against Clinical Isolates of Azole-Resistant Candida Albicans. *Lett. Appl. Microbiol.* 51, 351–357. doi:10.1111/j.1472-765X.2010.02900.x

Joung, D. K., Choi, S. H., Kang, O. H., Kim, S. B., Mun, S. H., Seo, Y. S., et al. (2015). Synergistic Effects of Oxyresveratrol in Conjunction with Antibiotics against Methicillin-Resistant *Staphylococcus aureus*. *Mol. Med. Rep.* 12, 663–667. doi:10.3892/mmr.2015.3345

Joung, D. K., Joung, H., Yang, D. W., Kwon, D. Y., Choi, J. G., Woo, S., et al. (2012). Synergistic Effect of Rhein in Combination with Ampicillin or Oxacillin against Methicillin-Resistant *Staphylococcus aureus*. *Exp. Ther. Med.* 3, 608–612. doi:10.3892/etm.2012.459

Joung, D. K., Kang, O. H., Seo, Y. S., Zhou, T., Lee, Y. S., Han, S. H., et al. (2016). Luteolin Potentiates the Effects of Aminoglycoside and β-lactam Antibiotics against Methicillin-Resistant *Staphylococcus aureus In Vitro*. *Exp. Ther. Med.* 11, 2597–2601. doi:10.3892/etm.2016.3212

Kang, H. K., Kim, H. Y., and Cha, J. D. (2011). Synergistic Effects between Silibinin and Antibiotics on Methicillin-Resistant *Staphylococcus aureus* Isolated from Clinical Specimens. *Biotechnol. J.* 6, 1397–1408. doi:10.1002/biot.201000422

Kaur, A., Sharma, P., and Capalash, N. (2018). Curcumin Alleviates Persistence of Acinetobacter Baumannii against Colistin. *Sci. Rep.* 8, 11029. doi:10.1038/s41598-018-29291-z

Khameneh, B., Iranshahy, M., Ghandadi, M., Ghoochi Atashbeyk, D., Fazly Bazzaz, B. S., and Iranshahi, M. (2015). Investigation of the Antibacterial Activity and Efflux Pump Inhibitory Effect of Co-loaded Piperine and Gentamicin Nanoliposomes in Methicillin-Resistant *Staphylococcus aureus*. *Drug Dev. Ind. Pharm.* 41, 989–994. doi:10.3109/03639045.2014.920025

Khan, S. N., Khan, S., Misba, L., Sharief, M., Hashmi, A., and Khan, A. U. (2019). Synergistic Fungicidal Activity with Low Doses of Eugenol and Amphotericin B against Candida Albicans. *Biochem. Biophys. Res. Commun.* 518, 459–464. doi:10.1016/j.bbrc.2019.08.053

Kifer, D., Mužinić, V., and Klarić, M. Š. (2016). Antimicrobial Potency of Single and Combined Mupirocin and Monoterpenes, Thymol, Menthol and 1,8-cineole against *Staphylococcus aureus* Planktonic and Biofilm Growth. *J. Antibiot. (Tokyo)* 69, 689–696. doi:10.1038/ja.2016.10

Kim, S. Y., Kim, J., Jeong, S. I., Jahng, K. Y., and Yu, K. Y. (2015). Antimicrobial Effects and Resistant Regulation of Magnolol and Honokiol on Methicillin-Resistant *Staphylococcus aureus*. *Biomed. Res. Int.* 2015, 283630. doi:10.1155/2015/283630

Kumar, L., Chhibber, S., and Harjai, K. (2013). Zingerone Inhibit Biofilm Formation and Improve Antibiofilm Efficacy of Ciprofloxacin against *Pseudomonas aeruginosa* PAO1. *Fitoterapia* 90, 73–78. doi:10.1016/j.fitote.2013.06.017

Kumar, S., and Pandey, A. K. (20132013). Chemistry and Biological Activities of Flavonoids: an Overview. *ScientificWorldJournal* 2013, 162750. doi:10.1155/2013/162750

Kuok, C. F., Hoi, S. O., Hoi, C. F., Chan, C. H., Fong, I. H., Ngok, C. K., et al. (2017). Synergistic Antibacterial Effects of Herbal Extracts and Antibiotics on Methicillin-Resistant *Staphylococcus aureus*: A Computational and Experimental Study. *Exp. Biol. Med. (Maywood)* 242, 731–743. doi:10.1177/1535370216689828

Lee, W. X., Basri, D. F., and Ghazali, A. R. (2017). Bactericidal Effect of Pterostilbene Alone and in Combination with Gentamicin against Human Pathogenic Bacteria. *Molecules* 22. doi:10.3390/molecules22030463

Li, B., Yao, Q., Pan, X. C., Wang, N., Zhang, R., Li, J., et al. (2011). Artesunate Enhances the Antibacterial Effect of {beta}-Lactam Antibiotics against *Escherichia coli* by Increasing Antibiotic Accumulation via Inhibition of the Multidrug Efflux Pump System AcrAB-TolC. *J. Antimicrob. Chemother.* 66, 769–777. doi:10.1093/jac/dkr017

Li, X., Song, Y., Wang, L., Kang, G., Wang, P., Yin, H., et al. (2021). A Potential Combination Therapy of Berberine Hydrochloride with Antibiotics against Multidrug-Resistant Acinetobacter Baumannii. *Front. Cell. Infect. Microbiol.* 11, 660431. doi:10.3389/fcimb.2021.660431

Li, Y., Huang, J., Li, L., and Liu, L. (2017). Synergistic Activity of Berberine with Azithromycin against Pseudomonas Aeruginosa Isolated from Patients with Cystic Fibrosis of Lung *In Vitro* and *In Vivo*. *Cell. Physiol. Biochem.* 42, 1657–1669. doi:10.1159/000479411

Li, Y., and Jiang, J. G. (2018). Health Functions and Structure-Activity Relationships of Natural Anthraquinones from Plants. *Food Funct.* 9, 6063–6080. doi:10.1039/c8fo01569d

Liang, R. M., Yong, X. L., Duan, Y. Q., Tan, Y. H., Zeng, P., Zhou, Z. Y., et al. (2014). Potent *In Vitro* Synergism of Fusidic Acid (FA) and Berberine Chloride (BBR) against Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA). *World J. Microbiol. Biotechnol.* 30, 2861–2869. doi:10.1007/s11274-014-1712-2

Liu, Q. Q., Han, J., Zuo, G. Y., Wang, G. C., and Tang, H. S. (2016). Potentiation Activity of Multiple Antibacterial Agents by Salvianolate from the Chinese Medicine Danshen against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *J. Pharmacol. Sci.* 131, 13–17. doi:10.1016/j.jphs.2015.10.009

Liu, T., Luo, J., Bi, G., Du, Z., Kong, J., and Chen, Y. (2020a). Antibacterial Synergy between Linezolid and Baicalein against Methicillin-Resistant *Staphylococcus aureus* Biofilm *In Vivo*. *Microb. Pathog.* 147, 104411. doi:10.1016/j.micpath.2020.104411

Liu, Y., Cui, Y., Lu, L., Gong, Y., Han, W., and Piao, G. (2020b). Natural Indole-Containing Alkaloids and Their Antibacterial Activities. *Arch. Pharm. Weinh.* 353, e2000120. doi:10.1002/ardp.202000120

Lu, X., Yang, X., Li, X., Lu, Y., Ren, Z., Zhao, L., et al. (2013). *In Vitro* activity of Sodium New Houttuyfonate Alone and in Combination with Oxacillin or Netilmicin against Methicillin-Resistant *Staphylococcus aureus*. *PLoS One* 8, e68053. doi:10.1371/journal.pone.0068053

Marino, M., Bersani, C., and Comi, G. (2001). Impedance Measurements to Study the Antimicrobial Activity of Essential Oils from Lamiaceae and Compositae. *Int. J. Food Microbiol.* 67, 187–195. doi:10.1016/s0168-1605(01)00447-0

Messier, C., and Grenier, D. (2011). Effect of Licorice Compounds Licochalcone A, Glabridin and Glycyrrhizic Acid on Growth and Virulence Properties of Candida Albicans. *Mycoses* 54, e801–6. doi:10.1111/j.1439-0507.2011.02028.x

Morita, Y., Nakashima, K., Nishino, K., Kotani, K., Tomida, J., Inoue, M., et al. (2016). Berberine Is a Novel Type Efflux Inhibitor Which Attenuates the MexXY-Mediated Aminoglycoside Resistance in *Pseudomonas aeruginosa*. *Front. Microbiol.* 7, 1223. doi:10.3389/fmicb.2016.01223

Mun, S. H., Lee, Y. S., Han, S. H., Lee, S. W., Cha, S. W., Kim, S. B., et al. (2015). *In Vitro* Potential Effect of Morin in the Combination with β-Lactam Antibiotics against Methicillin-Resistant *Staphylococcus aureus*. *Foodborne Pathog. Dis.* 12, 545–550. doi:10.1089/fpd.2014.1923

Novais, J. S., Moreira, C. S., Silva, A. C. J. A., Loureiro, R. S., Sá Figueiredo, A. M., Ferreira, V. F., et al. (2018). Antibacterial Naphthoquinone Derivatives Targeting Resistant Strain Gram-Negative Bacteria in Biofilms. *Microb. Pathog.* 118, 105–114. doi:10.1016/j.micpath.2018.03.024

Novy, P., Rondevaldova, J., Kourimska, L., and Kokoska, L. (2013). Synergistic Interactions of Epigallocatechin Gallate and Oxytetracycline against Various Drug Resistant *Staphylococcus aureus* Strains *In Vitro*. *Phytomedicine* 20, 432–435. doi:10.1016/j.phymed.2012.12.010

Novy, P., Urban, J., Leuner, O., Vadlejch, J., and Kokoska, L. (2011). *In Vitro* synergistic Effects of Baicalin with Oxytetracycline and Tetracycline against *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 66, 1298–1300. doi:10.1093/jac/dkr108

Obiang-Obounou, B. W., Kang, O. H., Choi, J. G., Keum, J. H., Kim, S. B., Mun, S. H., et al. (2011). *In Vitro* potentiation of Ampicillin, Oxacillin, Norfloxacin, Ciprofloxacin, and Vancomycin by Sanguinarine against Methicillin-Resistant *Staphylococcus aureus*. *Foodborne Pathog. Dis.* 8, 869–874. doi:10.1089/fpd.2010.0759

Pal, A., and Tripathi, A. (2020). Demonstration of Bactericidal and Synergistic Activity of Quercetin with Meropenem Among Pathogenic Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae*. *Microb. Pathog.* 143, 104120. doi:10.1016/j.micpath.2020.104120

Pal, A., and Tripathi, A. (2019). Quercetin Potentiates Meropenem Activity Among Pathogenic Carbapenem-Resistant *Pseudomonas aeruginosa* and Acinetobacter Baumannii. *J. Appl. Microbiol.* 127, 1038–1047. doi:10.1111/jam.14388

Phitaktim, S., Chomnawang, M., Sirichaiwetchakoon, K., Dunkhunthod, B., Hobbs, G., and Eumkeb, G. (2016). Synergism and the Mechanism of Action of the Combination of α-mangostin Isolated from Garcinia Mangostana L. And Oxacillin against an Oxacillin-Resistant Staphylococcus Saprophyticus. *BMC Microbiol.* 16, 195. doi:10.1186/s12866-016-0814-4

Piddock, L. J. (2012). The Crisis of No New Antibiotics-Wwhat Is the Way Forward? *Lancet Infect. Dis.* 12, 249–253. doi:10.1016/S1473-3099(11)70316-4

Pimchan, T., Maensiri, D., and Eumkeb, G. (2017). Synergy and Mechanism of Action of α-mangostin and Ceftazidime against Ceftazidime-Resistant Acinetobacter Baumannii. *Lett. Appl. Microbiol.* 65, 285–291. doi:10.1111/lam.12789

Pinheiro, P. F., Menini, L. A. P., Bernardes, P. C., Saraiva, S. H., Carneiro, J. W. M., Costa, A. V., et al. (2018). Semisynthetic Phenol Derivatives Obtained from Natural Phenols: Antimicrobial Activity and Molecular Properties. *J. Agric. Food Chem.* 66, 323–330. doi:10.1021/acs.jafc.7b04418

Pourahmad Jaktaji, R., and Mohammadi, P. (2018). Effect of Total Alkaloid Extract of Local Sophora Alopecuroides on Minimum Inhibitory Concentration and Intracellular Accumulation of Ciprofloxacin, and acrA Expression in Highly

Resistant *Escherichia coli* Clones. *J. Glob. Antimicrob. Resist* 12, 55–60. doi:10.1016/j.jgar.2017.09.005

Puvaca, N., Milenkovic, J., Galonja Coghill, T., Bursic, V., Petrovic, A., Tanaskovic, S., et al. (2021). Antimicrobial Activity of Selected Essential Oils against Selected Pathogenic Bacteria: *In Vitro* Study. *Antibiot. (Basel)* 10.

Qian, M., Tang, S., Wu, C., Wang, Y., He, T., Chen, T., et al. (2015). Synergy between Baicalein and Penicillins against Penicillinase-Producing *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 305, 501–504. doi:10.1016/j.ijmm.2015.05.001

Qiu, S., Sun, H., Zhang, A. H., Xu, H. Y., Yan, G. L., Han, Y., et al. (2014). Natural Alkaloids: Basic Aspects, Biological Roles, and Future Perspectives. *Chin. J. Nat. Med.* 12, 401–406. doi:10.1016/S1875-5364(14)60063-7

Qu, S., Dai, C., Shen, Z., Tang, Q., Wang, H., Zhai, B., et al. (2019). Mechanism of Synergy between Tetracycline and Quercetin against Antibiotic Resistant *Escherichia coli*. *Front. Microbiol.* 10, 2536. doi:10.3389/fmicb.2019.02536

Roudashti, S., Zeighami, H., Mirshahabi, H., Bahari, S., Soltani, A., and Haghi, F. (2017). Synergistic Activity of Sub-inhibitory Concentrations of Curcumin with Ceftazidime and Ciprofloxacin against *Pseudomonas aeruginosa* Quorum Sensing Related Genes and Virulence Traits. *World J. Microbiol. Biotechnol.* 33, 50. doi:10.1007/s11274-016-2195-0

Ruan, X., Deng, X., Tan, M., Yu, C., Zhang, M., Sun, Y., et al. (2021). *In Vitro* antibiofilm Activity of Resveratrol against Avian Pathogenic *Escherichia coli*. *BMC Vet. Res.* 17, 249. doi:10.1186/s12917-021-02961-3

Ruan, Z., Cui, J., He, Z., Guo, Y., Jia, X., and Huang, X. (2020). Synergistic Effects from Combination of Cryptotanshinone and Fosfomycin against Fosfomycin-Susceptible and Fosfomycin-Resistant *Staphylococcus aureus*. *Infect. Drug Resist* 13, 2837–2844. doi:10.2147/IDR.S255296

Sakagami, Y., Iinuma, M., Piyasena, K. G., and Dharmaratne, H. R. (2005). Antibacterial Activity of Alpha-Mangostin against Vancomycin Resistant Enterococci (VRE) and Synergism with Antibiotics. *Phytomedicine* 12, 203–208. doi:10.1016/j.phymed.2003.09.012

Shi, C., Li, M., Muhammad, I., Ma, X., Chang, Y., Li, R., et al. (2018). Combination of Berberine and Ciprofloxacin Reduces Multi-Resistant Salmonella Strain Biofilm Formation by Depressing mRNA Expressions of luxS, rpoE, and ompR. *J. Vet. Sci.* 19, 808–816. doi:10.4142/jvs.2018.19.6.808

Shi, G., Shao, J., Wang, T., Wu, D., and Wang, C. (2017). Mechanism of Berberine-Mediated Fluconazole-Susceptibility Enhancement in Clinical Fluconazole-Resistant *Candida tropicalis* Isolates. *Biomed. Pharmacother.* 93, 709–712. doi:10.1016/j.biopha.2017.06.106

Siriwong, S., Teethaisong, Y., Thumanu, K., Dunkhunthod, B., and Eumkeb, G. (2016). The Synergy and Mode of Action of Quercetin Plus Amoxicillin against Amoxicillin-Resistant Staphylococcus Epidermidis. *BMC Pharmacol. Toxicol.* 17, 39. doi:10.1186/s40360-016-0083-8

Siriwong, S., Thumanu, K., Hengpratom, T., and Eumkeb, G. (2015). Synergy and Mode of Action of Ceftazidime Plus Quercetin or Luteolin on Streptococcus Pyogenes. *Evid. Based Complement. Altern. Med.* 2015, 759459. doi:10.1155/2015/759459

Sousa Silveira, Z., Macêdo, N. S., Sampaio Dos Santos, J. F., Sampaio De Freitas, T., Rodrigues Dos Santos Barbosa, C., Júnior, D. L. S., et al. (2020). Evaluation of the Antibacterial Activity and Efflux Pump Reversal of Thymol and Carvacrol against *Staphylococcus aureus* and Their Toxicity in *Drosophila melanogaster*. *Molecules* 25. doi:10.3390/molecules25092103

Souza, C. R. M., Bezerra, W. P., and Souto, J. T. (2020). Marine Alkaloids with Anti-inflammatory Activity: Current Knowledge and Future Perspectives. *Mar. Drugs* 18. doi:10.3390/md18030147

Stapleton, P. D., and Taylor, P. W. (2002). Methicillin Resistance in *Staphylococcus aureus*: Mechanisms and Modulation. *Sci. Prog.* 85, 57–72. doi:10.3184/003685002783238870

Su, F., and Wang, J. (2018). Berberine Inhibits the MexXY-OprM Efflux Pump to Reverse Imipenem Resistance in a Clinical Carbapenem-Resistant *Pseudomonas aeruginosa* Isolate in a Planktonic State. *Exp. Ther. Med.* 15, 467–472. doi:10.3892/etm.2017.5431

Su, P. W., Yang, C. H., Yang, J. F., Su, P. Y., and Chuang, L. Y. (2015). Antibacterial Activities and Antibacterial Mechanism of Polygonum Cuspidatum Extracts against Nosocomial Drug-Resistant Pathogens. *Molecules* 20, 11119–11130. doi:10.3390/molecules200611119

Su, T., Qiu, Y., Hua, X., Ye, B., Luo, H., Liu, D., et al. (2020). Novel Opportunity to Reverse Antibiotic Resistance: To Explore Traditional Chinese Medicine with Potential Activity against Antibiotics-Resistance Bacteria. *Front. Microbiol.* 11, 610070. doi:10.3389/fmicb.2020.610070

Sudano Roccaro, A., Blanco, A. R., Giuliano, F., Rusciano, D., and Enea, V. (2004). Epigallocatechin-gallate Enhances the Activity of Tetracycline in Staphylococci by Inhibiting its Efflux from Bacterial Cells. *Antimicrob. Agents Chemother.* 48, 1968–1973. doi:10.1128/AAC.48.6.1968-1973.2004

Sundaramoorthy, N. S., Sivasubramanian, A., and Nagarajan, S. (2020). Simultaneous Inhibition of MarR by Salicylate and Efflux Pumps by Curcumin Sensitizes Colistin Resistant Clinical Isolates of Enterobacteriaceae. *Microb. Pathog.* 148, 104445. doi:10.1016/j.micpath.2020.104445

Teethaisong, Y., Sirichaiwetchakoon, N. K., Kupittayanant, P. S., Eumkeb, G., and Eumkeb, G. (2014). Synergistic Activity and Mechanism of Action of Stephania Suberosa Forman Extract and Ampicillin Combination against Ampicillin-Resistant *Staphylococcus aureus*. *J. Biomed. Sci.* 21, 90. doi:10.1186/s12929-014-0090-2

Teng, Z., Li, M., Shi, D., Deng, X., and Wang, J. (2018). Synergistic Interactions of Cryptotanshinone and Aminoglycoside Antibiotics against *Staphylococcus aureus In Vitro*. *J. Glob. Antimicrob. Resist* 13, 264–265. doi:10.1016/j.jgar.2018.05.013

Usman Amin, M., Khurram, M., Khan, T. A., Faidah, H. S., Ullah Shah, Z., Ur Rahman, S., et al. (2016). Effects of Luteolin and Quercetin in Combination with Some Conventional Antibiotics against Methicillin-Resistant *Staphylococcus aureus*. *Int. J. Mol. Sci.* 17. doi:10.3390/ijms17111947

Vázquez, N. M., Fiorilli, G., Cáceres Guido, P. A., and Moreno, S. (2016). Carnosic Acid Acts Synergistically with Gentamicin in Killing Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates. *Phytomedicine* 23, 1337–1343. doi:10.1016/j.phymed.2016.07.010

Vipin, C., Saptami, K., Fida, F., Mujeeburahiman, M., Rao, S. S., AthmikaArun, A. B., et al. (2020). Potential Synergistic Activity of Quercetin with Antibiotics against Multidrug-Resistant Clinical Strains of *Pseudomonas aeruginosa*. *PLoS One* 15, e0241304. doi:10.1371/journal.pone.0241304

Vivekanandan, L., Sheik, H., Singaravel, S., and Thangavel, S. (2018). Ameliorative Effect of Silymarin against Linezolid-Induced Hepatotoxicity in Methicillin-Resistant *Staphylococcus aureus* (MRSA) Infected Wistar Rats. *Biomed. Pharmacother.* 108, 1303–1312. doi:10.1016/j.biopha.2018.09.133

Wagner, H., and Ulrich-Merzenich, G. (2009). Synergy Research: Approaching a New Generation of Phytopharmaceuticals. *Phytomedicine* 16, 97–110. doi:10.1016/j.phymed.2008.12.018

Wang, G., Li, L., Wang, X., Li, X., Zhang, Y., Yu, J., et al. (2019). Hypericin Enhances β-lactam Antibiotics Activity by Inhibiting sarA Expression in Methicillin-Resistant *Staphylococcus aureus*. *Acta Pharm. Sin. B* 9, 1174–1182. doi:10.1016/j.apsb.2019.05.002

Wang, S., Kim, M. C., Kang, O. H., and Kwon, D. Y. (2020). The Mechanism of Bisdemethoxycurcumin Enhances Conventional Antibiotics against Methicillin-Resistant *Staphylococcus aureus*. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21217945

Wang, S., Kang, O. H., and Kwon, D. Y. (2021). Trans-Cinnamaldehyde Exhibits Synergy with Conventional Antibiotic against Methicillin-Resistant *Staphylococcus aureus*. *Int. J. Mol. Sci.* 22. doi:10.3390/ijms22052752

Wang, S. Y., Sun, Z. L., Liu, T., Gibbons, S., Zhang, W. J., and Qing, M. (2014). Flavonoids from Sophora Moorcroftiana and Their Synergistic Antibacterial Effects on MRSA. *Phytother. Res.* 28, 1071–1076. doi:10.1002/ptr.5098

Wang, Y. M., Kong, L. C., Liu, J., and Ma, H. X. (2018). Synergistic Effect of Eugenol with Colistin against Clinical Isolated Colistin-Resistant *Escherichia coli* Strains. *Antimicrob. Resist Infect. Control* 7, 17. doi:10.1186/s13756-018-0303-7

Wei, S., Yang, Y., Tian, W., Liu, M., Yin, S., and Li, J. (2020). Synergistic Activity of Fluoroquinolones Combining with Artesunate against Multidrug-Resistant *Escherichia coli*. *Microb. Drug Resist* 26, 81–88. doi:10.1089/mdr.2018.0463

Wojtyczka, R. D., Dziedzic, A., Kępa, M., Kubina, R., Kabała-Dzik, A., Mularz, T., et al. (2014). Berberine Enhances the Antibacterial Activity of Selected Antibiotics against Coagulase-Negative Staphylococcus Strains *In Vitro*. *Molecules* 19, 6583–6596. doi:10.3390/molecules19056583

Wu, S. C., Yang, Z. Q., Liu, F., Peng, W. J., Qu, S. Q., Li, Q., et al. (2019). Antibacterial Effect and Mode of Action of Flavonoids from Licorice against Methicillin-Resistant *Staphylococcus aureus*. *Front. Microbiol.* 10, 2489. doi:10.3389/fmicb.2019.02489

Wultanska, D., Piotrowski, M., and Pituch, H. (2020). The Effect of Berberine Chloride And/or its Combination with Vancomycin on the Growth, Biofilm Formation, and Motility of Clostridioides Difficile. *Eur. J. Clin. Microbiol. Infect. Dis.* 39, 1391–1399.

Xu, Y., Quan, H., Wang, Y., Zhong, H., Sun, J., Xu, J., et al. (2017). Requirement for Ergosterol in Berberine Tolerance Underlies Synergism of Fluconazole and Berberine against Fluconazole-Resistant Candida Albicans Isolates. *Front. Cell. Infect. Microbiol.* 7, 491. doi:10.3389/fcimb.2017.00491

Yang, J. F., Yang, C. H., Chang, H. W., Yang, C. S., Wang, S. M., Hsieh, M. C., et al. (2010). Chemical Composition and Antibacterial Activities of Illicium Verum against Antibiotic-Resistant Pathogens. *J. Med. Food* 13, 1254–1262. doi:10.1089/jmf.2010.1086

Yong, J., Zu, R., Huang, X., Ge, Y., and Li, Y. (2020). Synergistic Effect of Berberine Hydrochloride and Fluconazole against Candida Albicans Resistant Isolates. *Front. Microbiol.* 11, 1498. doi:10.3389/fmicb.2020.01498

Yu, H. H., Kim, K. J., Cha, J. D., Kim, H. K., Lee, Y. E., and You, N. Y. Y. O. (2005). Antimicrobial Activity of Berberine Alone and in Combination with Ampicillin or Oxacillin against Methicillin-Resistant *Staphylococcus aureus*. *J. Med. Food* 8, 454–461. doi:10.1089/jmf.2005.8.454

Zhang, H., Wang, K., Zhang, G., Ho, H. I., and Gao, A. (2010). Synergistic Anti-candidal Activity of Tetrandrine on Ketoconazole: an Experimental Study. *Planta Med.* 76, 53–61. doi:10.1055/s-0029-1185973

Zhao, W. H., Hu, Z. Q., Hara, Y., and Shimamura, T. (2002). Inhibition of Penicillinase by Epigallocatechin Gallate Resulting in Restoration of Antibacterial Activity of Penicillin against Penicillinase-Producing *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 46, 2266–2268. doi:10.1128/aac.46.7.2266-2268.2002

Zhou, X. Y., Ye, X. G., He, L. T., Zhang, S. R., Wang, R. L., Zhou, J., et al. (2016). *In Vitro* characterization and Inhibition of the Interaction between Ciprofloxacin and Berberine against Multidrug-Resistant *Klebsiella pneumoniae*. *J. Antibiot. (Tokyo)* 69, 741–746. doi:10.1038/ja.2016.15

Zhou, X. Z., Jia, F., Liu, X. M., Yang, C., Zhao, L., and Wang, Y. J. (2013). Total Alkaloids from Sophora Alopecuroides L. Increase Susceptibility of Extended-Spectrum β-lactamases Producing *Escherichia coli* Isolates to Cefotaxime and Ceftazidime. *Chin. J. Integr. Med.* 19, 945–952. doi:10.1007/s11655-011-0899-4

Zuo, G. Y., Han, Z. Q., Hao, X. Y., Han, J., Li, Z. S., and Wang, G. C. (2014). Synergy of Aminoglycoside Antibiotics by 3-Benzylchroman Derivatives from the Chinese Drug Caesalpinia Sappan against Clinical Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Phytomedicine* 21, 936–941. doi:10.1016/j.phymed.2014.03.004

Zuo, G. Y., Li, Y., Han, J., Wang, G. C., Zhang, Y. L., and Bian, Z. Q. (2012). Antibacterial and Synergy of Berberines with Antibacterial Agents against Clinical Multi-Drug Resistant Isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Molecules* 17, 10322–10330. doi:10.3390/molecules170910322

Zuo, G. Y., Li, Y., Wang, T., Han, J., Wang, G. C., Zhang, Y. L., et al. (2011). Synergistic Antibacterial and Antibiotic Effects of Bisbenzylisoquinoline Alkaloids on Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Molecules* 16, 9819–9826. doi:10.3390/molecules16129819

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 08 June 2022
doi: 10.3389/fphar.2022.865097

Modeling Kaempferol as a Potential Pharmacological Agent for COVID-19/PF Co-Occurrence Based on Bioinformatics and System Pharmacological Tools

Yong Jiang[1†], Yi-Zi Xie[2,3†], Chen-Wen Peng[2,3†], Kai-Nan Yao[4], Xue-Ying Lin[2,3], Shao-Feng Zhan[2], Hong-Fa Zhuang[2], Hui-Ting Huang[2], Xiao-Hong Liu[2], Xiu-Fang Huang[2,5]* and Hang Li[4]**

[1]Shenzhen Hospital of Integrated Traditional Chinese and Western Medicine, Shenzhen, China, [2]The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [3]The First Clinical Medical School, Guangzhou University of Chinese Medicine, Guangzhou, China, [4]Shenzhen Bao'an District Traditional Chinese Medicine Hospital, Guangzhou University of Chinese Medicine, Shenzhen, China, [5]Lingnan Medical Research Center of Guangzhou University of Chinese Medicine, Guangzhou, China

Objective: People suffering from coronavirus disease 2019 (COVID-19) are prone to develop pulmonary fibrosis (PF), but there is currently no definitive treatment for COVID-19/PF co-occurrence. Kaempferol with promising antiviral and anti-fibrotic effects is expected to become a potential treatment for COVID-19 and PF comorbidities. Therefore, this study explored the targets and molecular mechanisms of kaempferol against COVID-19/PF co-occurrence by bioinformatics and network pharmacology.

Methods: Various open-source databases and Venn Diagram tool were applied to confirm the targets of kaempferol against COVID-19/PF co-occurrence. Protein-protein interaction (PPI), MCODE, key transcription factors, tissue-specific enrichment, molecular docking, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to clarify the influential molecular mechanisms of kaempferol against COVID-19 and PF comorbidities.

Results: 290 targets and 203 transcription factors of kaempferol against COVID-19/PF co-occurrence were captured. Epidermal growth factor receptor (EGFR), proto-oncogene tyrosine-protein kinase SRC (SRC), mitogen-activated protein kinase 3 (MAPK3), mitogen-activated protein kinase 1 (MAPK1), mitogen-activated protein kinase 8 (MAPK8), RAC-alpha serine/threonine-protein kinase (AKT1), transcription factor p65 (RELA) and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA) were identified as the most critical targets, and kaempferol showed effective binding activities with the above critical eight targets. Further, anti-COVID-19/PF co-occurrence effects of kaempferol were associated with the regulation of inflammation, oxidative stress, immunity, virus infection, cell growth process and metabolism. EGFR, interleukin 17 (IL-17), tumor necrosis factor (TNF), hypoxia inducible factor 1 (HIF-1), phosphoinositide 3-kinase/AKT

OPEN ACCESS

Edited by:
Joan Villena García,
Universidad de Valparaíso, Chile

Reviewed by:
Lihong Peng,
Hunan University of Technology, China
Talha Bin Emran,
Begum Gulchemonara Trust University, Bangladesh

***Correspondence:**
Xiao-Hong Liu
rsclxh@gzucm.edu.cn
Xiu-Fang Huang
879172531@qq.com
Hang Li
drlihang@foxmail.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

Received: 29 January 2022
Accepted: 25 April 2022
Published: 08 June 2022

Citation:
Jiang Y, Xie Y-Z, Peng C-W, Yao K-N, Lin X-Y, Zhan S-F, Zhuang H-F, Huang H-T, Liu X-H, Huang X-F and Li H (2022) Modeling Kaempferol as a Potential Pharmacological Agent for COVID-19/PF Co-Occurrence Based on Bioinformatics and System Pharmacological Tools. Front. Pharmacol. 13:865097. doi: 10.3389/fphar.2022.865097

serine/threonine kinase (PI3K/AKT) and Toll-like receptor signaling pathways were identified as the key anti-COVID-19/PF co-occurrence pathways.

Conclusion: Kaempferol is a candidate treatment for COVID-19/PF co-occurrence. The underlying mechanisms may be related to the regulation of critical targets (EGFR, SRC, MAPK3, MAPK1, MAPK8, AKT1, RELA, PIK3CA and so on) and EGFR, IL-17, TNF, HIF-1, PI3K/AKT and Toll-like receptor signaling pathways. This study contributes to guiding development of new drugs for COVID-19 and PF comorbidities.

Keywords: kaempferol, pulmonary fibrosis, COVID-19, co-occurrence, bioinformatic analysis, system pharmacology

INTRODUCTION

The outbreak of coronavirus disease 2019 (COVID-19) in December 2019, with rising incidence and prevalence worldwide, has caused more than six million deaths (World Health Organization, 2022). Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the trigger for COVID-19 pandemic, and belongs to the same coronavirus lineage that causes SARS (Zhu et al., 2020). Common clinical symptoms of SARS-CoV-2 infection include fever, cough, tiredness, shortness of breath and even death with exacerbation (Wu and McGoogan, 2020). Independent risk factors associated with COVID-19 include hypertension, diabetes, chronic obstructive pulmonary disease, and cardiovascular and cerebrovascular diseases (Wang et al., 2020). Although vaccine use has reduced the incidence of COVID-19, vaccinated people are still at risk of contracting SARS-CoV-2 and the number of COVID-19 cases remains high (Soleimanpour and Yaghoubi, 2021). Drugs against SARS-CoV-2 have been developed that reduce the risk of COVID-19 developing into severe COVID-19, but drug-resistant variants of SARS-CoV-2 may still emerge (Hammond et al., 2022). These shows that COVID-19 remains a serious threat to global health.

Pulmonary fibrosis (PF) is a pathological event caused by acute and chronic interstitial lung injury. PF causes chronic dyspnea, long-term disability and affects the quality of life of the patients (Lechowicz et al., 2020). PF is characterized by alveolar epithelium damage, inflammation infiltration, myofibroblasts activation and excessive deposition of extracellular matrix (ECM) (Giacomelli et al., 2021). Of note, CT images of 62 COVID-19 patients in Wuhan show vacuolar sign in more than half of them (Zhou et al., 2020). Diffuse alveolar damage, fibroblast proliferation and fibrosis are also found in autopsies of COVID-19 patients (Schaller et al., 2020). Alveolar epithelial type II (ATII) cells show a decreasing trend in SARS-CoV-2 infected patients (Delorey et al., 2021). The spike (S) protein of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) expressed in ATII cells to enter host cells (Ziegler et al., 2020; Celik et al., 2021). Damaged ATII cells can release transforming growth factor-β (TGF-β) (Tatler and Jenkins, 2012), platelet derived growth factor (Antoniades et al., 1990), connective tissue growth factor (Pan et al., 2001) and interluekin-6 (IL-6) (Crestani et al., 1994), thereby activating lung fibroblasts to increase ECM deposition and promote the development of PF (Sisson et al., 2010). The above researches reveal that COVID-19 patients are at high risk of developing PF (George et al., 2020). Obviously, COVID-19/PF co-occurrence is a catastrophic threat to global health, and it is unclear whether the damage caused by COVID-19/PF co-occurrence can be reversed (John et al., 2021). Therefore, it is an urgent need to find an influential treatment for COVID-19/PF co-occurrence.

Pirfenidone is one of the FDA-approved anti-fibrotic agents to treat idiopathic pulmonary fibrosis (IPF). Compared with methylprednisolone alone, pirfenidone and methylprednisolone combination therapy improves PF in hospitalized patients diagnosed with severe COVID-19 pneumonia (Acat et al., 2021). However, pirfenidone cannot prevent or reverse the progression of PF, which also limits its use in COVID-19/PF co-occurrence (Lancaster et al., 2019; Noble et al., 2011). There is no reported effective treatment for COVID-19/PF co-occurrence so far, thus the discovery of effective drugs against COVID-19/PF co-occurrence will contribute to improving patient prognosis and reducing social burdens. Surprisingly, it is confirmed that natural products have the effect of suppressing viral replication and transcription, and can inhibit cytokine storm and improve immunodeficiency (An et al., 2021). Natural product is also increasingly recognized as an alternative source for inhibiting fibrosis (Bahri et al., 2017). Natural products can reduce fibrosis by inhibiting inflammation, myofibroblast activation, ECM accumulation and epithelial-mesenchymal transition (EMT) (Chen et al., 2018). Natural products are a treasure trove for discovering new therapeutic drugs for COVID-19/PF co-occurrence. A natural product with dual antiviral and antifibrotic effects may have the great potential to become a therapeutic agent for COVID-19 and PF comorbidities.

Kaempferol, a natural flavonoid that widely exists in many fruits, vegetables and herbal medicine, is known as an antimicrobial, anti-inflammatory and antioxidant compound (Devi et al., 2015; Imran et al., 2019; Ren et al., 2019). Main protease (Mpro), a potential drug target for treating COVID-19, is found to be potentially inhibited by kaempferol (Khaerunnisa et al., 2020; Mahmud et al., 2021). Moreover, it is reported that kaempferol may directly target

SARS-CoV-2 main protease (3CL pro) to perform anti-COVID effect (Shaldam et al., 2021; Zhang et al., 2021). Simultaneously, kaempferol inhibits the progression of silica-induced PF and attenuates fibrotic airway remodeling *via* modulating protease-activated receptor-1 activation (Gong et al., 2014; Liu et al., 2019). The above researches suggest that kaempferol has dual effects against COVID-19/PF co-occurrence, but the molecular mechanisms have not been investigated. Therefore, drug-target, disease-target and critical targets among COVID-19, PF and kaempferol were captured. Protein-protein interaction (PPI), MCODE, transcription factors, tissue-specific enrichment, molecular docking, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) analyses were performed. The detailed strategy of exploring the targets and mechanisms of kaempferol against COVID-19/PF co-occurrence by bioinformatics and network pharmacology is shown in **Figure 1**.

MATERIALS AND METHODS

Screening for Drug-Related Targets

The targets associated with kaempferol were retrieved from Comparative Toxicoomics Database (CTD, http://ctdbase.org/, accessed date: 3 September 2021) (Davis et al., 2021), Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://tcmspe.com/, accessed date: 2 September 2021) (Ru et al., 2014), Swiss Target Prediction (http://swisstargetprediction.ch/,

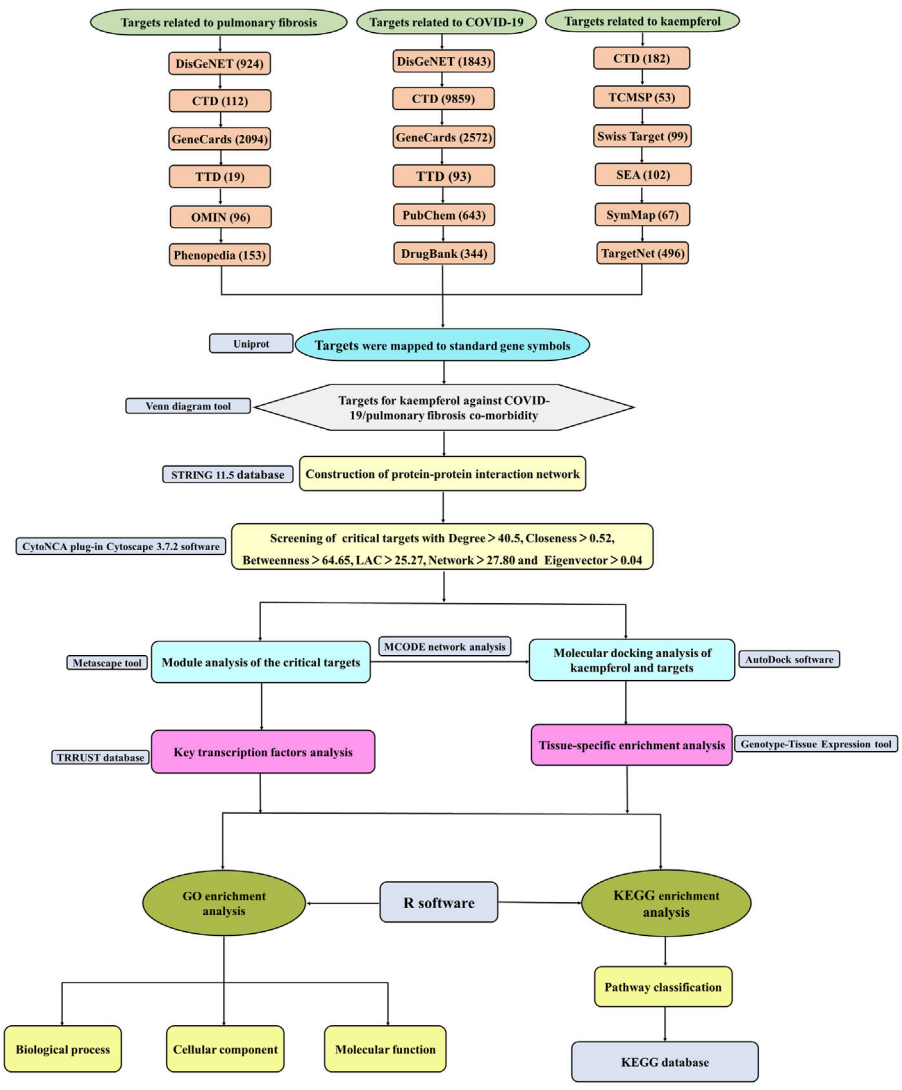


FIGURE 1 | The flow diagram of a pragmatic strategy for identifying the pharmacological mechanism of kaempferol against COVID-19/pulmonary fibrosis co-occurrence based on system pharmacology and bioinformatics analysis.

accessed date: 2 September 2021) (Daina et al., 2019), Similarity Ensemble Approach (SEA, https://sea.bkslab.org/, accessed date: 2 September 2021) (Keiser et al., 2007), SymMap (https://www.symmap.org/, accessed date: 2 September 2021) (Wu et al., 2019) and TargetNet (http://targetnet.scbdd.com/, accessed date: 2 September 2021) (Yao et al., 2016).

Collection of Disease-Related Targets

Targets related to COVID-19 were obtained from DisGeNET (https://www.disgenet.org/home/, accessed date: 2 September 2021) (Pinero et al., 2017), CTD, GeneCards (https://www.genecards.org/, accessed date: 3 September 2021) (Rebhan et al., 1997), Therapeutic Target Database (TTD, http://db.idrblab.net/ttd/, accessed date: 3 September 2021) (Wang et al., 2020), PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed date: 2 September 2021) (Kim et al., 2021) and DrugBank database (https://www.drugbank.com/, accessed date: 4 September 2021) (Wishart et al., 2018).

Six databases were used to obtained PF-related targets including DisGeNET, CTD, GeneCards, TTD, Online Mendelian Inheritance in Man (OMIM, https://omim.org/, accessed date: 2 September 2021) (Amberger et al., 2015) and Phenopedia (https://phgkb.cdc.gov/PHGKB/startPagePhenoPedia.action, accessed date: 2 September 2021) (Yu et al., 2010). Targets were mapped to standard symbols by using Uniprot database (https://www.uniprot.org/, accessed date: 2 September 2021) (UniProt, 2015).

Analysis of Overlapping Targets Between Drug and Diseases

The Venn package of R 3.6.2 software was used to draw the petal map. Venn diagram showing the intersection of the targets of kaempferol against COVID-19/PF co-morbidity was plotted by the Venn Diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and Microsoft Excel.

Protein-Protein Interaction Network Construction and Critical Targets Analysis

The shared targets between diseases and drug were put into the STRING 11.5 database (https://string-db.org/, accessed date: 6 September 2021) (Szklarczyk et al., 2021) to construct a PPI network. The organism was set to "*Homo sapiens*" and the minimum required interaction score was set to 0.4. Then the PPI network was visualized by Cytoscape 3.7.2 software (https://cytoscape.org/) (Otasek et al., 2019). The CytoNCA plug-in Cytoscape 3.7.2 software was applied to calculate topological parameters including degree, closeness, betweenness, LAC, network and eigenvector (Tang et al., 2015). Regarding the medians of topological parameters as the screening threshold, the overlapping targets above the threshold were identified as critical targets.

Module Analysis of Critical Targets

Metascape (http://metascape.org/, accessed date: 7 September 2021) was used to perform module analysis of critical targets (Zhou et al., 2019). MCODE score (Bader and Hogue, 2003) was applied to cluster the most significant modules. Code score was calculated on the connection density of the adjacent area, and the target in MCODE module with greater degree value was considered to play a more important role in treating COVID-19/PF co-morbidity. Of note, the top five targets with the highest degree values in MCODE modules were selected to perform molecular docking analysis.

Key Transcription Factors Analysis of Critical Targets

Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST, https://www.grnpedia.org/trrust/, accessed date: 7 September 2021) is a useful tool for predicting transcriptional regulatory network (Han et al., 2018). The TRRUST database provides abundant information of 8,444 transcription factors (TFs)-target network. Critical targets were input to TRRUST database with the species of "Human." The top 10 TFs ranking based on p value from small to large were selected to construct the TFs-target network by using Cytoscape 3.7.2 software.

Tissue-Specific Enrichment Analysis of Critical Targets

Genotype-Tissue Expression (GETx) (https://www.gtexportal.org/, accessed date: 7 September 2021) is an online tool to study genetic variation and expression of human tissues (Consortium, 2013). The top 50 targets ranking based on modules' degree values from high to low were selected for tissue-specific enrichment analysis. The heat map showed the correlation between different samples and targets, and more the important tissues corresponding to the targets would show darker colors.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analyses of Critical Targets

GO enrichment analysis included biological process (BP), molecular function (MF) and cellular component (CC), as well as KEGG pathway enrichment analysis were conducted in R 3.6.2 software. "Org.hs.eg.db" (https://www.bioconductor.org/packages/org.Hs.eg.db, accessed date: 7 September 2021) was used to match the gene ID corresponding to critical targets. Then "cluster Profiler" package (Wu et al., 2021) was used to perform enrichment analysis with the criteria of $p$valueCutoff = 0.05 and $q$valueCutoff = 0.05. Based on adjusted p value in ascending order, the top 20 enrichment results were selected to display as a bubble chart by bioinformatics tool (http://www.bioinformatics.com.cn/). Furthermore, the KEGG pathways were classified based on KEGG databases and visualized by hiplot (https://hiplot.com.cn/).

Molecular Docking Analysis of the Top Five Targets

Molecular docking is widely applied in drug detection and is often used to predict the relationship between targets and ligand.

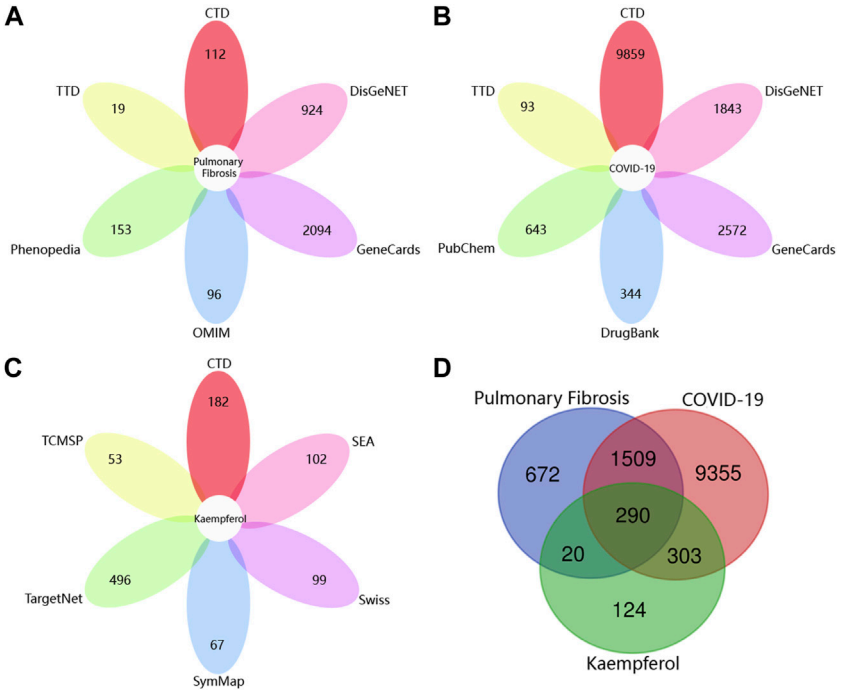


FIGURE 2 | Collection of targets related to drug and diseases from various open-source databases. **(A)** The number of pulmonary fibrosis-related targets from six open-source databases. **(B)** The number of targets related to COVID-19 from six open-source databases. **(C)** The number of targets associated with kaempferol from six open-source databases. **(D)** Venn diagram depicting common targets between COVID-19, pulmonary fibrosis and kaempferol.

Molecular docking was carried out between kaempferol and the top five targets *via* AutoDock software (Vina 1.5.6, http://autodock.scripps.edu/) (Shen et al., 2021; Trott and Olson, 2010), which was often used to calculate the molecular interaction force between protein and ligand. The small-molecule two-dimensional structure format information of kaempferol was obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and saved in the SDF format. The SDF molecular structure file of kaempferol was converted into a PDB file by Open Babel software. The three-dimensional structure of key target proteins was downloaded from the RCSB PDB database (https://www.rcsb.org/) (Rose et al., 2021). The Auto Dock Tools 1.5.6 software was used to convert the molecular structure document into PDBQT format and perform molecular docking. The PyMol 2.3.2 software was used to visualize the results with higher docking scores and calculate the corresponding RMSD values.

RESULTS

Targets of Kaempferol Against COVID-19/PF Co-Occurrence

As shown in **Figure 2A** unique PF-related targets were obtained from DisGeNET (924), CTD (112), GeneCards (2,094), TTD (19), OMIM (96) and Phenopedia (153). As shown in **Figure 2B**, 11,457 unique targets of COVID-19 were retrieved from DisGeNET (1,843), CTD (9,859), GeneCards (2,572), TTD (93), PubChem (643) and DrugBank (344). As shown in **Figure 2C**, 737 unique targets related to kaempferol were identified from CTD (182), TCMSP (53), Swiss Target Prediction (99), SEA (102), SymMap (67) and TargetNet (496). Finally, 290 targets of kaempferol against COVID-19/PF co-occurrence were obtained (**Figure 2D**).

Protein-Protein Interaction Network Construction and Critical Targets Acquisition

The nodes represented shared targets and the edges indicated protein-protein interactions between shared targets in PPI network. PPI network of 290 common targets shown in **Figure 3A** contained 290 nodes and 7,431 edges. Through the topological identification and calculation of PPI network, the medians of the topological parameter were degree = 40.5, closeness = 0.52, betweenness = 64.65, LAC = 25.27, network = 27.80 and eigenvector = 0.04. Then 115 critical targets with the topological parameters greater than the medians of above six topological factors were screened out to construct PPI network of critical targets. There were 115 nodes and 3,639 edges in the PPI network of critical targets as shown in **Figure 3B**.

Investigation of Important Modules

Module analysis was carried out by using Metascape tool and five functional clusters were shown in **Figures 4A–E**. Module 1 included 28 nodes and 132 edges with MCODE score = 4.714.

Module 2 contained 24 nodes and 205 edges with MCODE score = 8.541. Module 3 included 21 nodes and 62 edges with MCODE score = 2.952. Module 4 comprised of 4 nodes and 4 edges with MCODE score = 1.000. Module 5 included 3 nodes and 3 edges with MCODE score = 1.000. The top five targets with the highest degree scores were epidermal growth factor receptor (EGFR, degree = 23), proto-oncogene tyrosine-protein kinase SRC (SRC, degree = 21), mitogen-activated protein kinase 3 (MAPK3, degree = 21), mitogen-activated protein kinase 1 (MAPK1, degree = 21), mitogen-activated protein kinase 8 (MAPK8, degree = 20), RAC-alpha serine/thre onine-protein kinase (AKT1, degree = 20), transcription factor p65 (RELA, degree = 19) and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA, degree = 18).

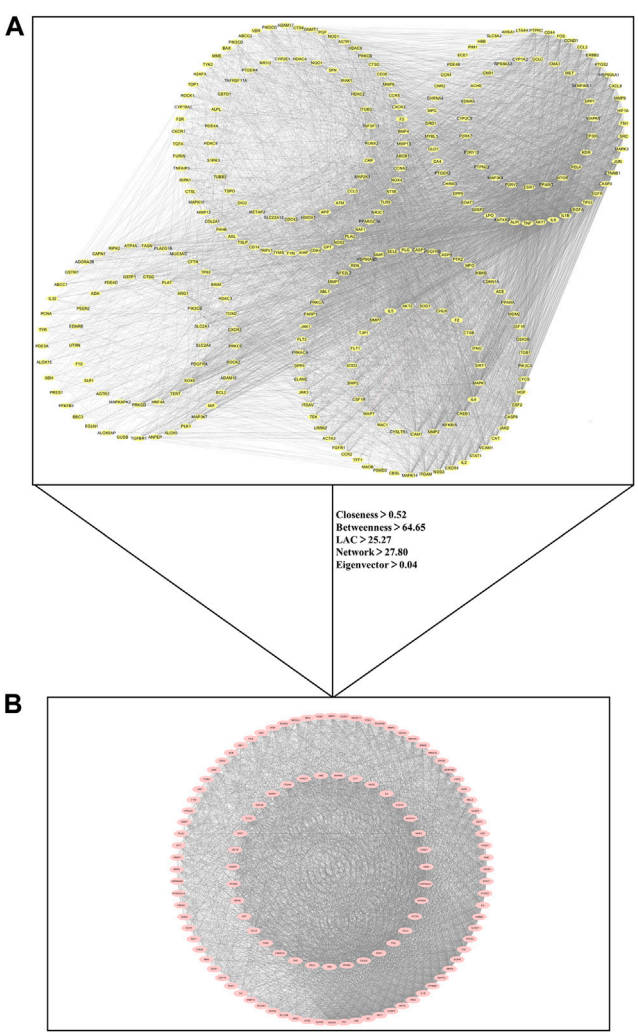


FIGURE 3 | Protein-protein interaction (PPI) network for critical targets of kaempferol against COVID-19/pulmonary fibrosis co-occurrence. Nodes represent targets and edges represent protein-protein interactions. **(A)** PPI network of 290 common targets between COVID-19, pulmonary fibrosis and kaempferol. **(B)** PPI network of 115 critical targets for kaempferol against COVID-19/pulmonary fibrosis co-occurrence.

Key Transcription Factors Acquisition

115 critical targets were input to the TRRUST database and 203 TFs were obtained. TFs-target network contained 97 nodes including 10 TFs, 87 targets and 278 edges (**Figure 5**). Red nodes represented TFs and purple nodes represented corresponding targets, and the edge indicated the relevance between TFs and corresponding targets. The size of the red node was negatively correlated with *p* value, the larger the size of the red node was, the more important it is in the TFs-target network. Especially, there were four critical targets that were also predicted as TFs, including signal transducerand activator of transcription 1 (STAT1), tumor protein P53 (TP53), JUN proto-oncogene, AP-1 transcription factor subunit (JUN) and RELA. The detailed information of the top 10 TFs were listed in **Table 1**.

Critical Targets Were Mostly Enriched in Lung Tissue

Tissues were represented on the abscissa and targets were indicated on the ordinate (**Figure 6**). The data was presented as a heat map and the color indicated the level of enrichment. The darker the color was, the higher the expression level of critical target in corresponding tissue was. The result indicated that most critical targets were highly expressed in lung tissue, especially fibronectin 1 (FN1), heat shock protein 90 alpha family class B member 1 (HSP90AB1), fos proto-oncogene, AP-1 transcription factor subunit (FOS), JUN, RAC family small GTPase 1 (RAC1), vascular endothelial growth factor A (VEGFA), ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1), RELA, heat shock protein 90 alpha family class A member 1 (HSP90AA1) and so on.

Gene Ontology Enrichment Analysis

2,958 GO terms were obtained, of which 2,705 belonged to GO-BP, 94 to GO-CC and 159 to GO-MF. The top 20 GO terms were respectively shown in **Figures 7A–C**. As for GO-BP, critical targets were mainly enriched in response to lipopolysaccharide, response to molecule of bacterial origin, response to oxidative stress, cellular response to biotic stimulus, response to antibiotic, regulation of cell-cell adhesion and so on. As for GO-MF, critical targets were mainly enriched in cytokine receptor binding, phosphatase binding, protein tyrosine kinase activity, growth factor receptor binding, protein phosphatase binding and so on. As for GO-CC, critical targets were mainly enriched in membrane raft, membrane microdomain, membrane region, focal adhesion, cell-substrate adherens junction and so on.

Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

174 KEGG terms were acquired and the top 20 KEGG terms were shown in **Figure 8**. Critical targets were mainly enriched in the EGFR tyrosine kinase inhibitor resistance, interleukin 17 (IL-17) signaling pathway, tumor necrosis factor (TNF) signaling pathway, Toll-like receptor signaling pathway, Yersinia infection, advanced glycation end product-receptor for advanced glycation end product (AGE-RAGE) signaling pathway in diabetic complications, hypoxia inducible factor 1 (HIF-1) signaling pathway, T cell receptor signaling pathway,

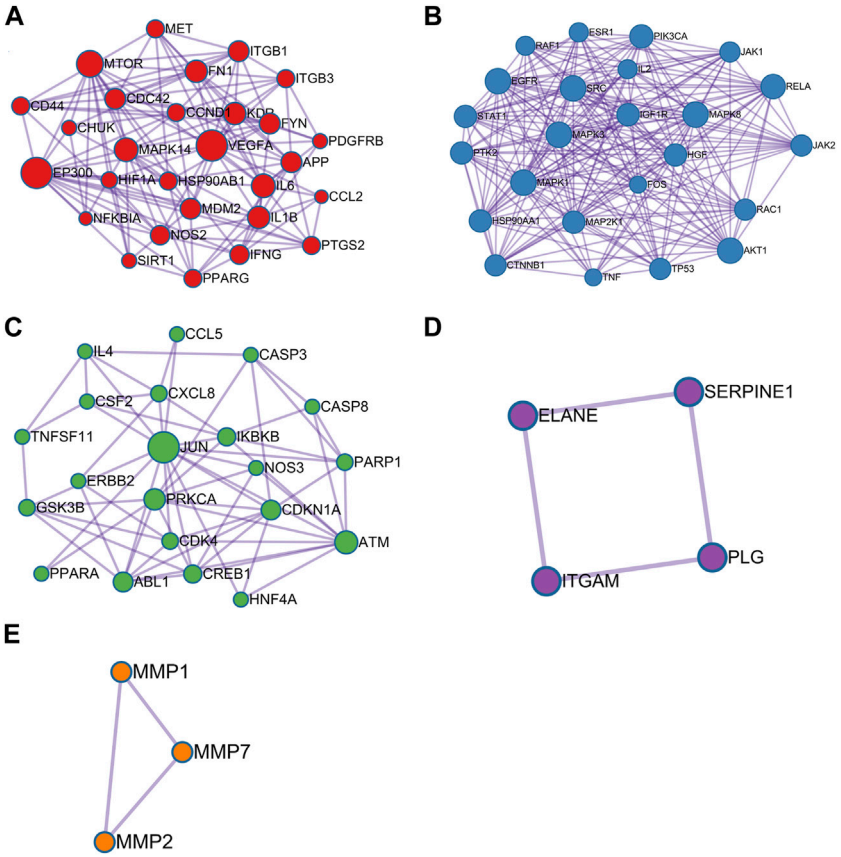


FIGURE 4 | Module analysis of 115 critical genes is performed by the Metascape tool. Each module demonstrats different biological process functions. **(A)** Module 1; **(B)** Module 2; **(C)** Module 3; **(D)** Module 4; **(E)** Module 5.

C-type lectin receptor signaling pathway, Th17 cell differentiation, phosphoinositide 3-kinase/AKT serine/threonine kinase (PI3K/Akt) signaling pathway and so on. The results of KEGG pathway enrichment analysis were classified into five types, containing inflammation, oxidative stress, immunity, virus infection, cell growth processes and metabolism (**Figure 9**).

Kaempferol Had Good Binding Activities With Critical Targets

To investigate whether kaempferol directly binds to EGFR, MAPK1, MAPK3, SRC, AKT1, MAPK8, RELA and PIK3CA (the top five targets with the highest degree values), molecular docking analysis was performed by Auto Dock Tools software. A binding energy less than 0 indicates spontaneous binding of ligand and receptor. The lower binding energy indicates a better binding effect. It is generally believed that binding energy < -5 kcal mol^{-1} indicates a good binding activity. Moreover, the stability of the simulated molecular docking systems was investigated by the root-mean-square deviation (RMSD), and it means the system is stable when RMSD is lower than 2 Å. The molecular docking results showed that the binding energies of kaempferol and the eight critical targets ranged from -6.23 to -8.15 kcal mol^{-1} (**Table 2**). All the simulated molecular docking reached the RMSD value range required for stability. The better docking result was selected for molecular docking visualization by using PyMol 2.3.2 software. The results showed that 2-5 hydrogen bonds could be formed between kaempferol and the eight critical targets (**Figure 10**). Molecular docking results proved that kaempferol had good binding activities with the eight critical targets.

DISCUSSION

The prevention and treatment of COVID-19 related complications are public concerns. COVID-19/PF co-occurrence is a common and threatening condition, and early intervention is important for improving prognosis of pulmonary complications caused by SARS-CoV-2 infection (Pan et al., 2020). Traditional natural products have the effect of inhibiting viral replication and transcription, reducing cytokine storm and ameliorating immunodeficiency (An, et al., 2021). Further, growing evidence shows that natural products are alternative sources for improving fibrosis (Bahri, et al., 2017). Therefore, it reveals that natural product is a treasure trove for discovering new therapeutic drugs. It has been confirmed that kaempferol alleviates H9N2 influenza virus-induced inflammation and

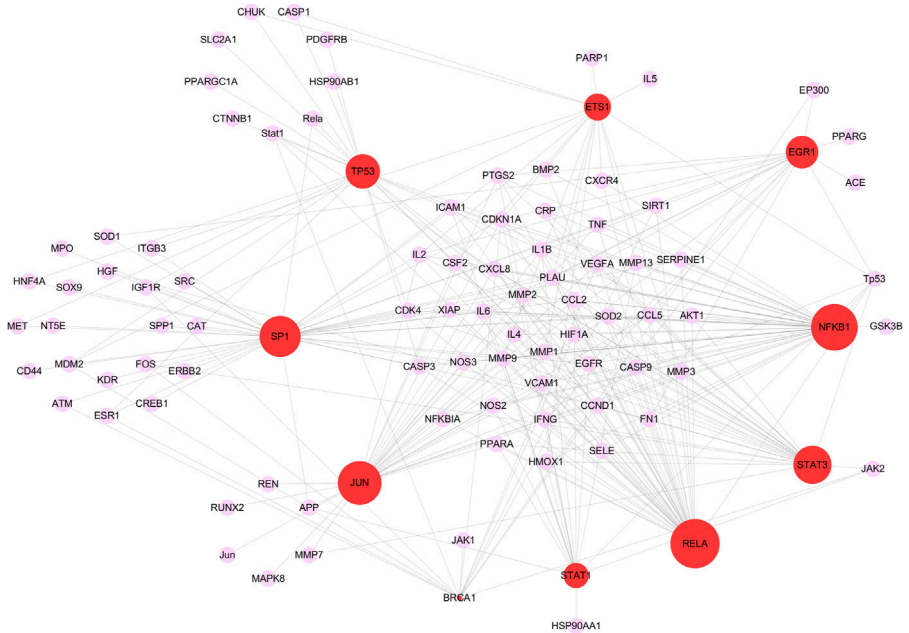


FIGURE 5 | The top 10 key transcription factors (TFs) of 115 critical targets. The red nodes represent TFs and the purple nodes represent corresponding targets. The edges represent the connection between TFs and targets. The sizes of red nodes present negative correlation with *p* values and a node with larger shape represents the more important role in treating COVID-19/pulmonary fibrosis co-occurrence.

TABLE 1 | Key transcription factors associated with critical targets.

Key transcription factors	Description	*p* value
RELA	V-rel *reticuloendotheliosis* viral oncogene homolog A (avian)	1.33E-49
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	3.34E-46
JUN	Jun proto-oncogene	2.28E-41
SP1	Sp1 transcription factor	2.52E-39
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	2.7E-33
TP53	Tumor protein p53	1.03E-26
EGR1	Early growth response 1	5.56E-23
ETS1	V-ets *erythroblastosis virus* E26 oncogene homolog 1 (avian)	3.68E-22
STAT1	Signal transducer and activator of transcription 1, 91 kDa	1.15E-21
BRCA1	Breast cancer 1, early onset	4.04E-21

acute lung injury (Zhang et al., 2017). Kaempferol can also inhibit the virus replication of the pseudorabies virus in mice (Li et al., 2021). Moreover, kaempferol is proved to inhibit the activity of the Japanese encephalitis virus in BHK-21 cells (Care et al., 2020). Except for the antiviral effect, the anti-PF effect of kaempferol is also verified by a silica-induced PF mice model (Liu, et al., 2019). The above evidences indicate that kaempferol with dual antiviral and anti-PF effects may be the promising medicine for treating COVID-19/PF co-occurrence. Thus, this study analyzed potential targets and mechanisms of kaempferol against COVID-19/PF co-occurrence by integrating bioinformatics and system pharmacological tools.

First, 290 common targets between kaempferol, COVID-19 and PF were obtained, and then 115 critical targets with greater topological parameters in the PPI network were screened out. The top five targets from the 115 critical targets were identified, including EGFR (degree = 23), SRC (degree = 21), MAPK3 (degree = 21), MAPK1 (degree = 21), MAPK8 (degree = 20), AKT1 (degree = 20), RELA (degree = 19) and PIK3CA (degree = 18). Computer modelling approaches show that kaempferol has a high binding affinity to 3CLpro (Shaldam, et al., 2021; Zhang, et al., 2021). *In vitro* experiment confirms that kaempferol has strong inhibitory effects on 3CLpro (Khan et al., 2021). Of note, except for the direct effect on virus-produced proteins, downstream molecules or signaling pathways during the pathologic process are also potential mechanisms for kaempferol against COVID-19/PF co-occurrence. Surprisely, molecular docking analysis found that kaempferol showed promising binding activities with the top five targets (EGFR, SRC, MAPK3, MAPK1, MAPK8, AKT1, RELA and PIK3CA). EGFR inhibitors are proved to have antiviral and antifibrotic effects based on the Viral Fibrotic score, indicating that EGFR

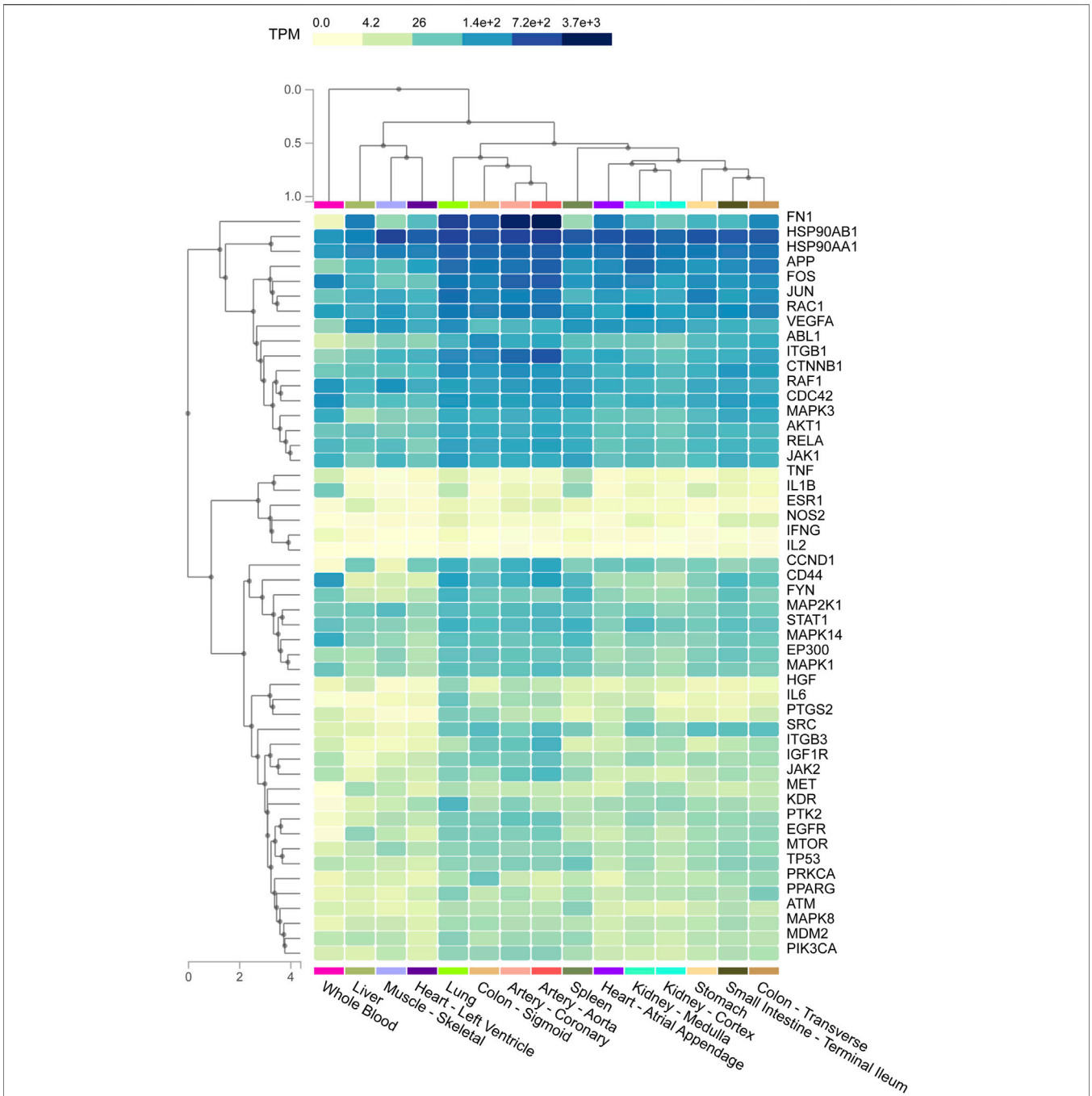


FIGURE 6 | The heat map shows the relationship between different tissue samples and critical targets. Column represents critical targets and row represents enriched tissues. The shades of colors represent the levels of enrichment of critical targets in tissues, and the darker the color indicates the more significant enrichment of targets in corresponding tissues.

may be a critical regulator of COVID-19/PF co-occurrence (Vagapova et al., 2021). SRC is involved in the pathogenesis of PF by regulating EMT, myofibroblast differentiation and inflammation.

Xu et al. (2020), and a recent study reports that targeting SRC reduces titers of SARS-CoV-2 (Meyer et al., 2021). AKT shows an increased trend in various fibrotic diseases (Lu et al., 2010; Huang et al., 2011), and it also increases in fibroblasts of bleomycin-induced IPF *in vivo* and *in vitro* (Vittal et al., 2005; Xia et al., 2008; Le Cras et al., 2010). Moreover, deficiency of AKT1 significantly inhibits viral RNA expression (Esfandiarei et al., 2004), and PI3K/AKT kinase inhibitors are found to suppress the replication of middle east respiratory syndrome (MERS) (Kindrachuk et al., 2015). The first identified member of the MAPK pathway is

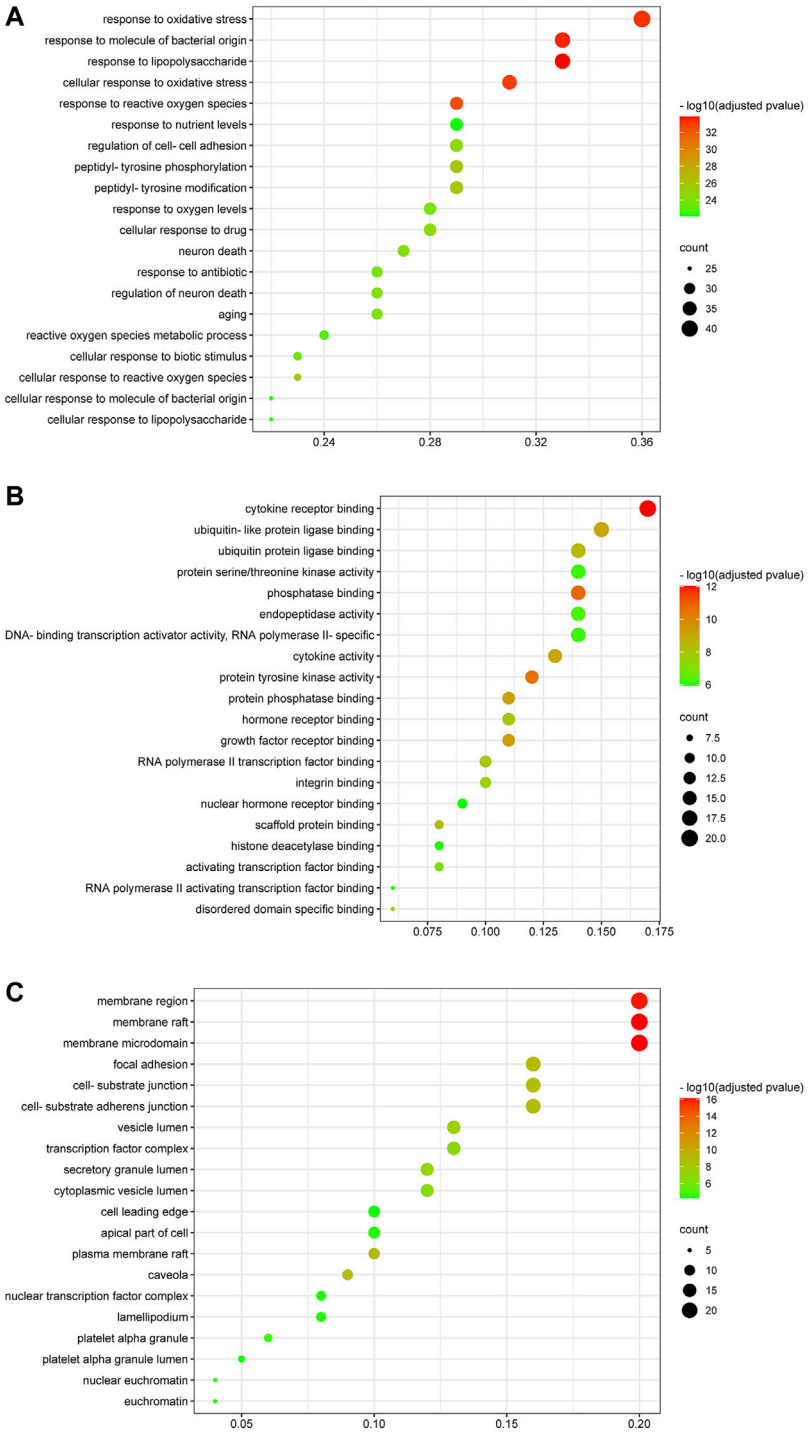


FIGURE 7 | Gene ontology enrichment analysis of critical targets. The size of the node represents the number of genes involved in the GO term, and the color from green to red indicates the –log10 (adjusted *p* value) from small to large. **(A)** Biological process enrichment results of critical targets. **(B)** Molecular function enrichment results of critical targets. **(C)** Cellular components enrichment results of critical targets.

extracellular signal-regulated kinase (ERK)1/2, which overexpresses in IPF (Antoniou et al., 2010). A study confirms that inhibition of ERK1/2 attenuates bleomycin-mediated PF by inhibiting EMT (Zou et al., 2020). In addition, MAPK is also involved in regulating virus replication, immune response and apoptosis of virus-infected cells (Bian et al., 2011; Gaur et al., 2011). It is worth noting that p38 MAPK inhibitor effectively prevents the phosphorylation of heat shock protein 27,

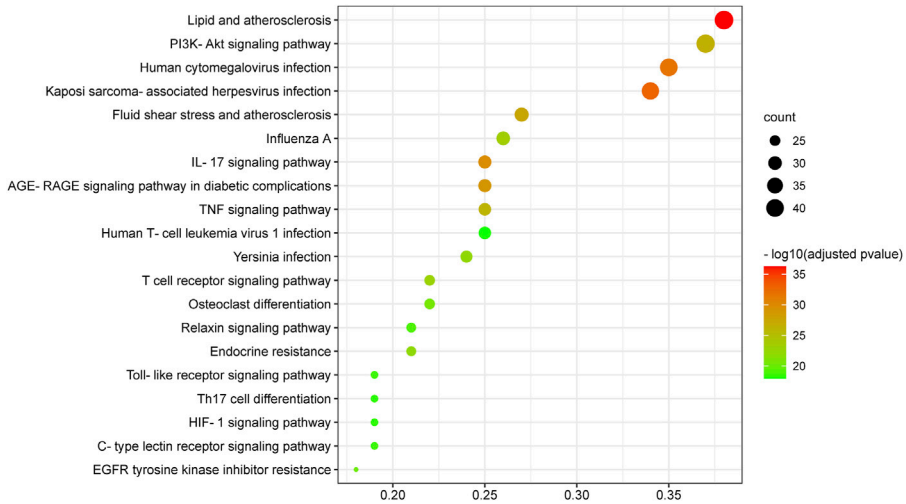


FIGURE 8 | Kyoto Encyclopedia of Genes and Genomes enrichment analysis of critical targets. The size of the node represents the number of genes involved in the enrichment pathway, and the color from green to red indicates the –log10 (adjusted *p* value) from small to large.

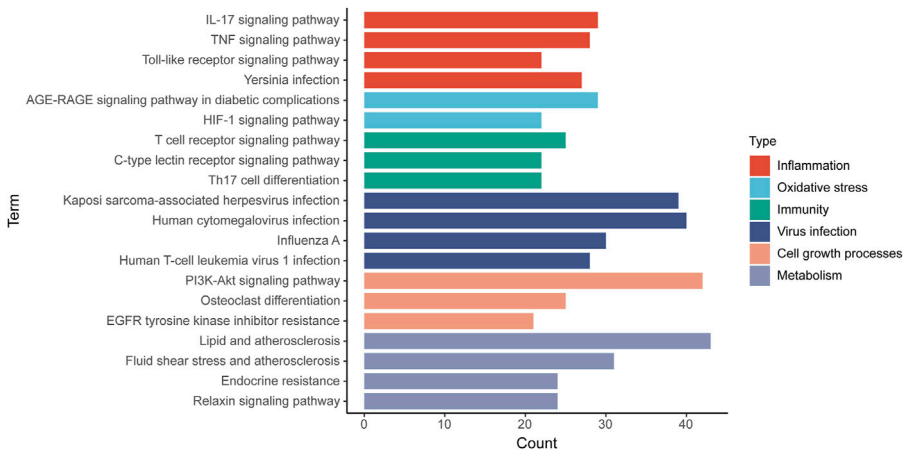


FIGURE 9 | The classification of KEGG pathways. The results of KEGG pathway enrichment analysis are classified into five types and a color represents a type. Column represents KEGG pathway terms and row represents the number of targets enriched on the pathways.

TABLE 2 | Molecular docking results of kaempferol with top eight critical targets.

Number	Target protein	PDB ID	RMSD	Binding energy (kcal/mol)
1	EGFR	5HG8	0.263	−7.530
2	MAPK1	6SLG	0.000	−6.170
3	MAPK3	4QTB	0.024	−6.750
4	SRC	1FMK	0.838	−7.720
5	AKT1	1UNQ	0.000	−6.550
6	MAPK8	2XRW	0.010	−6.420
7	RELA	6NV2	0.002	−6.230
8	PIK3CA	6PYS	0.048	−8.150

cathelicidin antimicrobial peptide response element-binding protein and eukaryotic initiation factor 4E in SARS-CoV infected cells (Mizutani et al., 2004). RELA regulates the interferon IFN response during SARS-CoV-2 infection (Yin et al., 2021), and inhibition of RELA contributes to improving PF (Hou et al., 2018). PIK3CA belongs to the lipid kinase family and is responsible for coordinating functions such as proliferation, vesicle trafficking, and protein synthesis in various cells (Maheshwari et al., 2017). The above results reveal that targeting the critical targets especially the top five targets may be the potential therapeutic approach for kaempferol against COVID-19/PF co-occurrence.

Abnormal TFs activation and subsequent abnormal pathogenic genes expression play important roles in disease progression. The top 10 TFs were identified from 115 critical targets, and RELA was the most significant TF with the smallest *p* value among the top 10 TFs. The activation of RELA, a subtype of nuclear factor kappa-B (NF-κB), enhances the expression of

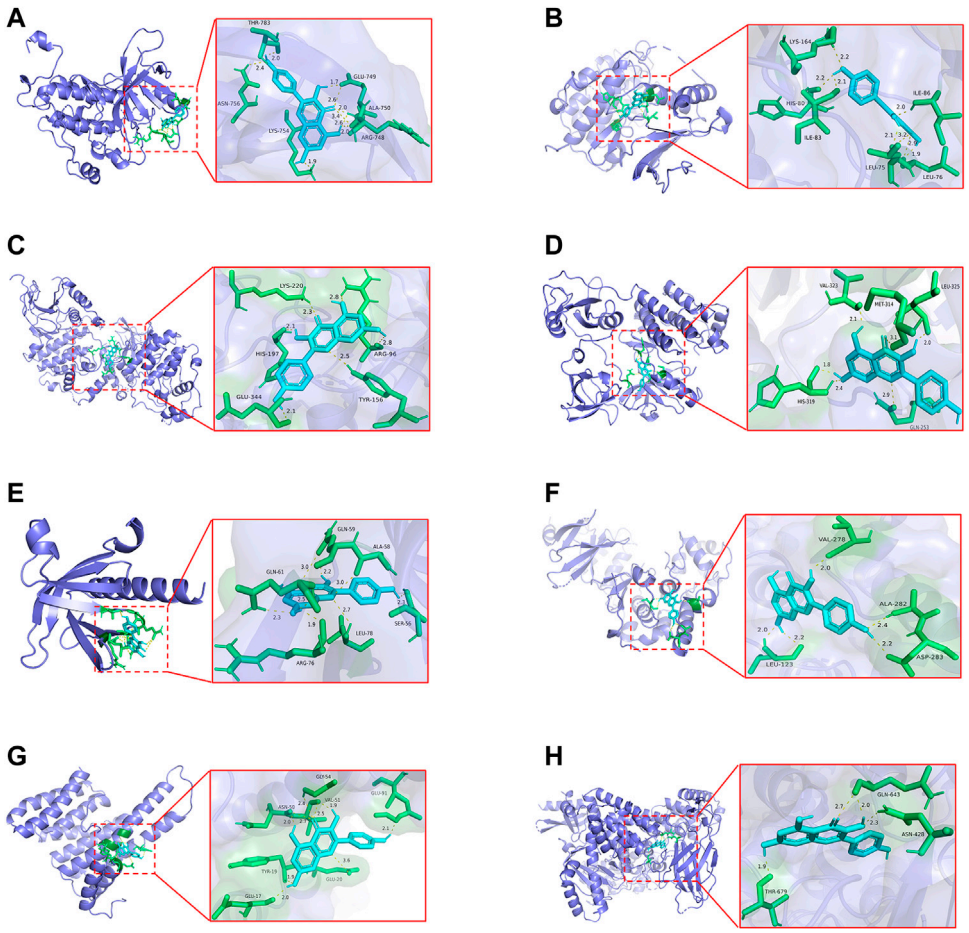


FIGURE 10 | The docking models of kaempferol with the identified the top eight critical targets. **(A)** Docking results of kaempferol and EGFR. **(B)** Docking results of kaempferol and MAPK1. **(C)** Docking results of kaempferol and MAPK3. **(D)** Docking results of kaempferol and SRC. **(E)** Docking results of kaempferol and AKT1. **(F)** Docking results of kaempferol and MAPK8. **(G)**Docking results of kaempferol and RELA. **(H)**Docking results of kaempferol and PIK3CA.

TGF-β1 (Rameshwar et al., 2000). TGF-β1 is a key pro-fibrotic factor that has been proved to promote the transition of fibroblast to myofibroblast in PF (Andersson-Sjoland et al., 2008; Goodwin and Jenkins, 2009). ACT001 (NF-κB inhibitor) attenuates PF through decreasing the transition of fibroblast to myofibroblast, inhibiting IL-6 production and fibronectin deposition (Jaffaret al., 2021). Increased inflammatory cytokines and chemokines levels result in spontaneous haemorrhage, thrombocytopenia and systemic inflammation, which are the main manifestations of the fatal cytokine syndrome in advanced COVID-19 patients (Song et al., 2020; Xu et al., 2020). The activation of NF-κB enhances the expression of inflammatory cytokines and chemokines, including IL-1, IL-6, IL-8 and TNF-α (Liao et al., 2005; Wang et al., 2007). Selective bruton tyrosine kinase inhibitor inhibits NF-κB at the RELA phosphorylation stage, which leads to the reduction of C-reactive protein and IL-6 and an improvement of oxygen saturation (Roschewski et al., 2020). Further, to explore the association between tissues and critical targets, tissue-specific enrichment analysis was performed. The results showed that FN1, HSP90AB1, HSP90AA1 and so on were significantly enriched in the lung tissues. One of the characteristics of PF is excessive deposition of ECM proteins such as fibronectin (Liu et al., 2017). Elevated fibronectin deposition has been found in the lung tissues of PF patients (Liu et al., 2019), and it has been suggested that SARS-CoV-2 infection may promote the fibronectin expression in alveolar epithelial cells (Xu et al., 2020). HSP90 plays an important role in the folding, maturation and stabilization of proteins, and is therefore required for replication of multiple DNA and RNA viruses (Nagy et al., 2011). HSP90 inhibitor could inhibit virus replication, thus inhibition of HSP90 may be an effective strategy against SARS-CoV-2 infection (Li et al., 2020). In addition, increasing evidence shows that HSP90 is closely related to fibrogenesis (Bellaye et al., 2014), and overexpression of HSP90 emerges as a hallmark pathological step indicating the fibrogenesis progress (Sontake et al., 2017; Bellaye et al., 2018). Immunohistochemistry study reveals that HSP90α and HSP90β are overexpressed in the lungs of IPF patients (Sibinska et al., 2017). HSP90α participates in the PF progress through promoting the phosphorylation of AKT in P38 and ERK

signaling pathways (Dong et al., 2017). The above descriptions indicate that targeting critical targets and TFs to regulate downstream genes may contribute to improving the condition of COVID-19/PF co-occurrence.

The biological process and molecular mechanisms of critical targets were further analyzed by GO and KEGG enrichment analyses. Critical targets were found to be strongly associated with regulation of virus infection, oxidative stress, inflammation, immune response and metabolic process. One of the characteristics of oxidative stress is the excessive production of reactive oxygen species (ROS) that damage lung tissues over time (Otoupalova et al., 2020). In response to lung tissues damage, lung fibroblasts proliferate and migrate to the damaged area to differentiate into myofibroblasts, causing increased fibronectin, type I and III collagen (Thannickal et al., 2004). Furthermore, oxidative stress participates in the pathogenesis of COVID-19, and SARS-CoV-2 infection induces oxidative stress through increasing the production of ROS and inhibiting antioxidant capacity mediated by the nuclear factor erythroid 2-related factor 2 in the host (Olagnier et al., 2020). Unfortunately, raised oxidative stress will induce inflammatory cascades that ultimately lead to in apoptosis, lung injury and dysregulated of immune responses (Delgado-Roche and Mesta, 2020). The GO result suggests that the effect of kaempferol against COVID-19/PF co-occurrence may be closely associated with the regulation of biological process of oxidative stress, inflammation, immune response and metabolic process.

Furthermore, it was pleasant to find that critical targets were mainly involved in oxidative stress, inflammation, cell growth process, metabolism, immunity and virus infection-related pathways. Among the KEGG pathways, IL-17 signaling pathway, TNF signaling pathway, Toll-like receptor signaling pathway, HIF-1 signaling pathway, EGFR tyrosine kinase inhibitor resistance and PI3K/Akt signaling pathway showed significant significance. IL-17 is found to be highly expressed in patients with COVID-19 and PF comorbidities (Nuovo et al., 2012; Jahaj et al., 2021). Circulating IL-17 is overexpressed in severe COVID-19 patients compared to severe non-COVID-19 patients (Jahaj, et al., 2021). IL-17 signaling pathway is closely related to T helper (Th)17 cell differentiation and exacerbates cytokine storm during SARS-CoV-2 infection (Wu and Yang, 2020). High levels of IL-17 are also found in the lung tissues of IPF patients, which demonstrates that IL-17 signaling pathway is related to IPF progress (Nuovo, et al., 2012). TLRs are pattern recognition receptors involved in the PF process by regulating inflammation and injury repair (Kim et al., 2011). Moreover, activation of Toll-like receptor signaling pathway promotes the overexpression of pro-inflammatory factors (Conti et al., 2020). And interaction between TLRs and viral particles is one of the reasons that causes death of COVID-19 patients (Patra et al., 2021). HIF-1, an important transcriptional factor in response to hypoxia, plays an important role in mammalian oxygen homeostasis and is involved in PF progress (Epstein et al., 2001; Xiong and Liu, 2017). Selective silence of HIF-1α in alveolar epithelial cells can inhibit the progression of bleomycin-induced PF (Weng et al., 2014). Dysregulation of HIF exacerbates edema and inflammation in the lung tissues of patients with ALI, which is associated with glycolysis and mitochondrial respiration (Eckle et al., 2013). Other study also shows that the viral ORF3a protein increases the expression of HIF-1α, which in turn aggravates SARS-CoV-2 infection and inflammatory response (Tian et al., 2021). Besides, EGFR has dual pro-fibrotic and anti-fibrotic effect, and cancer patients treated with EGFR tyrosine kinase inhibitors-monoclonal antibody present an elevated incidence of interstitial lung disease (Osawa et al., 2015). However, a study suggests that gefitinib can inhibit the progression of mice models of bleomycin-induced PF (Ishii et al., 2006). Spontaneous PF is observed in transgenic mice with high expression of EGFR ligands (Korfhagen et al., 1994; Perugorria et al., 2008), and EGFR ligands silencing contribute to improving PF (Madtes et al., 1999). In a word, these studies show that abnormal EGFR expression promotes the development of PF. Moreover, EGFR inhibits IFN-I production (Lupberger et al., 2013) and significantly increases during ALI (Finigan et al., 2012), indicating that EGFR is a potential targeted pathway for treating COVID-19. ALI caused by cytokine storm is the characteristic of COVID-19, and only the combination of TNF-α (an important subtype of TNF signaling pathway) and IFN-γ can induce inflammatory cell death during SARS-CoV-2 infection (Karki et al., 2021). In addition, TNF-α significantly increases in mice models of bleomycin-induced PF (Hou, et al., 2018). PI3K/AKT kinase inhibitors are confirmed to inhibit the replication of MERS (Kindrachuk, et al., 2015), and the inhibition of PI3K/AKT signaling pathway contributes to alleviating PF (Fang et al., 2020). The above researches illustrate that COVID-19 and PF share the common targeting pathways, and IL-17, TNF, HIF-1, EGFR, PI3K/AKT and Toll-like receptor signaling pathways were the critical mechanisms of kaempferol against COVID-19/PF co-occurrence.

CONCLUSION

This study is the first to elucidate the effect of kaempferol against COVID-19/PF co-occurrence by bioinformatics and systems pharmacology tools. The underlying mechanisms of kaempferol against COVID-19/PF co-occurrence may be related to bind to EGFR, SRC, MAPK3, MAPK1, MAPK8, AKT1, RELA and PIK3CA. Kaempferol might regulate inflammation, oxidative stress, immunity, virus infection, cell growth process and metabolism through targeting EGFR, IL-17, TNF, HIF-1, PI3K/AKT and Toll-like receptor signaling pathways to perform anti-COVID-19/PF co-occurrence effect. These findings suggest the possibility that kaempferol is a candidate compound to treat COVID-19/PF co-occurrence, but clinical, *in vivo* and *in vitro* experiments are needed to carry out to verify the predicted effect of kaempferol on COVID-19/PF co-occurrence in the future. This study contributes to providing effective strategy for exploring therapeutic approach for COVID-19/PF co-occurrence.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

HL, X-FH, and X-HL participated in the guidance and revision of the entire article. YJ conducted the writing and data analysis, Y-ZX and K-NY performed the literature searches and data analysis. C-WP, X-YL, S-FZ, and H-TH conducted the data analysis and revision of the article.

FUNDING

This work was supported by Traditional Chinese Medicine Bureau of Guangdong Province (20211344), This work was also supported by Shenzhen Baoan District Science and Technology Bureau (2020JD555). National Natural Science Foundation of China (82004141), Bao'an Traditional Chinese Medicine Development Foundation (2020KJCX-KTYJ-5), and Science, Technology, and Innovation Commission of Shenzhen Municipality (JCYJ20190808160407500). This work was supported by the Sanming Project of Medicine in Shenzhen (Grant No. SZZYSM202106006). This work was supported by the Science and Technology Program of Guangzhou, China (Grant no. 201904010235), and the National Natural Science Foundation of Guangdong, China (Grant no. 2020A1515010589 and Grant no. 2021A1515010146). This work was also supported by the "Double First-Class" and High-level University Discipline Collaborative Innovation Team Project of Guangzhou University of Chinese Medicine (Grant no. 2021XK16), Guangdong Provincial Department of Education Innovation Team Project (Grant no. 2018KCXTD007), the Key-Area Research and Development Program of Guangdong Province (Grant no. 2020B1111100002), the National Natural Science Foundation of China (Grant nos. 81973814 and 81904132), and the Technology Research of COVID-19 Treatment and Prevention and Special Project of Traditional Chinese Medicine Application-Research on the platform construction for the prevention and treatment of viral infectious diseases with traditional Chinese medicine (Grant no. 2020KJCX-KTYJ-130).

ACKNOWLEDGMENTS

We thank Lingnan Medical Research Center of Guangzhou University of Chinese Medicine and the Famous Traditional Chinese Medicine inheritance physician unit of X-HL of Guangdong for their support. We thank Professor Ling Jun Wang for his support in carrying out this work. We thank X-HL for her kind help in the initiation of this research and her selfless assistance.

REFERENCES

Acat, M., Yildiz Gulhan, P., Oner, S., and Turan, M. K. (2021). Comparison of Pirfenidone and Corticosteroid Treatments at the COVID-19 Pneumonia with the Guide of Artificial Intelligence Supported Thoracic Computed Tomography. *Int. J. Clin. Pract.* 75, e14961. doi:10.1111/ijcp.14961

Amberger, J. S., Bocchini, C. A., Schiettecatte, F., Scott, A. F., and Hamosh, A. (2015). OMIM.org: Online Mendelian Inheritance in Man (OMIM®), an Online Catalog of Human Genes and Genetic Disorders. *Nucleic Acids Res.* 43 (Database issue), D789–D798. doi:10.1093/nar/gku1205

An, X., Zhang, Y., Duan, L., JinZhao, S., Zhao, S., Zhou, R., et al. (2021). The Direct Evidence and Mechanism of Traditional Chinese Medicine Treatment of COVID-19. *Biomed. Pharmacother.* 137, 111267. doi:10.1016/j.biopha.2021.111267

Andersson-Sjöland, A., de Alba, C. G., Nihlberg, K., Becerril, C., Ramírez, R., Pardo, A., et al. (2008). Fibrocytes Are a Potential Source of Lung Fibroblasts in Idiopathic Pulmonary Fibrosis. *Int. J. Biochem. Cell. Biol.* 40 (10), 2129–2140. doi:10.1016/j.biocel.2008.02.012

Antoniades, H. N., Bravo, M. A., Avila, R. E., Galanopoulos, T., Neville-Golden, J., Maxwell, M., et al. (1990). Platelet-derived Growth Factor in Idiopathic Pulmonary Fibrosis. *J. Clin. Investig.* 86 (4), 1055–1064. doi:10.1172/JCI114808

Antoniou, K. M., Margaritopoulos, G. A., Soufla, G., Symvoulakis, E., Vassalou, E., Lymbouridou, R., et al. (2010). Expression Analysis of Akt and MAPK Signaling Pathways in Lung Tissue of Patients with Idiopathic Pulmonary Fibrosis (IPF). *J. Recept Signal Transduct. Res.* 30 (4), 262–269. doi:10.3109/10799893.2010.489227

Bader, G. D., and Hogue, C. W. (2003). An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks. *BMC Bioinforma.* 4, 2. doi:10.1186/1471-2105-4-2

Bahri, S., Ben Ali, R., Abidi, A., and Jameleddine, S. (2017). The Efficacy of Plant Extract and Bioactive Compounds Approaches in the Treatment of Pulmonary Fibrosis: A Systematic Review. *Biomed. Pharmacother.* 93, 666–673. doi:10.1016/j.biopha.2017.06.052

Bellaye, P. S., Burgy, O., Causse, S., Garrido, C., and Bonniaud, P. (2014). Heat Shock Proteins in Fibrosis and Wound Healing: Good or Evil? *Pharmacol. Ther.* 143 (2), 119–132. doi:10.1016/j.pharmthera.2014.02.009

Bellaye, P. S., Shimbori, C., Yanagihara, T., Carlson, D. A., Hughes, P., Upagupta, C., et al. (2018). Synergistic Role of HSP90α and HSP90β to Promote Myofibroblast Persistence in Lung Fibrosis. *Eur. Respir. J.* 51 (2). doi:10.1183/13993003.00386-2017

Bian, J., Wang, K., Kong, X., Liu, H., Chen, F., Hu, M., et al. (2011). Caspase- and P38-MAPK-dependent Induction of Apoptosis in A549 Lung Cancer Cells by Newcastle Disease Virus. *Arch. Virol.* 156 (8), 1335–1344. doi:10.1007/s00705-011-0987-y

Care, C., Sornjai, W., Jaratsittisin, J., Hitakarun, A., Wikan, N., Triwitayakorn, K., et al. (2020). Discordant Activity of Kaempferol towards Dengue Virus and Japanese Encephalitis Virus. *Molecules* 25 (5). doi:10.3390/molecules25051246

Celik, I., Yadav, R., Duzgun, Z., Albogami, S., El-Shehawi, A. M., FatimawaliEmran, F., et al. (2021). Interactions of the Receptor Binding Domain of SARS-CoV-2 Variants with hACE2: Insights from Molecular Docking Analysis and Molecular Dynamic Simulation. *Biology* 10 (9), 880. doi:10.3390/biology10090880

Chen, D. Q., Feng, Y. L., Cao, G., and Zhao, Y. Y. (2018). Natural Products as a Source for Antifibrosis Therapy. *Trends Pharmacol. Sci.* 39 (11), 937–952. doi:10.1016/j.tips.2018.09.002

Consortium, G. T. (2013). The Genotype-Tissue Expression (GTEx) Project. *Nat. Genet.* 45 (6), 580–585. doi:10.1038/ng.2653

Conti, P., Ronconi, G., Caraffa, A., Gallenga, C. E., Ross, R., Frydas, I., et al. (2020). Induction of Pro-inflammatory Cytokines (IL-1 and IL-6) and Lung Inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): Anti-inflammatory Strategies. *J. Biol. Regul. Homeost. Agents* 34 (2), 327–331. doi:10.23812/CONTI-E

Crestani, B., Cornillet, P., Dehoux, M., Rolland, C., Guenounou, M., and Aubier, M. (1994). Alveolar Type II Epithelial Cells Produce Interleukin-6 *In Vitro* and *In Vivo*. Regulation by Alveolar Macrophage Secretory Products. *J. Clin. Investig.* 94 (2), 731–740. doi:10.1172/JCI117392

Daina, A., Michielin, O., and Zoete, V. (2019). SwissTargetPrediction: Updated Data and New Features for Efficient Prediction of Protein Targets of Small Molecules. *Nucleic Acids Res.* 47 (W1), W357–W364. doi:10.1093/nar/gkz382

Davis, A. P., Grondin, C. J., Johnson, R. J., Sciaky, D., Wiegers, J., Wiegers, T. C., et al. (2021). Comparative Toxicogenomics Database (CTD): Update 2021. *Nucleic Acids Res.* 49 (D1), D1138–D1143. doi:10.1093/nar/gkaa891

Delgado-Roche, L., and Mesta, F. (2020). Oxidative Stress as Key Player in Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) Infection. *Arch. Med. Res.* 51 (5), 384–387. doi:10.1016/j.arcmed.2020.04.019

Delorey, T. M., Ziegler, C. G. K., Heimberg, G., Normand, R., Yang, Y., Segerstolpe, Å., et al. (2021). COVID-19 Tissue Atlases Reveal SARS-CoV-2 Pathology and Cellular Targets. *Nature* 595 (7865), 107–113. doi:10.1038/s41586-021-03570-8

Devi, K. P., Malar, D. S., Nabavi, S. F., Sureda, A., Xiao, J., Nabavi, S. M., et al. (2015). Kaempferol and Inflammation: From Chemistry to Medicine. *Pharmacol. Res.* 99, 1–10. doi:10.1016/j.phrs.2015.05.002

Dong, H., Luo, L., Zou, M., Huang, C., Wan, X., Hu, Y., et al. (2017). Blockade of Extracellular Heat Shock Protein 90α by 1G6-D7 Attenuates Pulmonary Fibrosis through Inhibiting ERK Signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 313 (6), L1006–L1015. doi:10.1152/ajplung.00489.2016

Eckle, T., Brodsky, K., Bonney, M., Packard, T., Han, J., Borchers, C. H., et al. (2013). HIF1A Reduces Acute Lung Injury by Optimizing Carbohydrate Metabolism in the Alveolar Epithelium. *PLoS Biol.* 11 (9), e1001665. doi:10.1371/journal.pbio.1001665

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., et al. (2001). *C. elegans* EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation. *Cell.* 107 (1), 43–54. doi:10.1016/s0092-8674(01)00507-4

Esfandiarei, M., Luo, H., Yanagawa, B., Suarez, A., Dabiri, D., Zhang, J., et al. (2004). Protein Kinase B/Akt Regulates Coxsackievirus B3 Replication through a Mechanism Which Is Not Caspase Dependent. *J. Virol.* 78 (8), 4289–4298. doi:10.1128/jvi.78.8.4289-4298.2004

Fang, L., Chen, H., Kong, R., and Que, J. (2020). Endogenous Tryptophan Metabolite 5-Methoxytryptophan Inhibits Pulmonary Fibrosis by Downregulating the TGF-B/smad3 and PI3K/AKT Signaling Pathway. *Life Sci.* 260, 118399. doi:10.1016/j.lfs.2020.118399

Finigan, J. H., Downey, G. P., and Kern, J. A. (2012). Human Epidermal Growth Factor Receptor Signaling in Acute Lung Injury. *Am. J. Respir. Cell. Mol. Biol.* 47 (4), 395–404. doi:10.1165/rcmb.2012-0100TR

Gaur, P., Munjhal, A., and Lal, S. K. (2011). Influenza Virus and Cell Signaling Pathways. *Med. Sci. Monit.* 17 (6), RA148–54. doi:10.12659/msm.881801

George, P. M., Wells, A. U., and Jenkins, R. G. (2020). Pulmonary Fibrosis and COVID-19: the Potential Role for Antifibrotic Therapy. *Lancet Respir. Med.* 8 (8), 807–815. doi:10.1016/s2213-2600(20)30225-3

Giacomelli, C., Piccarducci, R., Marchetti, L., Romei, C., and Martini, C. (2021). Pulmonary Fibrosis from Molecular Mechanisms to Therapeutic Interventions: Lessons from Post-COVID-19 Patients. *Biochem. Pharmacol.* 193, 114812. doi:10.1016/j.bcp.2021.114812

Gong, J. H., Cho, I. H., Shin, D., Han, S. Y., Park, S. H., and Kang, Y. H. (2014). Inhibition of Airway Epithelial-To-Mesenchymal Transition and Fibrosis by Kaempferol in Endotoxin-Induced Epithelial Cells and Ovalbumin-Sensitized Mice. *Lab. Investig.* 94 (3), 297–308. doi:10.1038/labinvest.2013.137

Goodwin, A., and Jenkins, G. (2009). Role of Integrin-Mediated TGFbeta Activation in the Pathogenesis of Pulmonary Fibrosis. *Biochem. Soc. Trans.* 37 (Pt 4), 849–854. doi:10.1042/BST0370849

Hammond, J., Leister-Tebbe, H., Gardner, A., Abreu, P., Bao, W., Wisemandle, W., et al. (2022). Oral Nirmatrelvir for High-Risk, Nonhospitalized Adults with Covid-19. *N. Engl. J. Med.* 386, 1397–1408. doi:10.1056/NEJMoa2118542

Han, H., Cho, J. W., Lee, S., Yun, A., Kim, H., Bae, D., et al. (2018). TRRUST V2: an Expanded Reference Database of Human and Mouse Transcriptional Regulatory Interactions. *Nucleic Acids Res.* 46 (D1), D380–D386. doi:10.1093/nar/gkx1013

Hou, J., Ma, T., Cao, H., Chen, Y., Wang, C., Chen, X., et al. (2018). TNF-α-induced NF-Kb Activation Promotes Myofibroblast Differentiation of LR-MSCs and Exacerbates Bleomycin-Induced Pulmonary Fibrosis. *J. Cell. Physiol.* 233 (3), 2409–2419. doi:10.1002/jcp.26112

Huang, J. F., Chuang, Y. H., Dai, C. Y., Yu, M. L., Huang, C. F., Hsiao, P. J., et al. (2011). Hepatic Akt Expression Correlates with Advanced Fibrosis in Patients with Chronic Hepatitis C Infection. *Hepatol. Res.* 41 (5), 430–436. doi:10.1111/j.1872-034X.2011.00786.x

Imran, M., Salehi, B., Sharifi-Rad, J., Aslam Gondal, T., Saeed, F., Imran, A., et al. (2019). Kaempferol: A Key Emphasis to its Anticancer Potential. *Molecules* 24 (12). doi:10.3390/molecules24122277

Ishii, Y., Fujimoto, S., and Fukuda, T. (2006). Gefitinib Prevents Bleomycin-Induced Lung Fibrosis in Mice. *Am. J. Respir. Crit. Care Med.* 174 (5), 550–556. doi:10.1164/rccm.200509-1534OC

Jaffar, J., Glaspole, I., Symons, K., and Westall, G. (2021). Inhibition of NF-Kb by ACT001 Reduces Fibroblast Activity in Idiopathic Pulmonary Fibrosis. *Biomed. Pharmacother.* 138, 111471. doi:10.1016/j.biopha.2021.111471

Jahaj, E., Vassiliou, A. G., Keskinidou, C., Gallos, P., Vrettou, C. S., Tsipilis, S., et al. (2021). Evaluating the Role of the Interleukin-23/17 Axis in Critically Ill COVID-19 Patients. *J. Pers. Med.* 11 (9). doi:10.3390/jpm11090891

John, A. E., Joseph, C., Jenkins, G., and Tatler, A. L. (2021). COVID-19 and Pulmonary Fibrosis: A Potential Role for Lung Epithelial Cells and Fibroblasts. *Immunol. Rev.* 302 (1), 228–240. doi:10.1111/imr.12977

Karki, R., Sharma, B. R., Tuladhar, S., Williams, E. P., Zalduondo, L., Samir, P., et al. (2021). Synergism of TNF-α and IFN-γ Triggers Inflammatory Cell Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes. *Cell.* 184 (1), 149–e17. doi:10.1016/j.cell.2020.11.025

Keiser, M. J., Roth, B. L., Armbruster, B. N., Ernsberger, P., Irwin, J. J., and Shoichet, B. K. (2007). Relating Protein Pharmacology by Ligand Chemistry. *Nat. Biotechnol.* 25 (2), 197–206. doi:10.1038/nbt1284

Khaerunnisa, S., Kurniawan, H., Awaluddin, R., Suhartati, S., and Soetjipto, S. (2020). *Potential Inhibitor of COVID-19 Main Protease (M^{Pro}) from Several Medicinal Plant Compounds by Molecular Docking Study* Medicine & Pharmacology. doi:10.20944/preprints202003.0226.v1Potential Inhibitor of COVID-19 Main Protease (Mpro) from Several Medicinal Plant Compounds by Molecular Docking Study

Khan, A., Heng, W., Wang, Y., Qiu, J., Wei, X., Peng, S., et al. (2021). In Silico and *In Vitro* Evaluation of Kaempferol as a Potential Inhibitor of the SARS-CoV-2 Main Protease (3CLpro). *Phytother. Res.* 35 (6), 2841–2845. doi:10.1002/ptr.6998

Kim, H. S., Go, H., Akira, S., and Chung, D. H. (2011). TLR2-mediated Production of IL-27 and Chemokines by Respiratory Epithelial Cells Promotes Bleomycin-Induced Pulmonary Fibrosis in Mice. *J. Immunol.* 187 (8), 4007–4017. doi:10.4049/jimmunol.1101654

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., et al. (2021). PubChem in 2021: New Data Content and Improved Web Interfaces. *Nucleic Acids Res.* 49 (D1), D1388–D1395. doi:10.1093/nar/gkaa971

Kindrachuk, J., Ork, B., Hart, B. J., Mazur, S., Holbrook, M. R., Frieman, M. B., et al. (2015). Antiviral Potential of ERK/MAPK and PI3K/AKT/mTOR Signaling Modulation for Middle East Respiratory Syndrome Coronavirus Infection as Identified by Temporal Kinome Analysis. *Antimicrob. Agents Chemother.* 59 (2), 1088–1099. doi:10.1128/AAC.03659-14

Korfhagen, T. R., Swantz, R. J., Wert, S. E., McCarty, J. M., Kerlakian, C. B., Glasser, S. W., et al. (1994). Respiratory Epithelial Cell Expression of Human Transforming Growth Factor-Alpha Induces Lung Fibrosis in Transgenic Mice. *J. Clin. Investig.* 93 (4), 1691–1699. doi:10.1172/JCI117152

Lancaster, L., Crestani, B., Hernandez, P., Inoue, Y., Wachtlin, D., Loaiza, L., et al. (2019). Safety and Survival Data in Patients with Idiopathic Pulmonary Fibrosis Treated with Nintedanib: Pooled Data from Six Clinical Trials. *BMJ Open Respir. Res.* 6 (1), e000397. doi:10.1136/bmjresp-2018-000397

Le Cras, T. D., Korfhagen, T. R., Davidson, C., Schmidt, S., Fenchel, M., Ikegami, M., et al. (2010). Inhibition of PI3K by PX-866 Prevents Transforming Growth Factor-Alpha-Induced Pulmonary Fibrosis. *Am. J. Pathol.* 176 (2), 679–686. doi:10.2353/ajpath.2010.090123

Lechowicz, K., Drożdżal, S., Machaj, F., Rosik, J., Szostak, B., Zegan-Barańska, M., et al. (2020). COVID-19: The Potential Treatment of Pulmonary Fibrosis Associated with SARS-CoV-2 Infection. *J. Clin. Med.* 9 (6). doi:10.3390/jcm9061917

Li, C., Chu, H., Liu, X., Chiu, M. C., Zhao, X., Wang, D., et al. (2020). Human Coronavirus Dependency on Host Heat Shock Protein 90 Reveals an Antiviral Target. *Emerg. Microbes Infect.* 9 (1), 1–27. doi:10.1080/22221751.2020.1850183

Li, L., Wang, R., Hu, H., Chen, X., Yin, Z., Liang, X., et al. (2021). The Antiviral Activity of Kaempferol against Pseudorabies Virus in Mice. *BMC Vet. Res.* 17 (1), 247. doi:10.1186/s12917-021-02953-3

Liao, Q. J., Ye, L. B., Timani, K. A., Zeng, Y. C., She, Y. L., Ye, L., et al. (2005). Activation of NF-kappaB by the Full-Length Nucleocapsid Protein of the

SARS Coronavirus. *Acta Biochim. Biophys. Sin. (Shanghai)* 37 (9), 607–612. doi:10.1111/j.1745-7270.2005.00082.x

Liu, G., Cooley, M. A., Jarnicki, A. G., Borghuis, T., Nair, P. M., Tjin, G., et al. (2019). Fibulin-1c Regulates Transforming Growth Factor-β Activation in Pulmonary Tissue Fibrosis. *JCI Insight* 5. doi:10.1172/jci.insight.124529

Liu, G., Cooley, M. A., Nair, P. M., Donovan, C., Hsu, A. C., Jarnicki, A. G., et al. (2017). Airway Remodelling and Inflammation in Asthma Are Dependent on the Extracellular Matrix Protein Fibulin-1c. *J. Pathol.* 243 (4), 510–523. doi:10.1002/path.4979

Liu, H., Yu, H., Cao, Z., Gu, J., Pei, L., Jia, M., et al. (2019). Kaempferol Modulates Autophagy and Alleviates Silica-Induced Pulmonary Fibrosis. *DNA Cell. Biol.* 38 (12), 1418–1426. doi:10.1089/dna.2019.4941

Lu, Y., Azad, N., Wang, L., Iyer, A. K., Castranova, V., Jiang, B. H., et al. (2010). Phosphatidylinositol-3-kinase/akt Regulates Bleomycin-Induced Fibroblast Proliferation and Collagen Production. *Am. J. Respir. Cell. Mol. Biol.* 42 (4), 432–441. doi:10.1165/rcmb.2009-0002OC

Lupberger, J., Duong, F. H., Fofana, I., Zona, L., Xiao, F., Thumann, C., et al. (2013). Epidermal Growth Factor Receptor Signaling Impairs the Antiviral Activity of Interferon-Alpha. *Hepatology* 58 (4), 1225–1235. doi:10.1002/hep.26404

Madtes, D. K., Elston, A. L., Hackman, R. C., Dunn, A. R., and Clark, J. G. (1999). Transforming Growth Factor-Alpha Deficiency Reduces Pulmonary Fibrosis in Transgenic Mice. *Am. J. Respir. Cell. Mol. Biol.* 20 (5), 924–934. doi:10.1165/ajrcmb.20.5.3526

Maheshwari, S., Miller, M. S., O'Meally, R., Cole, R. N., Amzel, L. M., and Gabelli, S. B. (2017). Kinetic and Structural Analyses Reveal Residues in Phosphoinositide 3-kinase α that Are Critical for Catalysis and Substrate Recognition. *J. Biol. Chem.* 292 (33), 13541–13550. doi:10.1074/jbc.M116.772426

Mahmud, S., Mita, M. A., Biswas, S., Paul, G. K., Promi, M. M., Afrose, S., et al. (2021). Molecular Docking and Dynamics Study to Explore Phytochemical Ligand Molecules against the Main Protease of SARS-CoV-2 from Extensive Phytochemical Datasets. *Expert Rev. Clin. Pharmacol.* 14 (10), 1305–1315. doi:10.1080/17512433.2021.1959318

Meyer, B., Chiaravalli, J., Gellenoncourt, S., Brownridge, P., Bryne, D. P., Daly, L. A., et al. (2021). Characterising Proteolysis during SARS-CoV-2 Infection Identifies Viral Cleavage Sites and Cellular Targets with Therapeutic Potential. *Nat. Commun.* 12 (1), 5553. doi:10.1038/s41467-021-25796-w

Mizutani, T., Fukushi, S., Saijo, M., Kurane, I., and Morikawa, S. (2004). Phosphorylation of P38 MAPK and its Downstream Targets in SARS Coronavirus-Infected Cells. *Biochem. Biophys. Res. Commun.* 319 (4), 1228–1234. doi:10.1016/j.bbrc.2004.05.107

Nagy, P. D., Wang, R. Y., Pogany, J., Hafren, A., and Makinen, K. (2011). Emerging Picture of Host Chaperone and Cyclophilin Roles in RNA Virus Replication. *Virology* 411 (2), 374–382. doi:10.1016/j.virol.2010.12.061

Noble, P. W., Albera, C., Bradford, W. Z., Costabel, U., Glassberg, M. K., Kardatzke, D., et al. (2011). Pirfenidone in Patients with Idiopathic Pulmonary Fibrosis (CAPACITY): Two Randomised Trials. *Lancet* 377 (9779), 1760–1769. doi:10.1016/S0140-6736(11)60405-4

Nuovo, G. J., Hagood, J. S., Magro, C. M., Chin, N., Kapil, R., Davis, L., et al. (2012). The Distribution of Immunomodulatory Cells in the Lungs of Patients with Idiopathic Pulmonary Fibrosis. *Mod. Pathol.* 25 (3), 416–433. doi:10.1038/modpathol.2011.166

Olagnier, D., Farahani, E., Thyrsted, J., Blay-Cadanet, J., Herengt, A., Idorn, M., et al. (2020). SARS-CoV2-mediated Suppression of NRF2-Signaling Reveals Potent Antiviral and Anti-inflammatory Activity of 4-Octyl-Itaconate and Dimethyl Fumarate. *Nat. Commun.* 11 (1), 4938. doi:10.1038/s41467-020-18764-3

Osawa, M., Kudoh, S., Sakai, F., Endo, M., Hamaguchi, T., Ogino, Y., et al. (2015). Clinical Features and Risk Factors of Panitumumab-Induced Interstitial Lung Disease: a Postmarketing All-Case Surveillance Study. *Int. J. Clin. Oncol.* 20 (6), 1063–1071. doi:10.1007/s10147-015-0834-3

Otasek, D., Morris, J. H., Bouças, J., Pico, A. R., and Demchak, B. (2019). Cytoscape Automation: Empowering Workflow-Based Network Analysis. *Genome Biol.* 20 (1), 185. doi:10.1186/s13059-019-1758-4

Otoupalova, E., Smith, S., Cheng, G., and Thannickal, V. J. (2020). Oxidative Stress in Pulmonary Fibrosis. *Compr. Physiol.* 10 (2), 509–547. doi:10.1002/cphy.c190017

Pan, L. H., Yamauchi, K., Uzuki, M., Nakanishi, T., Takigawa, M., Inoue, H., et al. (2001). Type II Alveolar Epithelial Cells and Interstitial Fibroblasts Express Connective Tissue Growth Factor in IPF. *Eur. Respir. J.* 17 (6), 1220–1227. doi:10.1183/09031936.01.00074101

Pan, Y., Guan, H., Zhou, S., Wang, Y., Li, Q., Zhu, T., et al. (2020). Initial CT Findings and Temporal Changes in Patients with the Novel Coronavirus Pneumonia (2019-nCoV): a Study of 63 Patients in Wuhan, China. *Eur. Radiol.* 30 (6), 3306–3309. doi:10.1007/s00330-020-06731-x

Patra, R., Chandra Das, N., and Mukherjee, S. (2021). Targeting Human TLRs to Combat COVID-19: A Solution? *J. Med. Virol.* 93 (2), 615–617. doi:10.1002/jmv.26387

Perugorria, M. J., Latasa, M. U., Nicou, A., Cartagena-Lirola, H., Castillo, J., Goñi, S., et al. (2008). The Epidermal Growth Factor Receptor Ligand Amphiregulin Participates in the Development of Mouse Liver Fibrosis. *Hepatology* 48 (4), 1251–1261. doi:10.1002/hep.22437

Piñero, J., Bravo, À., Queralt-Rosinach, N., Gutiérrez-Sacristán, A., Deu-Pons, J., Centeno, E., et al. (2017). DisGeNET: a Comprehensive Platform Integrating Information on Human Disease-Associated Genes and Variants. *Nucleic Acids Res.* 45 (D1), D833–D839. doi:10.1093/nar/gkw943

Rameshwar, P., Narayanan, R., Qian, J., Denny, T. N., Colon, C., and Gascon, P. (2000). NF-kappa B as a Central Mediator in the Induction of TGF-Beta in Monocytes from Patients with Idiopathic Myelofibrosis: an Inflammatory Response beyond the Realm of Homeostasis. *J. Immunol.* 165 (4), 2271–2277. doi:10.4049/jimmunol.165.4.2271

Rebhan, M., Chalifa-Caspi, V., Prilusky, J., and Lancet, D. (1997). GeneCards: Integrating Information about Genes, Proteins and Diseases. *Trends Genet.* 13 (4), 163. doi:10.1016/s0168-9525(97)01103-7

Ren, J., Lu, Y., Qian, Y., Chen, B., Wu, T., and Ji, G. (2019). Recent Progress Regarding Kaempferol for the Treatment of Various Diseases. *Exp. Ther. Med.* 18 (4), 2759–2776. doi:10.3892/etm.2019.7886

Roschewski, M., Lionakis, M. S., Sharman, J. P., Roswarski, J., Goy, A., Monticelli, M. A., et al. (2020). Inhibition of Bruton Tyrosine Kinase in Patients with Severe COVID-19. *Sci. Immunol.* 5 (48). doi:10.1126/sciimmunol.abd0110

Rose, Y., Duarte, J. M., Lowe, R., Segura, J., Bi, C., Bhikadiya, C., et al. (2021). RCSB Protein Data Bank: Architectural Advances towards Integrated Searching and Efficient Access to Macromolecular Structure Data from the PDB Archive. *J. Mol. Biol.* 433 (11), 166704. doi:10.1016/j.jmb.2020.11.003

Ru, J., Li, P., Wang, J., Zhou, W., Li, B., Huang, C., et al. (2014). TCMSP: a Database of Systems Pharmacology for Drug Discovery from Herbal Medicines. *J. Cheminform* 6, 13. doi:10.1186/1758-2946-6-13

Schaller, T., Hirschbühl, K., Burkhardt, K., Braun, G., Trepel, M., Märkl, B., et al. (2020). Postmortem Examination of Patients with COVID-19. *JAMA* 323 (24), 2518–2520. doi:10.1001/jama.2020.8907

Shaldam, M. A., Yahya, G., Mohamed, N. H., Abdel-Daim, M. M., and Al Naggar, Y. (2021). In Silico screening of Potent Bioactive Compounds from Honeybee Products against COVID-19 Target Enzymes. *Environ. Sci. Pollut. Res. Int.* 28 (30), 40507–40514. doi:10.1007/s11356-021-14195-9

Shen, L., Liu, F., Huang, L., Liu, G., Zhou, L., and Peng, L. (2021). VDA-RWLRLS: An Anti-SARS-CoV-2 Drug Prioritizing Framework Combining an Unbalanced Bi-random Walk and Laplacian Regularized Least Squares. *Comput. Biol. Med.* 140, 105119. doi:10.1016/j.compbiomed.2021.105119

Sibinska, Z., Tian, X., Korfei, M., Kojonazarov, B., Kolb, J. S., Klepetko, W., et al. (2017). Amplified Canonical Transforming Growth Factor-β Signalling via Heat Shock Protein 90 in Pulmonary Fibrosis. *Eur. Respir. J.* 49 (2). doi:10.1183/13993003.01941-2015

Sisson, T. H., Mendez, M., Choi, K., Subbotina, N., Courey, A., Cunningham, A., et al. (2010). Targeted Injury of Type II Alveolar Epithelial Cells Induces Pulmonary Fibrosis. *Am. J. Respir. Crit. Care Med.* 181 (3), 254–263. doi:10.1164/rccm.200810-1615OC

Soleimanpour, S., and Yaghoubi, A. (2021). COVID-19 Vaccine: where Are We Now and where Should We Go? *Expert Rev. Vaccines* 20 (1), 23–44. doi:10.1080/14760584.2021.1875824

Song, P., Li, W., Xie, J., Hou, Y., and You, C. (2020). Cytokine Storm Induced by SARS-CoV-2. *Clin. Chim. Acta* 509, 280–287. doi:10.1016/j.cca.2020.06.017

Sontake, V., Wang, Y., Kasam, R. K., Sinner, D., Reddy, G. B., Naren, A. P., et al. (2017). Hsp90 Regulation of Fibroblast Activation in Pulmonary Fibrosis. *JCI Insight* 2 (4), e91454. doi:10.1172/jci.insight.91454

Szklarczyk, D., Gable, A. L., Nastou, K. C., Lyon, D., Kirsch, R., Pyysalo, S., et al. (2021). The STRING Database in 2021: Customizable Protein-Protein Networks, and Functional Characterization of User-Uploaded Gene/measurement Sets. *Nucleic Acids Res.* 49 (D1), D605–D612. doi:10.1093/nar/gkaa1074

Tang, Y., Li, M., Wang, J., Pan, Y., and Wu, F. X. (2015). CytoNCA: a Cytoscape Plugin for Centrality Analysis and Evaluation of Protein Interaction Networks. *Biosystems* 127, 67–72. doi:10.1016/j.biosystems.2014.11.005

Tatler, A. L., and Jenkins, G. (2012). TGF-β Activation and Lung Fibrosis. *Proc. Am. Thorac. Soc.* 9 (3), 130–136. doi:10.1513/pats.201201-003AW

Thannickal, V. J., Toews, G. B., White, E. S., Lynch, J. P., 3rd, and Martinez, F. J. (2004). Mechanisms of Pulmonary Fibrosis. *Annu. Rev. Med.* 55, 395–417. doi:10.1146/annurev.med.55.091902.103810

Tian, M., Liu, W., Li, X., Zhao, P., Shereen, M. A., Zhu, C., et al. (2021). HIF-1α Promotes SARS-CoV-2 Infection and Aggravates Inflammatory Responses to COVID-19. *Signal Transduct. Target Ther.* 6 (1), 308. doi:10.1038/s41392-021-00726-w

Trott, O., and Olson, A. J. (2010). AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading. *J. Comput. Chem.* 31 (2), 455–461. doi:10.1002/jcc.21334

UniProt, C. (2015). UniProt: a Hub for Protein Information. *Nucleic Acids Res.* 43, D204–D212. doi:10.1093/nar/gku989

Vagapova, E. R., Lebedev, T. D., and Prassolov, V. S. (2021). Viral Fibrotic Scoring and Drug Screen Based on MAPK Activity Uncovers EGFR as a Key Regulator of COVID-19 Fibrosis. *Sci. Rep.* 11 (1), 11234. doi:10.1038/s41598-021-90701-w

Vittal, R., Horowitz, J. C., Moore, B. B., Zhang, H., Martinez, F. J., Toews, G. B., et al. (2005). Modulation of Prosurvival Signaling in Fibroblasts by a Protein Kinase Inhibitor Protects against Fibrotic Tissue Injury. *Am. J. Pathol.* 166 (2), 367–375. doi:10.1016/S0002-9440(10)62260-2

Wang, B., Li, R., Lu, Z., and Huang, Y. (2020). Does Comorbidity Increase the Risk of Patients with COVID-19: Evidence from Meta-Analysis. *Aging (Albany NY)* 12 (7), 6049–6057. doi:10.18632/aging.103000

Wang, W., Ye, L., Ye, L., Li, B., Gao, B., Zeng, Y., et al. (2007). Up-regulation of IL-6 and TNF-Alpha Induced by SARS-Coronavirus Spike Protein in Murine Macrophages via NF-kappaB Pathway. *Virus Res.* 128 (1-2), 1–8. doi:10.1016/j.virusres.2007.02.007

Wang, Y., Zhang, S., Li, F., Zhou, Y., Zhang, Y., Wang, Z., et al. (2020). Therapeutic Target Database 2020: Enriched Resource for Facilitating Research and Early Development of Targeted Therapeutics. *Nucleic Acids Res.* 48 (D1), D1031–D1041. doi:10.1093/nar/gkz981

Weng, T., Poth, J. M., Karmouty-Quintana, H., Garcia-Morales, L. J., Melicoff, E., Luo, F., et al. (2014). Hypoxia-induced Deoxycytidine Kinase Contributes to Epithelial Proliferation in Pulmonary Fibrosis. *Am. J. Respir. Crit. Care Med.* 190 (12), 1402–1412. doi:10.1164/rccm.201404-0744OC

Wishart, D. S., Feunang, Y. D., Guo, A. C., Lo, E. J., Marcu, A., Grant, J. R., et al. (2018). DrugBank 5.0: a Major Update to the DrugBank Database for 2018. *Nucleic Acids Res.* 46 (D1), D1074–D1082. doi:10.1093/nar/gkx1037

World Health Organization (2022). *WHO Coronavirus (COVID-19) Dashboard*. from Avaible at: https://covid19.who.int/.

Wu, D., and Yang, X. O. (2020). TH17 Responses in Cytokine Storm of COVID-19: An Emerging Target of JAK2 Inhibitor Fedratinib. *J. Microbiol. Immunol. Infect.* 53 (3), 368–370. doi:10.1016/j.jmii.2020.03.005

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., et al. (2021). clusterProfiler 4.0: A Universal Enrichment Tool for Interpreting Omics Data. *Innovation* 2 (3), 100141. doi:10.1016/j.xinn.2021.100141

Wu, Y., Zhang, F., Yang, K., Fang, S., Bu, D., Li, H., et al. (2019). SymMap: an Integrative Database of Traditional Chinese Medicine Enhanced by Symptom Mapping. *Nucleic Acids Res.* 47 (D1), D1110–D1117. doi:10.1093/nar/gky1021

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and Important Lessons from the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases from the Chinese Center for Disease Control and Prevention. *JAMA* 323 (13), 1239–1242. doi:10.1001/jama.2020.2648

Xia, H., Diebold, D., Nho, R., Perlman, D., Kleidon, J., Kahm, J., et al. (2008). Pathological Integrin Signaling Enhances Proliferation of Primary Lung Fibroblasts from Patients with Idiopathic Pulmonary Fibrosis. *J. Exp. Med.* 205 (7), 1659–1672. doi:10.1084/jem.20080001

Xiong, A., and Liu, Y. (2017). Targeting Hypoxia Inducible Factors-1α as a Novel Therapy in Fibrosis. *Front. Pharmacol.* 8, 326. doi:10.3389/fphar.2017.00326

Xu, J., Xu, X., Jiang, L., Dua, K., Hansbro, P. M., and Liu, G. (2020). SARS-CoV-2 Induces Transcriptional Signatures in Human Lung Epithelial Cells that Promote Lung Fibrosis. *Respir. Res.* 21 (1), 182. doi:10.1186/s12931-020-01445-6

Xu, X. W., Wu, X. X., Jiang, X. G., Xu, K. J., Ying, L. J., Ma, C. L., et al. (2020). Clinical Findings in a Group of Patients Infected with the 2019 Novel Coronavirus (SARS-Cov-2) outside of Wuhan, China: Retrospective Case Series. *BMJ* 368, m606. doi:10.1136/bmj.m606

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., et al. (2020). Pathological Findings of COVID-19 Associated with Acute Respiratory Distress Syndrome. *Lancet Respir. Med.* 8 (4), 420–422. doi:10.1016/S2213-2600(20)30076-X

Yao, Z. J., Dong, J., Che, Y. J., Zhu, M. F., Wen, M., Wang, N. N., et al. (2016). TargetNet: a Web Service for Predicting Potential Drug-Target Interaction Profiling via Multi-Target SAR Models. *J. Comput. Aided Mol. Des.* 30 (5), 413–424. doi:10.1007/s10822-016-9915-2

Yin, X., Riva, L., Pu, Y., Martin-Sancho, L., Kanamune, J., Yamamoto, Y., et al. (2021). MDA5 Governs the Innate Immune Response to SARS-CoV-2 in Lung Epithelial Cells. *Cell. Rep.* 34 (2), 108628. doi:10.1016/j.celrep.2020.108628

Yu, W., Clyne, M., Khoury, M. J., and Gwinn, M. (2010). Phenopedia and Genopedia: Disease-Centered and Gene-Centered Views of the Evolving Knowledge of Human Genetic Associations. *Bioinformatics* 26 (1), 145–146. doi:10.1093/bioinformatics/btp618

Zhang, R., Ai, X., Duan, Y., Xue, M., He, W., Wang, C., et al. (2017). Kaempferol Ameliorates H9N2 Swine Influenza Virus-Induced Acute Lung Injury by Inactivation of TLR4/MyD88-Mediated NF-Kb and MAPK Signaling Pathways. *Biomed. Pharmacother.* 89, 660–672. doi:10.1016/j.biopha.2017.02.081

Zhang, X., Gao, R., Zhou, Z., Tang, X., Lin, J., Wang, L., et al. (2021). A Network Pharmacology Based Approach for Predicting Active Ingredients and Potential Mechanism of Lianhuaqingwen Capsule in Treating COVID-19. *Int. J. Med. Sci.* 18 (8), 1866–1876. doi:10.7150/ijms.53685

Zhou, S., Wang, Y., Zhu, T., and Xia, L. (2020). CT Features of Coronavirus Disease 2019 (COVID-19) Pneumonia in 62 Patients in Wuhan, China. *AJR Am. J. Roentgenol.* 214 (6), 1287–1294. doi:10.2214/AJR.20.22975

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape Provides a Biologist-Oriented Resource for the Analysis of Systems-Level Datasets. *Nat. Commun.* 10 (1), 1523. doi:10.1038/s41467-019-09234-6

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* 382 (8), 727–733. doi:10.1056/NEJMoa2001017

Ziegler, C. G. K., Allon, S. J., Nyquist, S. K., Mbano, I. M., Miao, V. N., Tzouanas, C. N., et al. (2020). SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues. *Cell.* 181 (5), 1016–e19. doi:10.1016/j.cell.2020.04.035

Zou, M., Zhang, G., Zou, J., Liu, Y., Liu, B., Hu, X., et al. (2020). Inhibition of the ERK1/2-Ubiquitous Calpains Pathway Attenuates Experimental Pulmonary Fibrosis *In Vivo* and *In Vitro*. *Exp. Cell. Res.* 391 (1), 111886. doi:10.1016/j.yexcr.2020.111886

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 17 June 2022
doi: 10.3389/fphar.2022.888073

A Five-Dimensional Network Meta-Analysis of Chinese Herbal Injections for Treating Acute Tonsillitis Combined With Western Medicine

*Peiying Huang[1,2], Yin Li[3], Bixuan Huang[4], Shuai Zhao[2,5], Li Chen[2,5], Hansu Guan[6], Yan Chen[5], Yuchao Feng[2,5], Xiaoyan Huang[2,5], Yi Deng[2,5], Sisi Lei[1,2], Qihua Wu[1,2], Haobo Zhang[1,2], Zhongyi Zeng[7], Linsheng Zeng[7] and Bojun Chen[1,2,5]**

[1]The Second Clinical Medical School of Guangzhou University of Chinese Medicine, Guangzhou, China, [2]Guangdong Provincial Key Laboratory of Research on Emergency in Traditional Chinese Medicine, Clinical Research Team of Prevention and Treatment of Cardiac Emergencies with Traditional Chinese Medicine, Guangzhou, China, [3]The First Clinical Medical School of Guangzhou University of Chinese Medicine, Guangzhou, China, [4]Department of Nursing, Hubei University of Arts and Science, Xiangyang, China, [5]Emergency Department of Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou, China, [6]Emergency Department of the Third Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, [7]Shenzhen Traditional Chinese Medicine Hospital, Shenzhen, China

OPEN ACCESS

Edited by:
Carlos L. Cespedes-Acuña, University of Bio Bio Chillan Chile, Chile

Reviewed by:
Rao Sun, Huazhong University of Science and Technology, China
Xin Li, Shanghai University of Traditional Chinese Medicine, China

***Correspondence:**
Bojun Chen
719523476@qq.com

Specialty section:
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

Received: *02 March 2022*
Accepted: *06 May 2022*
Published: *17 June 2022*

Citation:
Huang P, Li Y, Huang B, Zhao S, Chen L, Guan H, Chen Y, Feng Y, Huang X, Deng Y, Lei S, Wu Q, Zhang H, Zeng Z, Zeng L and Chen B (2022) A Five-Dimensional Network Meta-Analysis of Chinese Herbal Injections for Treating Acute Tonsillitis Combined With Western Medicine. Front. Pharmacol. 13:888073. doi: 10.3389/fphar.2022.888073

Background: Acute tonsillitis has high morbidity. Chinese herbal injections (CHIs) were reported to be useful in treating acute tonsillitis and might reduce the probability of antibiotic resistance. Nevertheless, the optimal strategy for combining CHIs with western medicine (WM) to treat acute tonsillitis remains unclear.

Methods: We retrieved data from the following databases with retrieval time from inception to 11 January 2022: PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, Wanfang Database, Weipu Journal Database, and Chinese Biomedical Literature Database. Version 2 of the Cochrane risk-of-bias tool (ROB2) was used for evaluating the quality of the included studies. R 4.1.2, STATA 14.0, and Python 3.10.4 were employed for network meta-analysis, with 5-dimensional K-means cluster analysis, meta-regression analyses, sensitivity analyses, and subgroup analyses.

Results: A total of 110 randomized controlled trials including 12,152 patients were included. All the studies were rated as "high risk" and "some concerns". In terms of improving clinical effectiveness rate, Qingkailing injection + WM ranked ahead of other interventions (89.51%). Regarding reducing antipyretic time, Reduning injection + WM had the highest-ranking probability (68.48%). As for shortening sore throat relief time, Shuanghuanglian injection + WM ranked first (76.82%). Concerning shortening red and swollen tonsils relief time, Yanhuning injection + WM possessed the highest-ranking probability (89.17%). In terms of reducing tonsillar exudate relief time, Xuebijing injection + WM ranked ahead of the other interventions (94.82%). Additionally, the results of the cluster analysis suggested that Xuebijing injection + WM, Reduning injection + WM, and Yanhuning injection + WM were probably the best interventions. Furthermore, adverse drug reactions rate of Xuebijing injection + WM, Reduning injection + WM, Yanhuning

injection + WM, Qingkailing injection + WM, and Shuanghuanglian injection + WM were individually 0.00%, 3.11%, 3.08%, 4.29%, and 4.62%.

Conclusions: CHIs + WM have a better impact on patients with acute tonsillitis than WM alone. Xuebijing injection, Reduning injection, and Yanhuning injection might have potential advantages in treating the disease. Concerning adverse drug reactions, Xuebijing injection is presumably the optimal CHI. More high-quality studies are needed to further confirm our findings.

Systematic Review Registration: CRD42022303243; URL= https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=303243

Keywords: acute tonsillitis, Chinese herbal injections, western medicine, efficacy, 5-dimensional network meta-analysis

INTRODUCTION

Acute tonsillitis is a type of acute upper respiratory tract infection, with acute sore throat as the principal symptom, accompanied by fever, red and swollen tonsils, enlarged cervical lymph nodes, and may be associated with tonsil exudation (Bartlett et al., 2015; Windfuhr et al., 2016). The disease affects both sexes and all age groups, predominantly in school-aged children (Sidell and Shapiro, 2012). It is estimated that acute tonsillitis makes up approximately 1.3% of outpatient visits (Kocher and Selby, 2014), which generates a substantial workload for primary care physicians and places huge financial pressures on medical budget (Bird et al., 2014).

In 50%–80% of acute tonsillitis patients, the causative pathogens are viruses (e.g., Epstein–Barr virus, rhinovirus, respiratory syncytial virus, adenovirus, and coronavirus), while 5%–36% of cases are caused by bacteria, for the most part, Group A beta-haemolytic streptococci (Ebell et al., 2000; Bird et al., 2014). In western medicine (WM), symptomatic and supportive treatment is the mainstay of viral cases, such as fluid rehydration, antipyretic analgesics, local anesthetics, and corticosteroids. Antibiotics are used as prescribed when there is a possibility of bacterial infection. Although pathogen detection and Centor score/McIsaac score are helpful in the pathogen diagnosis, it remains difficult to distinguish between a bacterial or viral etiology clinically (Bird et al., 2014; Windfuhr et al., 2016). Standard use of antibiotics, therefore, is difficult to achieve for clinicians, which may bring the promotion of bacterial resistance as well as adverse drug reactions. Additionally, antipyretic analgesics, anesthetics, or corticosteroids are classically restrained in some patient populations due to their certain side effects, such as non-steroidal anti-inflammatory drugs in gastrointestinal bleeding and opioids in airway compromise (Bird et al., 2014). Tonsillectomy, a way to deal with recurrent acute tonsillitis, also be restrained during the acute phase and has limited long-term benefits (Morad et al., 2017).

Compared with WM, Traditional Chinese medicine presents the following advantages in treating acute tonsillitis: multiple mechanisms of action (e.g., antimicrobial, anti-inflammatory, antipyretic, pain relief), few contraindications, and low-cost treatment (Fan et al., 2017). Chinese herbal injections (CHIs) are intravenous injections prepared by extracting the active ingredients of traditional Chinese medicine, with the characteristics of rapid onset and improved bioavailability. Research showed that contrasted with WM alone, CHI combined with WM has better clinical efficiency, including more preferable relief of symptoms and shorter disease duration, which may reduce the adverse drug reactions of WM and decrease the risk of antibiotic resistance (Zhou et al., 2020). However, there is a wide variety of CHIs used for acute tonsillitis with few studies comparing them. We thus initiated a network meta-analysis to achieve these comparisons.

METHODS

This study was conducted following the PRISMA extension statement (Hutton et al., 2015) with a PRISMA checklist which is provided in **Supplementary File S1**.

Search Strategy

We searched relevant databases including PubMed, Embase, Web of Science, Cochrane Library, China National Knowledge Infrastructure, Wanfang Database, Weipu Journal Database, and Chinese Biomedical Literature Database from database inception to 11 January 2022. The search strategies are provided in **Supplementary File S2**.

Study Selection

Only randomized controlled trials (RCTs) which targeted the treatment of CHIs to acute tonsillitis were included. In the selected studies, CHIs plus WM should be compared with WM alone or/and another type of CHIs plus WM. Notably, each group within one included trial received the same treatment regimen of WM. No limitations were defined by age, sex, or race whereas patients with concurrent acute tonsillitis and infections at other sites (e.g., pneumonia) were excluded. The outcome of interest included clinical effectiveness rate (proportion of patients improving after treatment), antipyretic time, sore throat relief time, red and swollen tonsils relief time, tonsillar exudate relief time, and adverse drug reactions (ADRs). A study was admitted according to the inclusion criteria independently by two

reviewers. Discrepancies were resolved by consensus between the two reviewers or arbitrated by a third reviewer.

Data Extraction and Quality Assessment

Data regarding trial information (title, first-author, publication year, sample size, trial duration, interventions, and control), population characteristics (sex, age, and consistency of baseline), reported outcomes (response rate in categorical variables and means/standard deviation in continuous variables), information on methodology (blinding, random methods, and measurement of each indicator), and sponsorship from pharmaceutical companies, were extracted by two independent reviewers using Excel 356 software. The reviewers further used Version 2 of the Cochrane risk-of-bias tool for randomized trials (RoB 2) to assess the risk of bias for each outcome of the included RCTs through the following aspects: randomization process, deviations from intended interventions, missing outcome data, measurement of the outcome, and selection of the reported result (Sterne et al., 2019). Any discrepancies were resolved by discussions between the two reviewers, and if necessary, by arbitration by a third reviewer.

Data Analysis

In this study, the program was analyzed by a random-effects network meta-analysis within a Bayesian framework (Salanti, 2012; Mavridis and Salanti, 2013). Based on four Monte Carlo Markov Chains, 200,000 in iterations and 10,000 in annealing were set. Risk Ratio (RR)with 95% confidence interval (CI) was calculated as pooled effect measure for categorical variables while pooled effect measures of continuous variables were expressed as Mean Differences (MD) with 95%CI. A league table was generated to present the comparisons between each pair of interventions within each outcome. Surface under the cumulative ranking area curves (SUCRA) with mean ranking probabilities were used to summarize treatment hierarchy (Dias et al., 2013). Additionally, node-splitting method was performed to assess the inconsistency of the model by separating evidence on a particular comparison into direct and indirect evidence in outcome(s) with at least one closed loop (van Valkenhoef et al., 2016). A heatmap was closely employed to measure the contribution degree of each pair of interventions for overall inconsistency. Moreover, Global I^2-statistic was used to evaluate the heterogeneity of the estimated effect size (Higgins and Thompson, 2002). Network meta-regression in the context of a Bayesian framework was further conducted to examine the potential modification effects for outcome(s) with significant heterogeneity. Furthermore, subgroup network meta-analyses and sensitivity analyses were conducted to assess the robustness of the results and deal with heterogeneity. Comparison-adjusted funnel plots and Egger's test were used to explore potential publication bias in the outcomes with greater than or equal to 10 RCTs (Begg and Mazumdar, 1994; Stuck et al., 1998).

Additionally, for a comprehensive assessment of treatment effect of CHIs + WM, a 5-dimensional K-means cluster analysis based on the SUCRA values of the selected CHIs + WM within each outcome (clinical effectiveness rate, antipyretic time, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time) was performed (Wan et al., 1988). Missing values were replaced with the mean of the SUCRA values of each outcome. The steps of clustering were as follows: 1) Randomly selected K objects in the data space as the initial cluster centers. 2) According to the Euclidean distances among the SUCRA values and the pre-set cluster centers, the SUCRA values were divided into the cluster center (category) closest to them. 3) A value of the objective function was calculated using the mean of the SUCRA values in each category. Determine whether the values of the pre-set cluster center and the objective function were consistent. If so, output the result; if not, return to the second step to continue the iteration. Subsequently, principal component analysis (Karhunen-Loeve Transform) was used to convert the results of the 5-dimensional K-means cluster analysis into three dimensions via mapping and then visualize the results on a 3-dimensional axis (Bro and Smilde, 2014).

All analyses were performed using R 4.1.2 (gemtc package: network meta-analysis, heterogeneity, inconsistency, network meta-regression, subgroup analysis, and sensitivity analysis; ggplot2 package: SUCRA graphs), STATA 14.0 (publication bias), and Python 3.10.4 (sklearn package: 5-dimensional K-means cluster analysis, principal component analysis; matplotlib package: visualization of the results of principal component analysis).

RESULTS

Study Characteristics

Overall, 869 records were retrieved, in which 110 trials were finally included in the current analysis according to the predesigned criteria (see **Supplementary File S3** for the citations of the included studies). A flow chart of the literature search is provided in **Supplementary File S4**. All the selected trials were two-arm studies with publication years from 1998 to 2021, involving nine kinds of CHIs: Reduning injection (RDN, 31 RCTs), Tanreqing injection (TRQ, 23 RCTs), Xiyanping injection (XYP, 36 RCTs), Yanhuning injection (YHN, seven RCTs), Chuanhuning injection (CHN, one RCTs), Qingkailing injection (QKL, three RCTs), Shuanghuanglian injection (SHL, four RCTs), Xuebijing injection (XBJ, three RCTs), and Yuxingcao injection (YXC, four RCTs) (see **Supplementary File S5** for characteristics of the included CHIs). A total of 12,152 patients were included in the entire analysis, of whom 6,772 were male patients (56.78%). Overall, 103 (93.64%), 48 (43.64%), 28 (25.45%), 18 (16.36%), 33 (30.00%), and 44 (40.00%) studies, separately, contributed to the six outcomes, i.e., clinical effectiveness rate, antipyretic time, sore throat relief time, red and swollen tonsils relief time, tonsillar exudate relief time, and ADRs. The selected trails possessed consistent baselines and treatment duration of them ranging from 2 to 10 days. The details of the selected RCTs are shown in **Supplementary File S6**. Of the 110 RCTs included, the connections among the interventions were visualized as a network diagram within each outcome. The network graphs are depicted in **Figure 1**, in which the size of node represents the sample size and the thickness of the line between nodes represents the volume of studies.

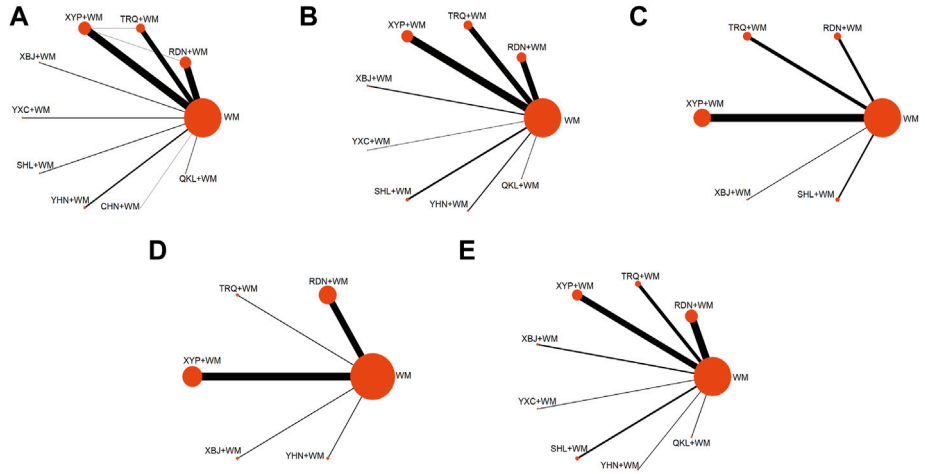


FIGURE 1 | Network graph of different interventions **(A)** Clinical effectiveness rate **(B)** Antipyretic time **(C)** Sore throat relief time **(D)** Red and swollen tonsils relief time **(E)** Tonsillar exudate relief time; WM, Western Medicine; RDN, Reduning injection; TRQ, Tanreqing injection; QKL, Qingkailing injection; XBJ, Xuebijing injection; SHL, Shuanghuanglian injection; YHN, Yanhuning injection; CHN, Chuanhuning injection; YXC, Yuxingcao injection; XYP, Xiyanping injection.

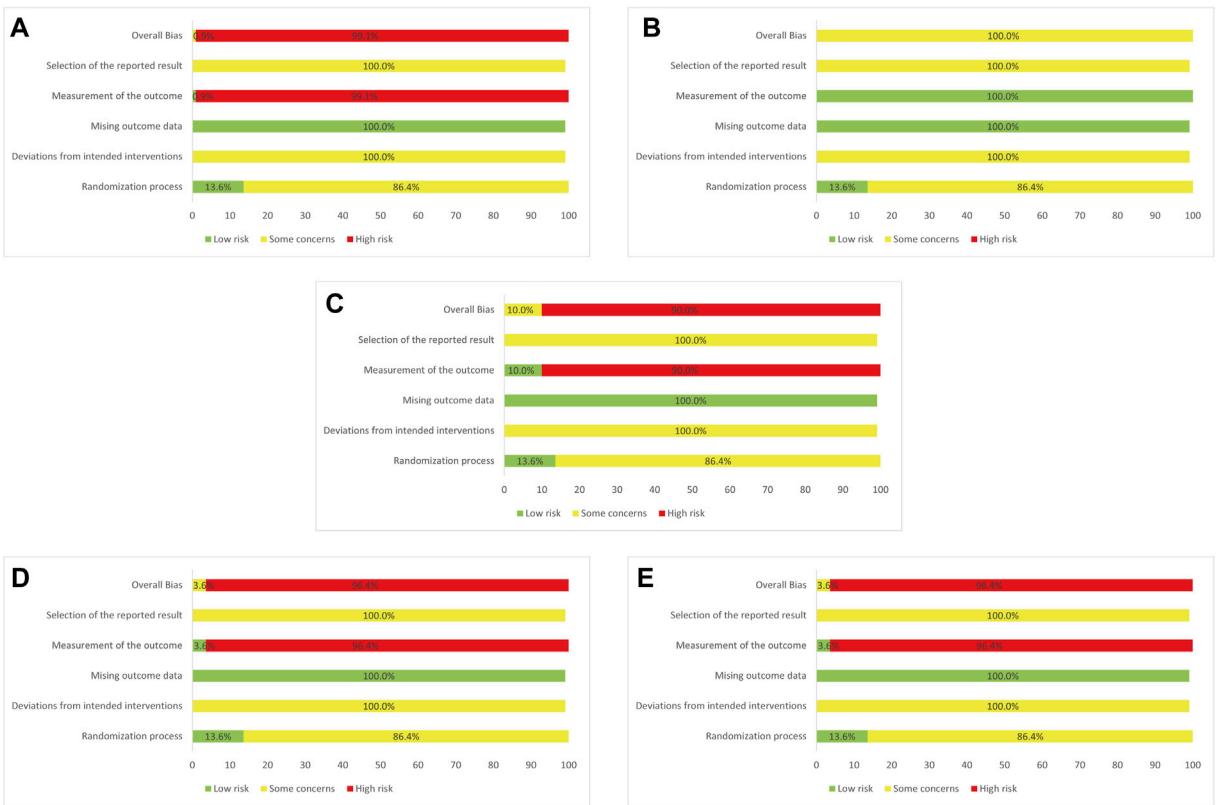


FIGURE 2 | Assessment of risk bias **(A)** Clinical effectiveness rate **(B)** Antipyretic time **(C)** Sore throat relief time **(D)** Red and swollen tonsils relief time **(E)** Tonsillar exudate relief time.

TABLE 1 | Relative effect sizes for each comparison.

Interventions	Clinical Effectiveness Rate, RR (95% CI)	Antipyretic Time, MD (95%CI)	Sore Throat Relief Time, MD (95%CI)	Red and Swollen Tonsils Relief Time, MD (95%CI)	Tonsillar Exudate Relief Time, MD (95%CI)
CHN + WM vs.					
QKL + WM	**0.02 (0.01, 0.62)**	—	—	—	—
RDN + WM	**0.02 (0.01, 0.71)**	—	—	—	—
SHL + WM	**0.02 (0.01, 0.70)**	—	—	—	—
TRQ + WM	**0.02 (0.01, 0.71)**	—	—	—	—
XBJ + WM	**0.02 (0.01, 0.63)**	—	—	—	—
XYP + WM	**0.02 (0.01, 0.68)**	—	—	—	—
YHN + WM	**0.02 (0.01, 0.69)**	—	—	—	—
YXC + WM	**0.02 (0.01, 0.70)**	—	—	—	—
WM	**0.02 (0.01, 0.81)**	—	—	—	—
QKL + WM vs.					
RDN + WM	**1.16 (0.98, 1.40)**	**−0.09 (−1.63, 1.45)**	—	—	**0.73 (−1.00, 2.46)**
SHL + WM	**1.13 (0.92, 1.41)**	**−0.28 (−1.95, 1.38)**	—	—	**0.22 (−1.70, 2.13)**
TRQ + WM	**1.15 (0.97, 1.39)**	**−0.53 (−2.07, 1.01)**	—	—	**−0.07 (−1.89, 1.75)**
XBJ + WM	**1.03 (0.83, 1.28)**	**−0.07 (−1.87, 1.73)**	—	—	1.66 (−0.36, 3.69)
XYP + WM	**1.10 (0.94, 1.33)**	**−0.54 (−2.06, 0.98)**	—	—	**0.24 (−1.50, 1.98)**
YHN + WM	**1.13 (0.94, 1.37)**	**−0.19 (−2.02, 1.64)**	—	—	**0.80 (−1.54, 3.14)**
YXC + WM	**1.13 (0.93, 1.39)**	**0.01 (−2.08, 2.08)**	—	—	**0.01 (−2.33, 2.34)**
WM	**1.32 (1.12, 1.59)**	**-1.67 (-3.15, -0.19)**	—	—	**−0.70 (−2.36, 0.96)**
RDN + WM vs.					
SHL + WM	0.98 (0.86, 1.10)	−0.19 (−1.08, 0.69)	0.04 (−0.82, 0.89)	—	−0.51 (−1.58, 0.56)
TRQ + WM	0.99 (0.95, 1.04)	−0.44 (−1.07, 0.18)	−0.22 (−0.88, 0.42)	−0.45 (−1.83, 0.94)	−0.79 (−1.69, 0.10)
XBJ + WM	0.89 (0.77, 1.00)	0.02 (−1.10, 1.14)	−0.40 (−1.56, 0.74)	−0.79 (−2.18, 0.61)	0.94 (−0.32, 2.21)
XYP + WM	**0.96 (0.92, 0.99)**	−0.44 (−1.02, 0.12)	−0.29 (−0.85, 0.27)	−0.53 (−1.20, 0.17)	−0.49 (−1.22, 0.25)
YHN + WM	0.97 (0.90, 1.05)	−0.10 (−1.26, 1.06)	—	0.51 (−0.96, 1.99)	0.07 (−1.65, 1.79)
YXC + WM	0.98 (0.88, 1.08)	0.09 (−1.44, 1.62)	—	—	−0.73 (−2.45, 1.00)
WM	**1.14 (1.11, 1.18)**	**−1.57 (−2.01, −1.15)**	**−1.40 (−1.88, −0.93)**	**−1.28 (−1.79, −0.79)**	**−1.43 (−1.92, −0.93)**
SHL + WM vs.					
TRQ + WM	1.02 (0.90, 1.16)	−0.25 (−1.14, 0.65)	−0.26 (−1.10, 0.58)	—	−0.28 (−1.49, 0.93)
XBJ + WM	0.91 (0.76, 1.08)	0.21 (−1.08, 1.51)	−0.44 (−1.71, 0.83)	—	1.45 (−0.04, 2.96)
XYP + WM	0.98 (0.87, 1.11)	−0.25 (−1.11, 0.61)	−0.33 (−1.09, 0.45)	—	0.02 (−1.07, 1.12)
YHN + WM	0.99 (0.87, 1.15)	0.10 (−1.22, 1.42)	—	—	0.58 (−1.32, 2.49)
YXC + WM	1.01 (0.86, 1.17)	0.29 (−1.37, 1.95)	—	—	−0.22 (−2.12, 1.69)
WM	**1.17 (1.04, 1.32)**	**−1.38 (−2.15, −0.61)**	**−1.44 (−2.15, −0.73)**	—	−0.92 (−1.86, 0.04)
TRQ + WM vs.					
XBJ + WM	0.89 (0.77, 1.01)	0.46 (−0.67, 1.59)	−0.18 (−1.32, 0.96)	−0.34 (−2.17, 1.50)	**1.73 (0.36, 3.12)**
XYP + WM	0.96 (0.92, 1.00)	0.01 (−0.59, 0.58)	−0.07 (−0.59, 0.47)	−0.09 (−1.44, 1.31)	0.30 (−0.62, 1.23)
YHN + WM	0.98 (0.90, 1.06)	0.34 (−0.82, 1.50)	—	0.96 (−0.93, 2.86)	0.86 (−0.95, 2.68)
YXC + WM	0.98 (0.88, 1.09)	0.53 (−1.01, 2.07)	—	—	0.06 (−1.74, 1.88)
WM	**1.15 (1.11, 1.19)**	**−1.13 (−1.59, −0.69)**	**−1.18 (−1.62, −0.73)**	−0.84 (−2.13, 0.45)	−0.64 (−1.38, 0.11)
XBJ + WM vs.					
XYP + WM	1.07 (0.95, 1.24)	**−1.13 (−1.5, −0.76)**	0.11 (−0.97, 1.21)	0.25 (−1.12, 1.66)	**−1.43 (−2.72, −0.15)**
YHN + WM	1.09 (0.95, 1.28)	**−1.13 (−1.5, −0.76)**	—	1.30 (−0.61, 3.20)	−0.87 (−2.88, 1.15)
YXC + WM	1.10 (0.94, 1.30)	**−1.13 (−1.5, −0.76)**	—	—	−1.66 (−3.69, 0.35)
WM	**1.28 (1.14, 1.48)**	**−1.6 (−2.64, −0.56)**	−1.00 (−2.05, 0.05)	−0.50 (−1.81, 0.80)	**−2.36 (−3.53, −1.21)**
XYP + WM vs.					
YHN + WM	1.02 (0.94, 1.09)	0.35 (−0.79, 1.49)	—	1.05 (−0.43, 2.49)	0.56 (−1.18, 2.29)
YXC + WM	1.03 (0.92, 1.13)	0.54 (−0.98, 2.05)	—	—	−0.24 (−1.98, 1.50)
WM	**1.20 (1.16, 1.23)**	**−1.13 (−1.50, −0.76)**	**−1.11 (−1.41, −0.82)**	**−0.75 (−1.24, −0.30)**	**−0.94 (−1.48, −0.40)**
YHN + WM vs.					
YXC + WM	1.01 (0.89, 1.13)	0.19 (−1.63, 2.01)	—	—	−0.80 (−3.13, 1.53)

(Continued on following page)

TABLE 1 | (*Continued*) Relative effect sizes for each comparison.

Interventions	Clinical Effectiveness Rate, RR (95% CI)	Antipyretic Time, MD (95%CI)	Sore Throat Relief Time, MD (95%CI)	Red and Swollen Tonsils Relief Time, MD (95%CI)	Tonsillar Exudate Relief Time, MD (95%CI)
WM	**1.17 (1.10, 1.26)**	**−1.48 (−2.55, −0.41)**	—	**−1.80 (−3.19, −0.41)**	−1.50 (−3.15, 0.15)
YXC + WM vs.					
WM	**1.17 (1.06, 1.29)**	**−1.67 (−3.14, −0.20)**	—	—	−0.70 (−2.35, 0.95)

Note: Bold RR/MD (95% CI) indicates a statistically significant difference; RR, risk ratio; MD, mean differences; 95% CI, 95% Confidence Interval; WM, western medicine; RDN, reduning injection; TRQ, tanreqing injection; QKL, qingkailing injection; XBJ, xuebijing injection; SHL, shuanghuanglian injection; YHN, yanhuning injection; CHN, chuanhuning injection; YXC, yuxingcao injection; XYP, xiyanping injection.

Methodological Quality and Risk of Bias Results

Regarding methodologies of the selected trials, 17 RCTs (15.45%) reported specific details of randomized approaches. Allocation concealment was reported in 13.64% of the cases, and these trials were evaluated as "low risk" in "randomization process". By contrast, no clear information was reported in all the trials about a predesigned protocol or appropriate analysis that was used to estimate the effect of assignment to intervention, which made both "selection of the reported result" and "deviation from intended interventions" rated as "some concerns". "Missing outcomes data" was generally a low risk of bias as all the outcomes were comprehensively described with specific number of patients involved in the assessment. Additionally, one RCT (0.91%) showed itself as a double-blind trial while three RCTs (3.60%) blinded trial performers and 10 RCTs (10.00%) blinded trial participants. The differences in blinding among the included trials arrived at the results that in "measurement of the outcome", severally, 99.1%, 0.00%, 90.00%, 96.4%, and 96.4% of the selected studies in clinical effectiveness rate, antipyretic time, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time were rated as "high risk" and thus this part of the studies were assessed as "high risk" in "overall bias" (see **Figure 2** for risk of bias assessment).

Network Meta-Analysis

Clinical Effectiveness Rate

Nine CHIs (QKL, RDN, SHL, TRQ, XBJ, XYP, YHN, YXC, and CHN) were involved in the evaluation of clinical effectiveness rate. An improved effect of clinical effectiveness rate was detected for all types of the included CHIs + WM (apart from CHN + WM) vs. WM, while CHN + WM obtained a worse effect than other interventions. XYP + WM improved clinical effectiveness rate as compared with RDN + WM. No such evident effect was observed in any other comparison (see **Table 1** for between-intervention differences). According to SUCRA, QKL + WM (89.51%), XBJ + WM (87.39%), and XYP + WM (69.15%) ranked first, second, and third, respectively, whereas RDN + WM (38.25%), WM (11.13%), and CHN + WM (0.03%) separately ranked eighth, ninth, and 10th. More details about SUCRA and its rank probability are individually shown in **Figure 3** and **Table 2**.

Antipyretic Time

Eight CHIs (QKL, RDN, SHL, TRQ, XBJ, XYP, YHN, and YXC) were involved in the evaluation of antipyretic time. In this clinical indicator, shortening was statistically significant for QKL + WM, RDN + WM, SHL + WM, TRQ + WM, XBJ + WM, XYP + WM, YHN + WM, and YXC + WM, as compared with WM. In addition, XBJ + WM was superior to XYP + WM, YHN + WM, and YXC + WM. No significant association was found with other comparators (see **Table 1** for between-intervention differences). Based on SUCRA, RDN + WM (68.48%), YXC + WM (66.37%), and QKL + WM (66.35%) ranked first, second, and third, respectively, whereas TRQ + WM (35.60%), XYP + WM (34.68%), and WM (0.42%) separately ranked seventh, eighth, and ninth. More details about SUCRA and its rank probability are individually shown in **Figure 3** and **Table 2**.

Sore Throat Relief Time

Five CHIs (RDN, SHL, TRQ, XBJ, and XYP) were involved in the evaluation of sore throat relief time. RDN + WM, SHL + WM, TRQ + WM, and XYP + WM statistically reduced sore throat relief time as compared with WM. No such evident effect was observed with other pairwise interventions (see **Table 1** for between-intervention differences). According to SUCRA, SHL + WM (76.82%), RDN + WM (76.77%), and TRQ + WM (54.58%) ranked first, second, and third, respectively, whereas XYP + WM (46.61%), XBJ + WM (44.62%), and WM (0.61%) severally ranked fourth, fifth, and sixth. More details about SUCRA and its rank probability are individually shown in **Figure 3** and **Table 2**.

Red and Swollen Tonsils Relief Time

Five CHIs (RDN, TRQ, XBJ, YHN, and XYP) were involved in the evaluation of red and swollen tonsils relief time. RDN + WM, XYP + WM, and YHN + WM showed a significant decrease in red and swollen tonsils relief time as compared with WM, whereas no significant association was found in any other comparison (see **Table 1** for between-intervention differences). According to SUCRA, YHN + WM (89.17%), RDN + WM (76.14%), and TRQ + WM (50.19%) ranked first, second, and third, respectively, whereas XYP + WM (44.63%), XBJ + WM (33.74%), and WM (6.12%) individually ranked fourth, fifth, and sixth. More details

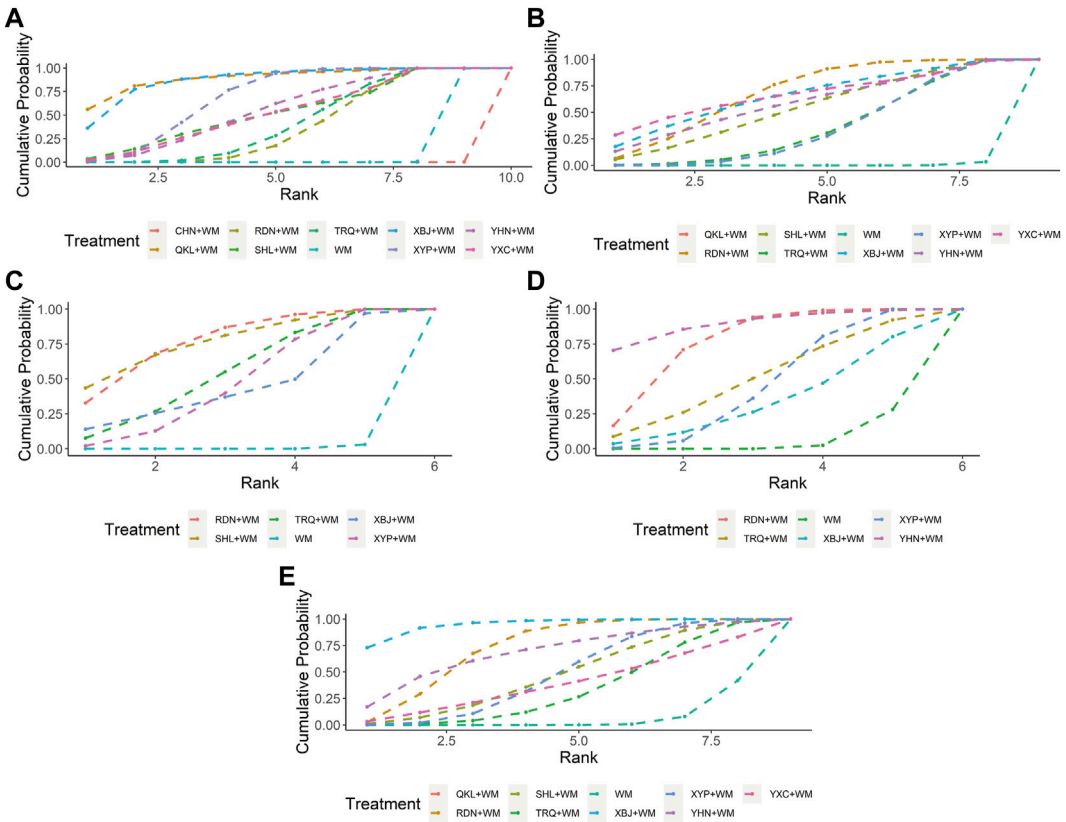


FIGURE 3 | Plots of the surface under the cumulative ranking curves for all interventions **(A)** Clinical effectiveness rate **(B)** Antipyretic time **(C)** Sore throat relief time **(D)** Red and swollen tonsils relief time **(E)** Tonsillar exudate relief time; WM, Western Medicine; RDN, Reduning injection; TRQ, Tanreqing injection; QKL, Qingkailing injection; XBJ, Xuebijing injection; SHL, Shuanghuanglian injection; YHN, Yanhuning injection; CHN, Chuanhuning injection; YXC, Yuxingcao injection; XYP, Xiyanping injection.

about SUCRA and its rank probability are individually shown in **Figure 3** and **Table 2**.

Tonsillar Exudate Relief Time

Eight CHIs (QKL, RDN, SHL, TRQ, XBJ, XYP, YHN, and YXC) were involved in the appraisal of tonsillar exudate relief time. RDN + WM, XBJ + WM, and XYP + WM were associated with a significant reduction in tonsillar exudate relief time as compared with WM. In comparison with XBJ + WM, both TRQ + WM and XYP + WM obtained a worse effect (see **Table 1** for between-intervention differences). According to SUCRA, XBJ + WM (94.82%), RDN + WM (73.02%), and YHN + WM (68.92%) ranked first, second, and third, respectively, whereas YXC + WM (39.03%), TRQ + WM (33.54%), and WM (6.31%) separately ranked seventh, eighth, and ninth. More details about SUCRA and its rank probability are individually shown in **Figure 3** and **Table 2**.

Adverse Drug Reactions

ADRs were monitored in 44 RCTs (40.00%), of which 24 studies (21.82%) reported the number of affected patients in detail whereas 20 studies (18.18%) presented no ADRs during the treatment. No ADRs were observed in the reported 52 patients using XBJ (0.00%). The ADRs rate for RDN, TRQ, XYP, YHN, YXC, QKL, and SHL were 3.11%, 0.88%, 2.99%, 3.08%, 2.78%, 4.29%, and 4.62%, respectively, without fatal reactions. The ADRs are further detailed in **Table 3**.

5-Dimensional K-Means Cluster Analysis

A 5-dimensional K-means cluster analysis was conducted to comprehensively compare the effects of the interventions on the five outcomes (clinical effectiveness rate, antipyretic time, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time). The results reduced to three dimensions by principal component analysis are shown in **Figure 4**. Upon visual inspection, all interventions were clustered into three categories, in which XBJ + WM, RDN + WM, and YHN + WM were classified as a category with optimal treatment effect while WM alone was as a category with worst curative effect.

Inconsistency, Heterogeneity, and Publication Bias

Assessment of inconsistency by node-splitting method indicated that inconsistency was not detected in clinical effectiveness rate as *p*-values in all the comparisons were greater than 0.05 (**Supplementary File S7**). The heatmap revealed that the

TABLE 2 | Ranking probabilities of surface under the cumulative ranking area curves (SUCRA) for five outcomes.

Interventions	Clinical Effectiveness Rate (%)	Antipyretic Time (%)	Sore Throat Relief Time (%)	Red and Swollen Tonsils Relief Time (%)	Tonsillar Exudate Relief Time (%)
QKL + WM	89.51	66.35			39.07
RDN + WM	38.25	68.48	76.77	76.14	73.02
SHL + WM	53.37	53.81	76.82		47.25
TRQ + WM	42.15	35.60	54.58	50.19	33.54
XBJ + WM	87.39	65.45	44.62	33.74	94.82
XYP + WM	69.15	34.68	46.61	44.63	48.04
YHN + WM	56.08	58.84		89.17	68.92
YXC + WM	52.95	66.37			39.03
CHN + WM	0.03				
WM	11.13	0.42	0.61	6.12	6.31

Note: WM, western medicine; RDN, reduning injection; TRQ, tanreqing injection; QKL, qingkailing injection; XBJ, xuebijing injection; SHL, shuanghuanglian injection; YHN, yanhuning injection; CHN, chuanhuning injection; YXC, yuxingcao injection; XYP, xiyanping injection.

pooled effect size in "RDN + WM vs. XYP + WM" had the greatest contribution to the inconsistency of clinical effectiveness rate (see **Supplementary File S8** for contribution degree of inconsistency). Regarding heterogeneity, Global I^2-statistic was 18.07%, 96.54%, 94.12%, 95.96%, and 96.63% for clinical effectiveness rate, antipyretic time, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time, individually. Upon visual inspection, the funnel plots showed unremarkable asymmetry on both sides of the centerline, which did not suggest a significant risk of publication bias in our sample of the included studies. Nevertheless, quantitative detection of publication bias (Egger's test) demonstrated that the p value for antipyretic time was 0.006 (<0.05), while the p values for clinical effectiveness rate, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time were respectively 0.434, 0.360, 0.424 and 0.400, suggesting that there were small-study effects in the outcome of antipyretic time. The funnel plots are shown in **Figure 5** and the results of Egger's test are provided in **Supplementary File S9**.

Meta-Regression Analyses, Sensitivity Analyses, and Subgroup Analyses

Since there are statistical heterogeneities according to the Global I^2, four outcomes (antipyretic time, sore throat relief time, red and swollen tonsils relief time, and tonsillar exudate relief time) were analyzed by network meta-regression with patients' age and publication year as the covariates. The results suggested that red and swollen tonsils relief time would decrease by 0.7 days per year's change in patients' age, whereas the covariates were not statistically significant in the remaining outcomes (**Supplementary File S10**). Sensitivity analysis, a network meta-analysis in selected studies published after 2010, indicated that the overall results were robust (**Supplementary File S11**). Subgroup analyses were performed according to patients' age, tonsil suppuration, and treatment regimen of WM. As the number of studies targeting adult patients, patients without suppurative tonsillitis, and patients treated with WM apart from penicillins/cephalosporins was too small to achieve subgroup analyses, the subgroup analyses were finally conducted in studies with pediatric patients, patients with suppurative tonsillitis, patients treated by penicillins, and patients received cephalosporins as the treatment regimen of WM. In the subgroup of children, compared to the overall results, XBJ + WM ranked first in clinical effectiveness rate (78.30%) while QKL + WM was not included in this outcome analysis (**Supplementary File S12**). The subgroup of patients with suppurative tonsillitis demonstrated that, as compared with the overall results, XBJ + WM ranked first (94.87%) in decreasing antipyretic time (**Supplementary File S13**). Compared to the overall results, the subgroup for patients who received penicillins as the treatment regimen of WM indicated that XYP + WM ranked first (81.19%) in reducing red and swollen tonsils relief time and YHN + WM possessed the highest-ranking probability (89.78%) in decreasing tonsillar exudate relief time, while XBJ + WM was not included in the tonsillar exudate relief time analysis (**Supplementary File S14**). In the subgroup for patients who received cephalosporins as the treatment regimen of WM, as compared with the overall results, XBJ + WM ranked ahead of other interventions in the outcomes of clinical effectiveness rate (99.35%) and antipyretic time (95.41%); TRQ + WM ranked first (77.97%) in reducing sore throat relief time; RDN + WM has the highest-ranking probability (85.13%) in decreasing red and swollen tonsils relief time; SHL + WM and YHN + WM were individually not included in the sore throat relief time analysis and red and swollen tonsils relief time analysis (**Supplementary File S15**).

DISCUSSION

In the theory of Traditional Chinese medicine, acute tonsillitis is classified as acute nippled moth, which is predominantly caused by pathogenic qi that is associated with heat-toxicity (Gao et al., 2017). Therefore, in the position of the Chinese medicine theory, the main strategy of treating acute tonsillitis is to clear heat and detoxify (Gao et al., 2017). In the current study, all the included CHIs have the efficacy of clearing heat or detoxifying and thus are used for the treatment of acute

TABLE 3 | Details of adverse drug reactions.

	Reduning Injection	Tanreqing Injection	Xiyanping Injection	Yanhuning Injection	Yuxingcao Injection	Qingkailing Injection	Shuanghuanglian Injection
Nausea and vomiting	1.29% (10/773)		1.03% (11/1067)		2.78% (2/72)	1.43% (1/70)	3.08% (2/65)
Diarrhea	0.91% (7/773)	0.44% (1/277)	0.47% (5/1067)				
Dizziness	0.26% (2/773)		0.37% (4/1067)				
Rash	0.65% (5/773)	0.44% (1/277)	1.03% (11/1067)	3.08% (2/65)		2.86% (2/70)	1.54% (1/65)
Chills			0.09% (1/1067)				
Total	3.11% (24/773)	0.88% (2/277)	2.99% (32/1067)	3.08% (2/65)	2.78% (2/72)	4.29% (3/70)	4.62% (3/65)

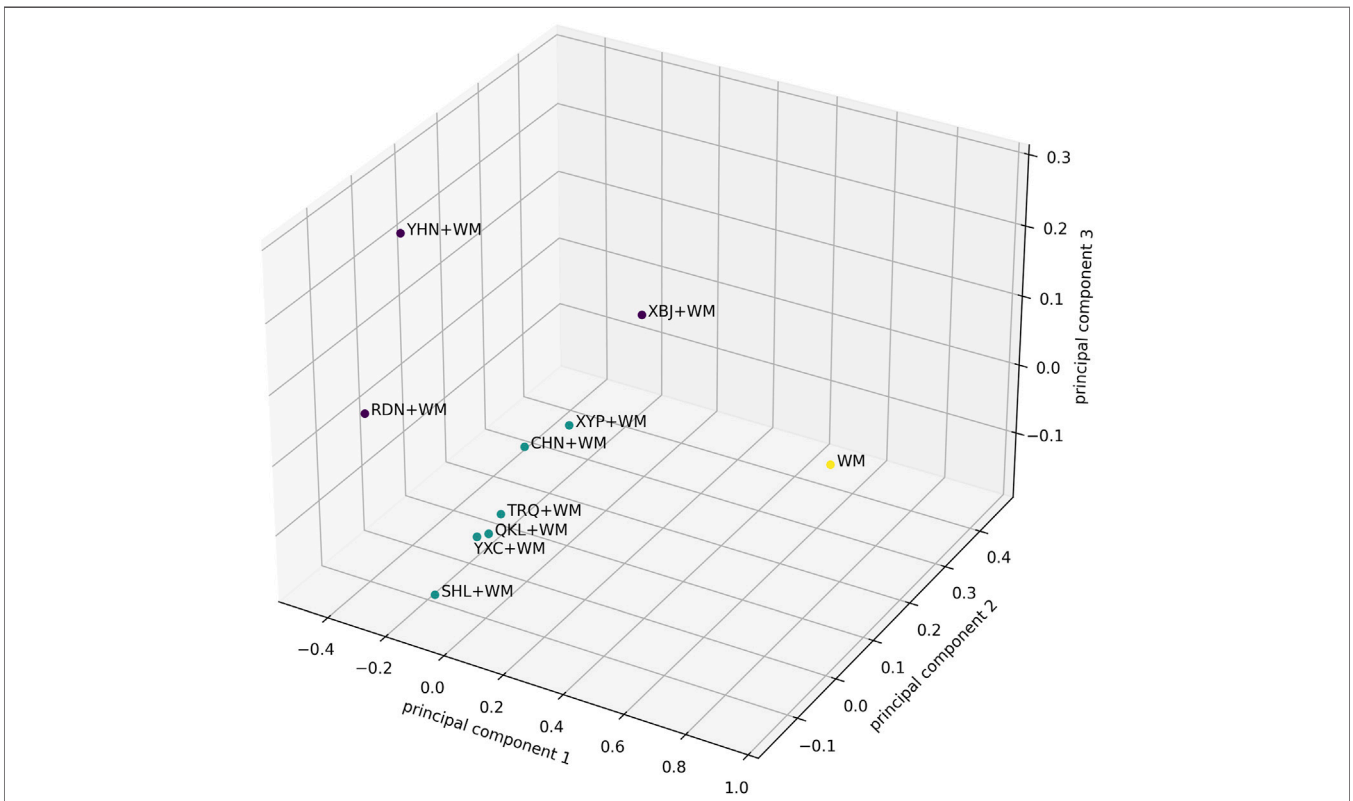


FIGURE 4 | The results of the 5-Dimensional K-means cluster analysis were reduced to three dimensions by principal component analysis. The purple dots represent the best category of curative effect, while the green dots are the second and the yellow dots are the worst. The axes represent the three principal components in the principal component analysis.

tonsillitis clinically. Among the CHIs, this network meta-analysis (SUCRA) suggested that QKL, XBJ, RDN, SHL, and YHN might have potential advantages in treating the disease, in which XBJ, RDN, and YHN deserved more attention based on the cluster analysis. Simultaneously, XBJ may be the optimal CHI for acute tonsillitis considering ADRs.

According to our findings, QKL + WM showed good performance in improving clinical effectiveness rate as well as resolving fever through pairwise comparison and ranking probability. QKL is mainly prepared from baicalin, *Isatis tinctoria* L [Brassicaceae], *Lonicera japonica* Thunb [Caprifoliaceae], and *Gardenia jasminoides* J.Ellis [Rubiaceae]. Similar to our study, some studies found that QKL has a significant antipyretic effect, which was associated with repairing the perturbed pathways of lipid metabolism and amino acid metabolism (Gao et al., 2013; Qin et al., 2016). An *in vitro* experiment confirmed an inhibitory efficacy on resistant bacteria containing blaNDM-1 by QKL and especially its active ingredient, baicalin; an experiment also indicated the significant inhibition of QKL plus antibiotics to multidrug-resistant bacteria (Shang et al., 2013). Besides, QKL was confirmed to possess its potent reduction in inhibiting damage caused by infection, e.g.,

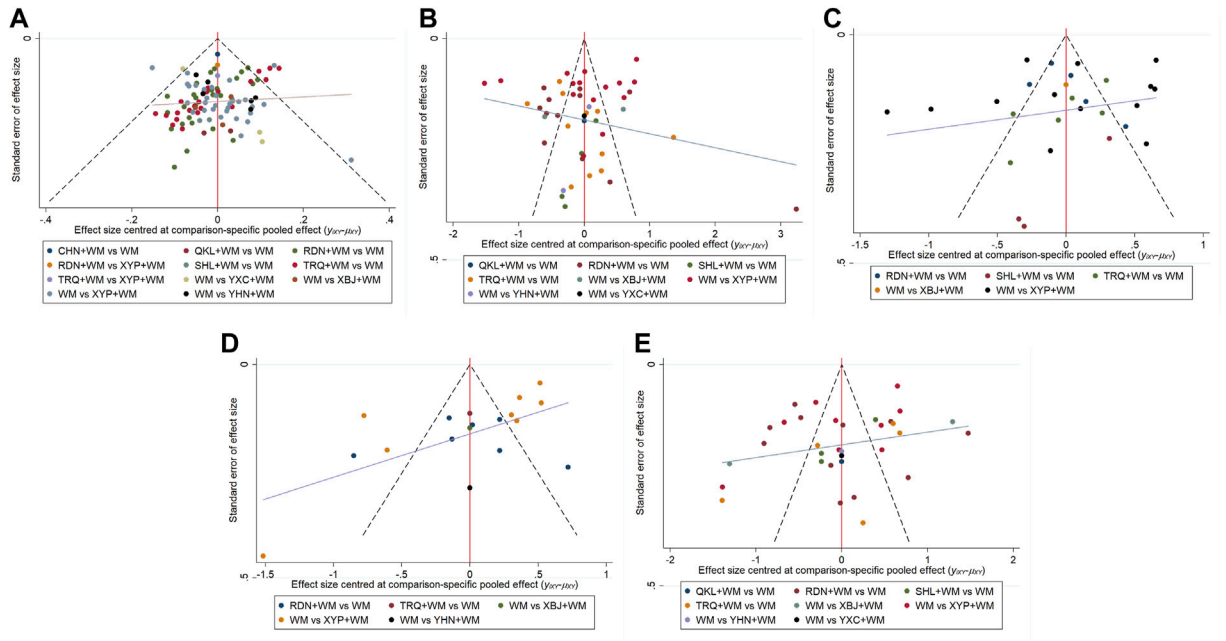


FIGURE 5 | Funnel plots **(A)** Clinical effectiveness rate **(B)** Antipyretic time **(C)** Sore throat relief time **(D)** Red and swollen tonsils relief time **(E)** Tonsillar exudate relief time.

leucopenia and thrombocytopenia (Yi et al., 2021). These pharmacological mechanisms may be tied to the efficacies of QKL to acute tonsillitis.

Apart from QKL, the injections that were prepared from traditional heat-clearing and detoxifying Chinese herbs in our study also included RDN, SHL, and YHN. We found that RDN + WM, SHL + WM, and YHN + WM exerted superior effects in lowering body temperature, shortening sore throat relief time, and reducing red and swollen tonsils relief time, separately. The antipyretic mechanism of RDN might be related to the regulation of biosynthesis as well as sphingolipid metabolism of valine, leucine, and isoleucine (Gao et al., 2020). In addition, RDN was reported to possess anti-inflammatory and antiviral effects (Cao et al., 2015; Xie et al., 2020; Xu et al., 2021), which might work in treating acute tonsillitis. SHL is made from active ingredients of *Forsythia suspensa* (Thunb.) Vahl [Oleaceae], *Lonicera japonica* Thunb [Caprifoliaceae], and *Scutellaria baicalensis* Georgi [Lamiaceae]. Under some *in vitro* and *in vivo* experiments, the injection also had benefit of inhibiting viruses, in which the pathogens might be the perpetrator of acute tonsillitis, e.g., SARS-CoV-2 and influenza A virus H5N1(Tang et al., 2018; Su et al., 2020). Moreover, SHL could inhibit NF-kappaB-mediated production of pro-inflammatory cytokines and chemokines, thereby reducing the inflammatory response to microbial infection (Chen et al., 2002). YHN originates from *Andrographis paniculata* (Burm.f.) Nees [Acanthaceae], a Chinese herbal medicine possessing primary effects of clearing heat and detumescence. Pharmacological research showed that YHN has strong inhibitory effects on respiratory syncytial virus, Coxsackie virus, Epstein-Barr virus, and rotavirus (Liu et al., 2007; Han, 2012; Guan and Cao, 2013; Huang et al., 2013), among which some viruses might cause acute tonsillitis. Additionally, YHN has therapeutic effects on SD rats with upper respiratory tract infection modeled by beta-hemolytic *streptococcus* via inhibiting the expression of IL-1β, IL-6β, and TNF-α(Liang et al., 2012), while beta-hemolytic *streptococcus* is the main bacterium causing acute tonsillitis.

Unlike the CHIs mentioned above consisting of heat-clearing and detoxifying Chinese herbs as raw materials, XBJ, another included intravenous Chinese medicine preparation, derived from a traditional formulation called "*Xuefuzhuyu* Decoction" which does not contain any heat-clearing and detoxifying Chinese herb but Chinese herb activating blood circulation and removing stasis, whereas has the functions of dispelling blood stasis and detoxification. Pharmacological analysis research had demonstrated that the main constituents of XBJ including paeoniflorin, senkyunolide I, safflor yellow A, danshensu, uridine, rosmarinic acid, beta-ocimene-X, gallic acid, protocatechualdehyde, hydroxysafflor yellow A, and oxypaeoniflorin, etc. via ultra-high-performance liquid chromatography (Ji et al., 2010; Jiang et al., 2013), in which the active ingredients play anti-infection and immunomodulatory effects by acting on targets/pathways such as COX-2, IKK-2, 5-LOX, NF-κB, MAPK, eNOS, iNOS, A2AR, and MIF(Ma et al., 2009; Jiang et al., 2013). These pharmacological mechanisms may be related to the treatment of acute tonsillitis with XBJ. Indeed, XBJ has played

a vital role in treating sepsis or septic shock as a result of its anti-inflammatory effect as well as immunomodulatory function, and thus the injection has been included in the treatment guidelines of sepsis in China (Branch, 2015). In clinical, XBJ has also been used to treat acute tonsillitis, a disease that is classified as an infectious disease as sepsis. As indicated in our study, XBJ was revealed as the potential optimal CHIs in shortening tonsillar exudate relief time and possibly even the best CHI for the comprehensive treatment of acute tonsillitis, which was consistent with the results of a previous network meta-analysis targeting CHIs plus WM in the treatment of acute tonsillitis in children (involving 65 RCTs as well as six CHIs). In that study, XBJ possessed the highest-ranking probability regarding antipyretic time, sore throat relief time, and red and swollen tonsils relief time, whereas had a similar ranking for clinical effectiveness rate and tonsillar exudate relief time with our subgroup analyses of children (Zhou et al., 2020).

In addition to clinical efficacy, the adverse reactions of CHIs are also attention-worthy. In the current study, we reported both the incidence and types of ADRs for seven CHIs. Although the studies we included did not monitor the occurrence of fatal ADRs, the safety of CHIs remains a concern; how to reduce the occurrence of ADRs in CHIs deserves our attention. Risk factors for ADRs in CHIs, in this case, may provide some recommendations. A retrospective study showed that ADRs are more likely to occur in children or combine with cephalosporin when using QKL (Wu et al., 2018). In addition, ADRs to XBJ are related to vehicle type, dosage, older age, and drug combination (e.g., reduced glutathione, aspirin-DL-lysine, and torsemide) (Wang et al., 2019), while the history of drug allergy, abnormal liver and kidney function, traditional Chinese medicine dialectical medication, dispensing time, drip rate, and drug combination might play roles in ADRs of SHL through multi-factor analysis (Pang and Zhang, 2018). Besides, children are a high-risk group for ADRs with YHN, and off-label drug use is responsible for ADRs in RDN (Huang, 2018; Yu et al., 2019). Anyhow, CHIs should be used more regulated and cautiously, especially in children.

Strength and Limitation

The major strength of the current study included comprehensive search strategies and analyses. Furthermore, we performed sensitivity analyses to assess the robustness of the results and carried out network meta-regression as well as subgroup analyses to address the heterogeneity of the selected studies. Meanwhile, a 5-dimensional K-means cluster analysis was employed to comprehensively compare the treatment effects of the selected CHIs on the five outcomes. However, some limitations to this study should be mentioned. First, all the outcomes were rated as "high risk" and "some concerns", for which the results should be interpreted cautiously. Secondary, the WM treatment regimens of the included studies were inconsistent; hence, the results should be interpreted with caution. Third, all the studies were conducted in China and the results may not be generalizable. Finally, several CHIs were associated with small numbers of RCTs (CHN, one RCTs; QKL, three RCTs; SHL, four RCTs; XBJ, three RCTs; YXC, four RCTs) and the interpretation of the results might be restricted.

CONCLUSION

CHIs combined with WM have more favorable effects than WM alone in treating acute tonsillitis. QKL, XBJ, RDN, SHL, and YHN deserve more attention when facing patients with acute tonsillitis. Taking ADRs into consideration, XBJ was probably the best CHI for the disease. More evidence, however, is required to support these suggestions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PH, and BC done conception and design of the study. YF and SL performed the literature search, screening, and extraction. XH, YC, and YD verified the data. QW and HZ evaluated the quality of the included RCTs. PH, YL, BH, LC, HG, ZZ, and LZ performed the network meta-analysis. PH wrote the original draft. BC and SZ interpreted the results, incorporated comments for the co-authors, and finalized the manuscript. All the authors approved the final version of the manuscript.

FUNDING

This work was funded by the National Natural Science Foundation of China (Grant NO.81273961 and NO.81303117), Science and Technology Foundation of Shenzhen City (Grant No. JCYJ20190812164009243), and Guangdong Medical Research Foundation (Grant No. B2020135).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.888073/full#supplementary-material

REFERENCES

Bartlett, A., Bola, S., and Williams, R. (2015). Acute Tonsillitis and its Complications: An Overview. *J. R. Nav. Med. Serv.* 101 (1), 69–73. doi:10.1136/jrnms-101-69

Begg, C. B., and Mazumdar, M. (1994). Operating Characteristics of a Rank Correlation Test for Publication Bias. *Biometrics* 50 (4), 1088–1101. doi:10.2307/2533446

Bird, J. H., Biggs, T. C., and King, E. V. (2014). Controversies in the Management of Acute Tonsillitis: An Evidence-Based Review. *Clin. Otolaryngol.* 39 (6), 368–374. doi:10.1111/coa.12299

Branch, C. M. A. I. M. (2015). Guidelines for the Treatment of Severe Sepsis/Septic Shock in China (2014). *Chin. Crit. Care Med.* 27 (6), 401–426. doi:10.3760/j.issn.2095-4352.2015.06.001

Bro, R., and Smilde, A. K. (2014). Principal Component Analysis. *Anal. Methods* 6 (9), 2812–2831. doi:10.1039/C3AY41907J

Cao, Z. Y., Chang, X. J., Zhao, Z. P., Cao, L., and Xiao, W. (2015). Antiviral Effects of Reduning Injection against Enterovirus 71 and Possible Mechanisms of Action. *Chin. J. Nat. Med.* 13 (12), 881–888. doi:10.1016/s1875-5364(15)30093-5

Chen, X., Howard, O. M., Yang, X., Wang, L., Oppenheim, J. J., and Krakauer, T. (2002). Effects of Shuanghuanglian and Qingkailing, Two Multi-Components of Traditional Chinese Medicinal Preparations, on Human Leukocyte Function. *Life Sci.* 70 (24), 2897–2913. doi:10.1016/s0024-3205(02)01541-2

Dias, S., Sutton, A. J., Ades, A. E., and Welton, N. J. (2013). Evidence Synthesis for Decision Making 2: A Generalized Linear Modeling Framework for Pairwise and Network Meta-Analysis of Randomized Controlled Trials. *Med. Decis. Mak.* 33 (5), 607–617. doi:10.1177/0272989x12458724

Ebell, M. H., Smith, M. A., Barry, H. C., Ives, K., and Carey, M. (2000). The Rational Clinical Examination. Does This Patient Have Strep Throat? *JAMA* 284 (22), 2912–2918. doi:10.1001/jama.284.22.2912

Fan, C. Z., Miao, Q., Zhang, Q., Fan, M. R., Liao, X., Liu, J., et al. (2017). Advantages and Evidence of Chinese Medicine in Prevention and Treatment of Adult Acute Tonsillitis. *Zhongguo Zhong Yao Za Zhi* 42 (8), 1430–1438. doi:10.19540/j.cnki.cjcmm.2017.0039

Gao, G., Jin, J., Liu, N., Zhao, P., Wang, C., and Liu, Q. (2017). The Progress of Traditional Chinese Medicine in the Treatment of Acute Tonsillitis in Children. *J. Emerg. Traditional Chin. Med.* 26 (02), 268–270. doi:10.3969/j.issn.1004-745X.2017.02.026

Gao, X., Guo, M., Peng, L., Zhao, B., Su, J., Liu, H., et al. (2013). UPLC Q-TOF/MS-Based Metabolic Profiling of Urine Reveals the Novel Antipyretic Mechanisms of Qingkailing Injection in a Rat Model of Yeast-Induced Pyrexia. *Evid. Based Complement. Altern. Med.* 2013, 864747. doi:10.1155/2013/864747

Gao, X., Huang, C., Geng, T., Chen, X., Wang, J., Liu, J., et al. (2020). Serum and Urine Metabolomics Based on UPLC-Q-TOF/MS Reveals the Antipyretic Mechanism of Reduning Injection in a Rat Model. *J. Ethnopharmacol.* 250, 112429. doi:10.1016/j.jep.2019.112429

Guan, Y., and Cao, S. (2013). Observation on the Curative Effect of Yanhuning Injection in the Treatment of Rotavirus Diarrhea in Children. *Chin. Med. Innov.* 10 (7), 2. doi:10.3969/j.issn.1674-4985.2013.07.012

Han, H. (2012). In Andrographolide Study on the Effect of Ribavirin Reducing the CVA16 Replication. *J. Community Med.* 10 (12), 4. CNKI:SUN:SQYX.0.2012-12-016.

Higgins, J. P., and Thompson, S. G. (2002). Quantifying Heterogeneity in a Meta-Analysis. *Stat. Med.* 21 (11), 1539–1558. doi:10.1002/sim.1186

Huang, K., Xu, D., Cao, Y., Ma, F., Zhang, G., Liu, R., et al. (2013). Comparison of the Effects of Recombinant Human Interferon α1b, Yanhuning and Xiyanping against Respiratory Syncytial Virus Infection. *Chin. J. Exp. Clin. Infect. Dis. (Electronic Version)* 7 (6), 5. doi:10.3877/cma.j.issn.1674-1358.2013.06.004

Huang, S. (2018). Related Factors and Countermeasures of Adverse Reactions in Yanhuning Injection. *Smart Healthc.* 4 (26), 2. doi:10.19335/j.cnki.2096-1219.2018.26.061

Hutton, B., Salanti, G., Caldwell, D. M., Chaimani, A., Schmid, C. H., Cameron, C., et al. (2015). The PRISMA Extension Statement for Reporting of Systematic Reviews Incorporating Network Meta-Analyses of Health Care Interventions: Checklist and Explanations. *Ann. Intern Med.* 162 (11), 777–784. doi:10.7326/m14-2385

Ji, L., Huang, H., Jiang, M., Bai, G., and Luo, G. (2010). Simultaneous HPLC Determination of 11 Essential Compounds in Xuebijing Injection. *China J. Chin. Materia Medica* 35 (18), 2395–2398. doi:10.4268/cjcmm20101807

Jiang, M., Zhou, M., Han, Y., Xing, L., Zhao, H., Dong, L., et al. (2013). Identification of NF-κB Inhibitors in Xuebijing Injection for Sepsis Treatment Based on Bioactivity-Integrated UPLC-Q/TOF. *J. Ethnopharmacol.* 147 (2), 426–433. doi:10.1016/j.jep.2013.03.032

Kocher, J. J., and Selby, T. D. (2014). Antibiotics for Sore Throat. *Am. Fam. Physician* 90 (1), 23–24.

Liang, L., Pu, J., Gao, H., and Tai, G. (2012). Effect of Yanhuning Injection on Animal Models with Acute Pharyngitis and its Mechanism. *Drug Eval. Res.* 35 (003), 165–168. CNKI:SUN:YWPJ.0.2012-03-007.

Liu, X., Zhan, S., Wang, Q., Ye, S., Zhou, L., Li, L., et al. (2007). Comparative Study on the Inhibition of EB Virus Antigen Expression by Common Andrographis Herb Extract versus Aciclovir. *Chin. J. Infect. Chemother.* 7 (6), 3. doi:10.3321/j.issn:1009-7708.2007.06.015

Ma, S.-T., Liu, P.-X., Long, W., Yu, J., and Xu, Y. (2009). Effects of the Multi-Target Capability of Xuebijing and its Inflammatory Pharmacodynamic Material Basis. *Acta Physico-Chimica Sin.* 25 (10), 2080–2086. doi:10.3866/PKU.WHXB20090907

Mavridis, D., and Salanti, G. (2013). A Practical Introduction to Multivariate Meta-Analysis. *Stat. Methods Med. Res.* 22 (2), 133–158. doi:10.1177/0962280211432219

Morad, A., Sathe, N. A., Francis, D. O., McPheeters, M. L., and Chinnadurai, S. (2017). Tonsillectomy Versus Watchful Waiting for Recurrent Throat Infection: A Systematic Review. *Pediatrics* 139 (2), e20163490. doi:10.1542/peds.2016-3490

Pang, S., and Zhang, X. (2018). Incidence of Adverse Reactions of Traditional Chinese Medicine Injection and its Influencing Factors. *Int. Med. Health Guid. News* 024 (020), 3179–3182. doi:10.3760/cma.j.issn.1007-1245.2018.20.042

Qin, L., Zhang, Z., Guo, M., Zhang, Q., Wang, Q., Lu, Z., et al. (2016). Plasma Metabolomics Combined with Lipidomics Profiling Reveals the Potential Antipyretic Mechanisms of Qingkailing Injection in a Rat Model. *Chem. Biol. Interact.* 254, 24–33. doi:10.1016/j.cbi.2016.05.022

Salanti, G. (2012). Indirect and Mixed-Treatment Comparison, Network, or Multiple-Treatments Meta-Analysis: Many Names, Many Benefits, Many Concerns for the Next Generation Evidence Synthesis Tool. *Res. Synth. Methods* 3 (2), 80–97. doi:10.1002/jrsm.1037

Shang, W., Wang, X. S., Zou, D. Y., Zhang, Z. N., Liao, X. R., and Yuan, J. (2013). Antimicrobial Effects of Qingkailing Injection Extract and Combination Therapy of Qingkailing Injection and Antibiotics on Bacteria Carrying blaNDM-1 Resistance Gene. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 33 (4), 506–509. CNKI:SUN:ZZXJ.0.2013-04-024.

Sidell, D., and Shapiro, N. L. (2012). Acute Tonsillitis. *Infect. Disord. Drug Targets* 12 (4), 271–276. doi:10.2174/187152612801319230

Sterne, J. A. C., Savović, J., Page, M. J., Elbers, R. G., Blencowe, N. S., Boutron, I., et al. (2019). RoB 2: A Revised Tool for Assessing Risk of Bias in Randomised Trials. *BMJ* 366, l4898. doi:10.1136/bmj.l4898

Stuck, A. E., Rubenstein, L. Z., and Wieland, D. (1998). Bias in Meta-Analysis Detected by a Simple, Graphical Test. Asymmetry Detected in Funnel Plot Was Probably Due to True Heterogeneity. *BMJ* 316 (7129), 469–461. doi:10.1136/bmj.316.7129.469

Su, H. X., Yao, S., Zhao, W. F., Li, M. J., Liu, J., Shang, W. J., et al. (2020). Anti-SARS-CoV-2 Activities *In Vitro* of Shuanghuanglian Preparations and Bioactive Ingredients. *Acta Pharmacol. Sin.* 41 (9), 1167–1177. doi:10.1038/s41401-020-0483-6

Tang, Y., Wang, Z., Huo, C., Guo, X., Yang, G., Wang, M., et al. (2018). Antiviral Effects of Shuanghuanglian Injection Powder against Influenza a Virus H5N1 *In Vitro* and *In Vivo*. *Microb. Pathog.* 121, 318–324. doi:10.1016/j.micpath.2018.06.004

van Valkenhoef, G., Dias, S., Ades, A. E., and Welton, N. J. (2016). Automated Generation of Node-Splitting Models for Assessment of Inconsistency in Network Meta-Analysis. *Res. Synth. Methods* 7 (1), 80–93. doi:10.1002/jrsm.1167

Wan, S. J., Wong, S. K. M., and Prusinkiewicz, P. (1988). An Algorithm for Multidimensional Data Clustering. *ACM Trans. Math. Softw.* 14 (2), 153–162. doi:10.1145/45054.45056

Wang, C., Shi, Q. P., Ding, F., Jiang, X. D., Tang, W., Yu, M. L., et al. (2019). Reevaluation of the Post-Marketing Safety of Xuebijing Injection Based on Real-World and Evidence-Based Evaluations. *Biomed. Pharmacother.* 109, 1523–1531. doi:10.1016/j.biopha.2018.10.190

Windfuhr, J. P., Toepfner, N., Steffen, G., Waldfahrer, F., and Berner, R. (2016). Clinical Practice Guideline: Tonsillitis I. Diagnostics and Nonsurgical Management. *Eur. Arch. Otorhinolaryngol.* 273 (4), 973–987. doi:10.1007/s00405-015-3872-6

Wu, B. L., He, W. X., Ke, M., Shang-Guan, X. F., He, G. F., and Huang, R. (2018). A Retrospective Analysis on 1330 Adverse Event Reports of Qingkailing in China: Further Perception of its Risks and Rational Use. *Curr. Med. Sci.* 38 (6), 1103–1108. doi:10.1007/s11596-018-1990-2

Xie, F., Xie, M., Yang, Y., Zhang, M., Xu, X., Liu, N., et al. (2020). Assessing the Anti-Inflammatory Mechanism of Reduning Injection by Network Pharmacology. *Biomed. Res. Int.* 2020, 6134098. doi:10.1155/2020/6134098

Xu, X., Zhang, J., Zheng, W., Yang, Z., Zhao, X., Wang, C., et al. (2021). Efficacy and Safety of Reduning Injection in the Treatment of COVID-19: A Randomized, Multicenter Clinical Study. *Ann. Palliat. Med.* 10 (5), 5146–5155. doi:10.21037/apm-20-2121

Yi, Y., Li, C. Y., Zhao, Y., Tian, J. Z., Wang, L. M., Pan, C., et al. (2021). Study on Synergistic Effect of Qingkailing Injection and Shengmai Injection on Organ Injury in Endotoxemia Rats. *Zhongguo Zhong Yao Za Zhi* 46 (16), 4193–4200. doi:10.19540/j.cnki.cjcmm.20210524.404

Yu, S., Wang, M., Ding, Y., Ye, L., and Chen, D. (2019). Analysis of 1452 Cases of Adverse Drug Reactions of Reduning Injection in Jiangsu Province. *Chin. J. Pharmacoepidemiol.* 28 (12), 800–804. CNKI:SUN:YWLX.0.2019-12-006.

Zhou, J., Jin, L., Tao, M., and Yuan, H. (2020). Network Meta-Analysis of Chinese Herbal Injections in Adjuvant Treating Suppurative Tonsillitis in Children. *Chin. Tradit. Pat. Med.* 42 (03), 648–656. doi:10.3969/j.issn.1001-1528.2020.03.020

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 23 August 2022
DOI 10.3389/fphar.2022.911667

OPEN ACCESS

EDITED BY
Joan Villena García,
Universidad de Valparaíso, Chile

REVIEWED BY
Ming-Ling Kuo,
Chang Gung University, Taiwan
Chang Xu,
Yanbian University Medical College, China

*CORRESPONDENCE
Li He,
hel@shchildren.com.cn
Sheng Guo,
guosheng@shchildren.com.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

RECEIVED 03 April 2022
ACCEPTED 05 July 2022
PUBLISHED 23 August 2022

CITATION
Zheng J, Wu Q, Zhang L, Zou Y, Wang M, He L and Guo S (2022), Anti-inflammatory activities of Qingfei oral liquid and its influence on respiratory microbiota in mice with ovalbumin-induced asthma.
Front. Pharmacol. 13:911667.
doi: 10.3389/fphar.2022.911667

Anti-inflammatory activities of Qingfei oral liquid and its influence on respiratory microbiota in mice with ovalbumin-induced asthma

Jun Zheng[1†], Qian Wu[1†], Liang Zhang[1], Ya Zou[2], Meifen Wang[3], Li He[1*] and Sheng Guo[4*]

[1]Department of Traditional Chinese Medicine, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, [2]Department of Emergency Medicine, Putuo Hospital, Shanghai University of Traditional Medicine, Shanghai, China, [3]Department of Pediatrics, Sanmen People's Hospital, Taizhou, Zhejiang, China, [4]Department of Endocrine, Genetics and Metabolism, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Dysbiosis of respiratory microbiota is closely related to the pathophysiological processes of asthma, including airway inflammation. Previous studies have shown that Qingfei oral liquid (QF) can alleviate airway inflammation and airway hyper-responsiveness in respiratory syncytial virus-infected asthmatic mice, but its effect on the respiratory microbiota is unknown. We therefore aimed to observe the effects of QF on airway inflammation and respiratory microbiota in ovalbumin (OVA)-induced asthmatic mice. We also explored the potential mechanism of QF in reducing airway inflammation by regulating respiratory microbiota. Hematoxylin and eosin as well as periodic acid-Schiff staining were performed to observe the effects of QF on lung pathology in asthmatic mice. Cytokine levels in bronchoalveolar lavage fluid (BALF) specimens were also measured. Changes in respiratory microbiota were analyzed using 16S rRNA gene sequencing, followed by taxonomical analysis. In order to verify the metagenomic function prediction results, the expression of key proteins related to the MAPK and NOD-like receptor signaling pathways in the lung tissues were detected by immunohistochemistry. The current study found that QF had a significant anti-inflammatory effect in the airways of asthmatic mice. This is mainly attributed to a reduction in lung pathology changes and regulating cytokine levels in BALF. Analysis of the respiratory microbiota in asthmatic mice showed that the abundance of Proteobacteria at the phylum level and *Pseudomonas* at the genus level increased significantly and QF could significantly regulate the dysbiosis of respiratory microbiota in asthmatic mice. Metagenomic functional prediction showed that QF can downregulate the MAPK and Nod-like receptor signaling pathways. Immunohistochemical results showed that QF could downregulate the expression of p-JNK, p-P38, NLRP3, Caspase-1, and IL-1β, which are all key proteins in the signaling pathway of lung tissue. Our study therefore concluded that QF may reduce airway inflammation in asthmatic mice by regulating respiratory microbiota, and to the possibly downregulate MAPK and Nod-like receptor signaling pathways as its underlying mechanism.

KEYWORDS

airway inflammation, asthma, metagenomic functional prediction, Qingfei oral liquid, respiratory microbiota

1 Introduction

Bronchial asthma is a heterogeneous disease characterized by chronic airway inflammation and airway hyper-responsiveness. The major symptoms of asthma, ranging from mild symptoms to life-threatening attacks, include coughing, wheezing, shortness of breath, and chest tightness (Licari et al., 2018). Although asthma can occur at any age, it is particularly common in children. Epidemiological investigations have shown that the global incidence of asthma in children has increased from 11.1% to 13.2% over the last 10 years (Asher et al., 2020). At present, there are still a number of children worldwide with asthma that is not effectively controlled for various reasons (Murphy et al., 2020).

Airway inflammation is considered the most important pathophysiological process in asthma. T helper (Th) lymphocytes play an important role in airway inflammation (Tumes et al., 2017). Previous studies have shown that asthma can be divided into at least two distinct molecular phenotypes based on the degree of Th2 inflammation, which have been described as Th2-high and Th2-low (Robinson et al., 2017). Th2 cells produce interleukin (IL)-4, IL-13, and IL-5, eventually leading to the accumulation of eosinophils in the lungs (Finkelman et al., 2010). In recent years, studies have shown that severely asthmatic patients with a Th2-low phenotype have been noted to display predominantly neutrophilic inflammation of their airways (Ramakrishnan et al., 2019). Moreover, Th17 cells and their cytokines (IL-17 and IL-6, among others) are implicated in the development of neutrophilic inflammation. However, $CD4^+CD25^+$ regulatory T cells and their cytokines (IL-10 and TGF-β, among others) were significantly reduced in patients with asthma (Gandhi et al., 2020).

Following the development of high-throughput sequencing technology, the relationship between changes in the human microbiota and asthma has attracted increasing attention. The respiratory microbiota, which exhibits the mucosal surface of the respiratory tract, is a direct participant in the local mucosal immunity of the respiratory system and is closely related to the occurrence and development of asthma (Budden et al., 2019). Our recent study also found that dynamic changes in the respiratory microbiota are closely associated with the progression of chronic asthma, including airway inflammation and airway remodeling (Zheng et al., 2021). Huang et al. (2015) showed that an increase in Proteobacteria abundance in the respiratory tract can promote neutrophil aggregation, which may affect the patient's response to corticosteroid therapy. A study of the composition of nasal microbiota showed that the increased abundance of *Streptococcus*, *Haemophilus*, and *Moraxella* was an important risk factor for predicting wheezing in preschoolers younger than 5 years, which persisted until a school-going age (Teo et al., 2018). In a mouse model study, *Prevotella* was found to significantly reduce neutrophil aggregation in the lung and production of Toll-like receptor (TLR) 2-mediated pro-inflammatory cytokines, thereby reducing lung inflammation, compared with *Haemophilus influenzae* (Larsen et al., 2015). In addition, some respiratory microbes can also play a role in reducing lung inflammation by producing metabolites (such as short-chain fatty acids) (Segal et al., 2017a; Segal et al., 2017b). These findings indicate that alterations in respiratory microbiota can affect the phenotype and severity of airway inflammation in patients with asthma by driving local mucosal immune responses. Furthermore, it is probable that the regulation of dysbiosis of respiratory microbiota can improve airway inflammation in asthma to some extent.

In recent years, an increasing number of studies have found that Traditional Chinese Medicine (TCM) compounds play a role in asthma prevention and treatment (Tsang et al., 2018; Cui et al., 2020). Qingfei oral liquid (QF), founded by Wang Shouchuan, a famous professor at Nanjing University of Chinese Medicine in China, has been used for the prevention and treatment of asthma in children for decades, and has achieved satisfactory results. It contains TCM concoction of *Ephedra sinica* Stapf [Ephedraceae], *Prunus armeniaca* var. Armeniaca [Rosaceae], and *Scutellaria baicalensis* Georgi [Lamiaceae], among others. A previous study by our team found that QF can reduce airway inflammation and airway hyper-responsiveness in respiratory syncytial virus (RSV)-infected asthmatic mice (Jing et al., 2020; Yu et al., 2021), but its effect on respiratory microbiota has not been observed. In view of this, the aim of the current study was to investigate the anti-inflammatory activities of QF and its effect on respiratory microbiota in mice with ovalbumin (OVA)-induced asthma and to explore the possible mechanism of QF in alleviating airway inflammation by regulating respiratory microbiota. The purpose of this study was to provide new knowledge for the prevention and treatment of asthma and provide a theoretical basis for the effective intervention of QF.

2 Materials and methods

2.1 Reagents and drugs

We obtained OVA from Sigma-Aldrich Chemical Co. (grade V; St. Louis, MO, United States). Mouse IL-4, IL-6, IL-10, and IL-17A ELISA kits and Imject Alum were purchased from Thermo Fisher Scientific (Rockford, IL, United States). Mouse total IgE and OVA-specific IgE ELISA kits were purchased from BioLegend (San Diego, CA, United States). Antibodies against

TABLE 1 Composition of Qingfei oral liquid (QF).

Latin name (Chinese name)	Parts & form used	Weight use (g)
Ephedra sinica Stapf (Ephedraceae) (Ma Huang)	Stem	3
Prunus armeniaca var. Armeniaca (Rosaceae) (Ku Xin Ren)	Dried ripe seed	10
Gypsum Fibrosum (Sheng Shi Gao)	Mineral	20
Scutellaria baicalensis Georgi (Lamiaceae) (Huang Qin)	Dried root	6
Morus alba L. (Moraceae) (Sang Bai Pi)	Dried bark of root	10
Lepidium apetalum Willd. (Brassicaceae) (Ting Li Zi)	Dried ripe seed	10
Kitagawia praeruptora (Dunn) Pimenov (Apiaceae) (Qian Hu)	Dried root	10
Reynoutria japonica Houtt. (Polygonaceae) (Hu Zhang)	Dried root and rhizome	12
Salvia miltiorrhiza Bunge (Lamiaceae) (Dan Shen)	Dried root and rhizome	10

NLRP3, Caspase-1, and IL-1β were obtained from Abcam Co. (Cambridge, MA, United States). Antibodies against p-P38, and p-JNK were obtained from CST Co. (Boston, MA, United States).

We obtained QF from the Chinese Pharmacy of Longhua Hospital, Shanghai University of Traditional Chinese Medicine. The standard dose of QF was 91 g (weight of total herb mixtures) and the composition of it was presented in Table 1. The extraction and quality control of QF were performed by the Department of Pharmacology at the Shanghai University of Traditional Chinese Medicine and Shanghai Fudan Fuda Science & Technology Co., Ltd. (Shanghai, China).

2.2 UHPLC-MS/MS conditions

Samples were analyzed by suspending 100 mg of each compound in 10 ml of 50% methanol aqueous solution in a 15 ml centrifugal tube. The tube was then sonicated for 30 min, whereafter 1 ml of supernatant was centrifuged at 14,000 rpm for 5 min. Following centrifugation, the supernatant was filtered through a 0.22 μm microporous membrane, and placed into sample vials for UHPLC-MS/MS analysis. Control samples were processed under the same conditions.

The LC conditions were as follows. Column: ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm); Column temperature: 35°C; Injection volume: 10 μl; Flow rate: 0.3 ml/min; Mobile Phase A: (deionized water with 0.1% formic acid); Phase B (acetonitrile with 0.1% formic acid). Specific gradient elution conditions were as follows: 0 min, 0% B; 10 min, 30% B; 25 min, 40% B; 30 min, 50% B; 40 min, 70% B; 45 min, 100% B; 60 min, 100% B; 60.5 min, 0% B and 70 min, 0% B.

The MS conditions were as follows. Q Exactive Orbitrap high resolution mass spectrometry was used to analyze sample. The detection mode was full MS-DDMS2, and positive and negative ion modes were scanned. Acquisition range: 100–1,200 Da, MS1 resolution: 70,000, MS2 resolution: 17,500, capillary voltage: ± 3.2 kV, capillary temp: 320°C, Aux gas heater temp: 350°C, sheath gas flow rate: 40 L/min, auxiliary gas flow rate: 15 L/min, AGC target: 1e6, TopN: 5, Full MS-ddMS2 NCE: 30, 40, 50.

2.3 Animal experiments

Female BALB/c mice (n = 36) aged 4–6 weeks and weighing 17.5–20.5 g were obtained from Shanghai Sippr-BK Laboratory Animal Co., Ltd. (Shanghai, China). All mice were maintained in a specific-pathogen-free grade animal room under controlled conditions with a 12 h light/dark cycle at a temperature of 24 ± 2°C with a relative appropriate humidity. Animal experiments were conducted in accordance with institutional guidelines for animal research. The Center for Laboratory Animals, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China authorized this experimental protocol (approval no. 2017Y003).

A mouse model of OVA-induced asthma was constructed as previously described, with slight modifications (Temelkovski et al., 1998). First, 36 mice were divided into two groups: control (n = 6) and OVA (n = 30). Mice in the OVA group were sensitized by intraperitoneal injection of 200 μl of a solution composed of 20 μg OVA dissolved in 100 μl sterile saline and 100 μl Imject Alum (containing 4 mg aluminum hydroxide (net weight)) on days 0, 7, and 14. Then, 30 mice from the OVA group were randomly divided into five groups: OVA group (no treatment, n = 6), OVA + QFH group (high-dose QF, n = 6), OVA + QFM group (medium-dose QF, n = 6), OVA + QFL group (low-dose QF, n = 6), and OVA + BUD group (budesonide aerosol inhalation, n = 6). For the OVA challenge, the mice were exposed to OVA aerosol (2.5% w/v) OVA solution in sterile saline administered using a PARI PRONEB Ultra compressor (Pari Proneb, Midlothian, WA, United States) for 30 min on days 21–24 and 28–31. The mice in the OVA + QFH group, the OVA + QFM group and the OVA + QFL group were given 1.82 g/d, 0.91 g/d and 0.455 g/d QF (Herbs dry weight), respectively by

intragastric administration 30 min before OVA challenge. One hour prior to the OVA challenge, mice in the OVA + BUD group were nebulized with budesonide (1 mg budesonide in 3 ml normal saline) for 30 min. Mice in the OVA group (no treatment) were administered an equivalent volume of distilled water intragastrical, followed by nebulization with normal saline for 30 min before OVA challenge. Mice in the control group were injected intraperitoneally with saline and an Imject Alum emulsion and then exposed to an aerosol of sterile saline without OVA, according to the same schedule. The mice were sacrificed within 24 h of final nebulization. After the mice were euthanized, nasal lavage fluid (NLF), bronchoalveolar lavage fluid (BALF), and left lower lung tissue were collected and preserved.

2.4 Histological analysis and immunohistochemical staining of lung tissue

First, we ligated the left lower lung and removed it after complete bronchoalveolar lavage. The lungs were harvested and infused with 4% paraformaldehyde for 24 h. Sections (4 μm-thick) were embedded in paraffin and then subjected to hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining to evaluate airway inflammation and mucus production in the lung tissues. Pathological changes were observed under a light microscope at ×200 magnification. To grade the extent of lung inflammation and goblet cell hyperplasia, a semiquantitative scoring system was used as previously described (Kujur et al., 2015; Kubo et al., 2019).

To observe the expression levels of related proteins in lung tissues, ligated left lung tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm-thick) were deparaffinized and rehydrated. The sections were pretreated with 0.3% H_2O_2 in methanol for 15 min to quench the endogenous peroxide activity and boiled at 100°C for 20 min in a 10% citrate buffer to unmask the antigens. Sections were incubated in primary antibodies (anti-NLRP3 1:200, anti-Caspase-1 1:1,000, and anti-IL-1β 1:500, anti-p-P38 1:200, and anti-p-JNK 1:100) at 4°C overnight and stained with HRP-labelled anti-rabbit IgG (1:1,000). After washing, DAB substrate was applied to the sections. Representative images of each slide were acquired at ×200 magnification for morphometric and comparative analysis.

2.5 Collection of bronchoalveolar lavage fluid and nasal lavage fluid

After euthanasia of the mice by cervical dislocation, the lungs were washed four times with sterile saline, using endotracheal intubation to the lower respiratory tract (LRT) (0.5 ml per round) to obtain samples for BALF. Then, a sterile leather hose was reinserted to rinse the nasal cavity with normal saline (0.5 ml per round) for 3–4 times and to collect the NLF. Both BALF and NLF were filtered once with a 0.22 μm filter. The filtered BALF was centrifuged at 1,000 g for 15 min, and the supernatants were collected for cytokine detection. A filter membrane was used for DNA extraction. All specimens were stored at −80°C.

2.6 Cytokine and IgE measurement

All BALF specimens were collected as described in Section 2.5. The levels of total IgE and OVA-specific IgE, IL-4, IL-6, IL-17A, and IL-10 in BALF were measured using ELISA kits according to the manufacturer's instructions.

2.7 16S rRNA gene sequencing and bioinformatics analysis

To explore the effect of QF on the respiratory microbiota of asthmatic mice, 16S rRNA gene sequencing was performed using NLF and BALF specimens from the control, OVA, OVA + QFM, and OVA + BUD groups. Total genomic DNA was extracted using the OMEGA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States), according to the manufacturer's instructions, and stored at −80°C until needed for analysis. The forward primer 338F (5-ACTCCTACGGGAGGCAGCA-3) and reverse primer 806R (5-GGACTACHVGGGTWTCTAAT-3) were used to amplify the V3–V4 region of the bacterial 16S rRNA genes. After the individual quantification step, the amplicons were pooled in equal quantities and paired-end sequencing (2 × 300 bp) was performed using Illumina MiSeq (Illumina, San Diego, CA, United States) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The procedures for DNA extraction, PCR amplification, and sequencing were based on previous studies[10] of our team.

Microbiome bioinformatic analysis was performed using QIIME 2 2020.11 with minor modifications (Hall and Beiko, 2018). Briefly, raw sequence data were demultiplexed using the demux plugin, followed by primer cutting using the Cutadapt plugin. The sequences were subjected to quality filtering, denoising, and merging, and chimeras were removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants were used to construct a phylogeny using FastTree (Price et al., 2009). We used the q2-diversity plugin to compute different α diversity metrics using Pielou's evenness indices and β diversity metrics using weighted UniFrac distance matrices. Principal coordinates analysis (PCoA) with weighted UniFrac distance matrices was used to study community composition. Taxonomy was assigned using a naive Bayes classifier pre-trained on the Greengenes 13_8_99% OTUs 16S rRNA gene full-length sequences and the q2-feature-

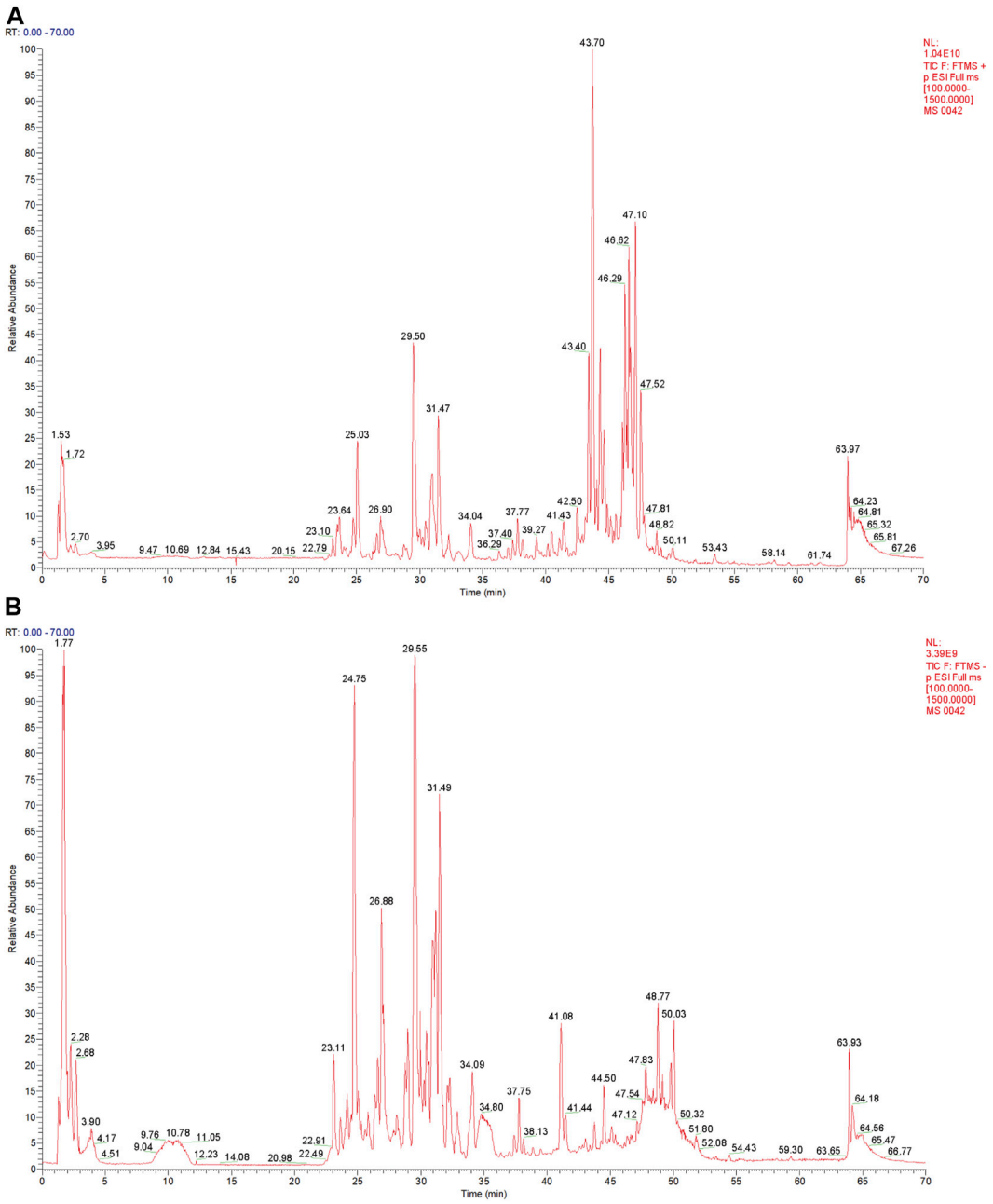


FIGURE 1
Total ion chromatography (TIC) of the extract of Qingfei oral liquid (QF). **(A)** TIC of the extract of QF in positive ion mode; **(B)** Total ion chromatography of the extract of QF in negative ion mode.

classifier plugin (Bokulich et al., 2018). The bar chart of the composition of respiratory microbiota was completed using Wekemo Bioincloud (https://www.bioincloud.tech). Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used to detect differentially abundant taxa across groups using default parameters. The PICRUSt software package (Langille et al., 2013) was used for metagenomic functional prediction analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al., 2004) were used to identify metagenomic contents. LEfSe and PICRUSt analyses were performed using resources available at http://huttenhower.sph.harvard.edu/galaxy/. The STAMP software (Parks et al., 2014) was used to analyze the predicted metagenome and identify pathways associated with airway inflammation.

TABLE 2 The information of top 20 compounds of the extract of Qingfei oral liquid (QF).

No.	Compound name	Formula	Mass deviation/ ppm	Molecular weight	Rt/ min	Match score	Area/ $\times10^{10}$	Relative amount/ %
1	Baicalin	C21H18O11	0.6	446.09	29.7	89.1	8.3	19.23
2	Wogonoside	C22H20O11	0.8	460.1	31.7	84.2	4.94	11.44
3	Amygdalin	C20H27NO11	0.6	457.16	24.9	93.6	4.36	10.11
4	Oroxylin A-7-O-β-D-glucuronide	C22H20O11	0.8	460.1	31.1	84.8	2.41	5.59
5	Cryptotanshinone	C19H20O3	1.3	296.14	44.8	87.4	1.91	4.42
6	Emodin-8-O-β-D-glucopyranoside	C21H20O10	0.2	432.11	31.4	92.9	1.57	3.63
7	Tanshinone IIA	C19H18O3	1.0	294.13	46.9	88.9	1.33	3.08
8	Sucrose	C12H22O11	0.2	342.12	1.9	93.6	1.29	3
9	Polydatin	C20H22O8	0.5	390.13	27.1	89.3	1.24	2.87
10	Emodin	C15H10O5	0.3	270.05	41.3	88.3	1.21	2.81
11	Praeruptorin C	C24H28O7	0.2	445.21	47.3	86.5	1.17	2.72
12	Praeruptorin A	C21H22O7	0.2	170.08	43.9	83.2	1.01	2.34
13	Baicalein	C15H10O5	0.4	270.05	34.2	88.6	0.89	2.06
14	Stachyose	C24H42O21	0.6	666.22	1.9	91	0.75	1.73
15	Chrysosplenetin B	C19H18O8	0.6	374.1	38.0	72.2	0.69	1.61
16	Decursinol	C14H14O4	0.5	246.09	41.6	76.3	0.63	1.46
17	Tinnevellin glucoside	C20H24O9	0.4	408.14	31.2	75.5	0.61	1.42
18	Resveratrol	C14H12O3	0.2	196.05	27.1	91.9	0.6	1.38
19	Wogonin	C16H12O5	0.3	284.07	37.6	92.6	0.44	1.03
20	Taxifolin	C15H12O7	0.3	304.06	26.5	86.1	0.42	0.96

2.8 Statistical analysis

The expression levels of cytokines and related proteins were compared by analysis of variance using GraphPad Prism 8.0, and SPSS 24.0. The differences between the four groups were tested using one-way analysis of variance (ANOVA), and the variance between the two groups was compared using an Tukey's multiple comparisons test. The Kruskal–Wallis test was used to estimate intergroup differences in α diversity metrics, β diversity metrics, and LEfSe analysis. The Wilcoxon test was used to compare subclasses. The predicted metagenome was analyzed using White's non-parametric t-test for comparisons between the two groups. Statistical tests used in the study were two-sided, and a p value ≤0.05 was considered to indicate statistical significance.

3 Results

3.1 Composition of Qingfei oral liquid

The QF extract was analyzed using UHPLC-MS/MS. The TIC in the positive and negative ion modes of the QF extract are shown in Figures 1A,B, respectively. Analysis of the top 20 compounds sorted by MS response values. The source of the active QF component was based on the Chinese Pharmacopoeia 2015 edition, the literature search was based on PubMed, and the compound data search was based on PubChem, Chemical Book, and SciFinder databases. The information on the top 20 compounds is presented in Table 2. The TIC of the blank samples are shown in Supplementary Figure S1.

3.2 Administration of Qingfei oral liquid attenuated airway inflammation in lung pathology in ovalbumin-induced asthmatic mice

To investigate the effect of QF on airway inflammation in OVA-induced asthmatic mice, lungs were stained with H&E and PAS. Compared to control mice, OVA-challenged mice showed obvious inflammatory cell infiltration around the small airways, bronchial wall thickening, and constriction (Figures 2A,B). Treatment of OVA-challenged mice with QF and budesonide significantly alleviated airway inflammation. In the budesonide and QFM group, the number of inflammatory cells infiltrating the airway was significantly lower than in the OVA group, as was the airway wall thickness and the inflammation score. For OVA-challenged mice treated with QF, medium-dose QF had the best effect (Figures 2A,B). PAS staining showed that the

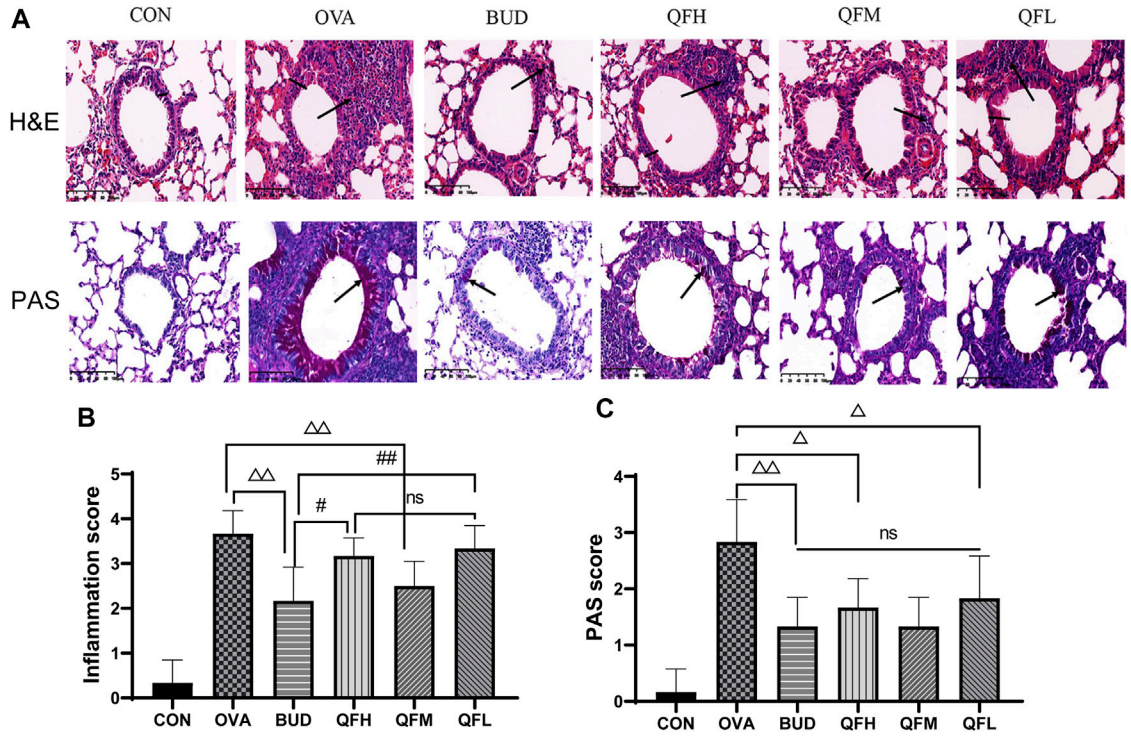


FIGURE 2
Effect of Qingfei oral liquid (QF) on pulmonary pathology in ovalbumin (OVA)-induced asthmatic mice. **(A)** Representative hematoxylin and eosin (H&E) staining were performed to observe airway inflammation in asthmatic mice (200×; the black arrows indicate the aggregation of inflammatory cells, and the black vertical line indicates the airway wall thickness). Periodic acid-Schiff (PAS) staining indicating the mucus-producing goblet cells around the small airways (200×; similar to the purple area indicated by the black arrow). **(B)** Inflammation scores are based on H&E staining. **(C)** The PAS scores indicating the mucus-producing goblet cells around the small airways. Data are expressed as mean ± standard deviation (SD) (one-way ANOVA followed by Tukey's test). $n = 6$, $^{\triangle}p \leq 0.05$ vs. the OVA group, $^{\triangle\triangle}p \leq 0.01$ vs. the OVA group; $^{\#}p \leq 0.05$ vs. the budesonide (BUD) group, $^{\#\#}p \leq 0.01$ vs. the BUD group, ns: no difference.

number of mucus-secreting goblet cells increased in OVA-challenged mice compared to that in control mice (Figures 2A,C). The budesonide and QF groups significantly reduced airway mucus secretion when compared to the OVA group at different doses, but there was no significant difference in the PAS score between the treatment groups. We can deduce that QF and budesonide alleviated these pathological changes. These results showed that QF can significantly alleviate airway inflammation in the lung pathology of asthmatic mice.

3.3 Administration of Qingfei oral liquid decreased inflammatory cytokines in bronchoalveolar lavage fluid of mice with ovalbumin-induced asthma

Total IgE, OVA-specific IgE, IL-4, IL-6, IL-17A, and IL-10 levels in BALF were measured using ELISA (Figure 3). Compared with the control group, the OVA group had significantly higher levels of total IgE (Figure 3A), OVA-specific IgE (Figure 3B), IL-4 (Figure 3C), IL-6 (Figure 3D), and IL-17A (Figure 3E), but lower levels of IL-10 (Figure 3F). Similar to the OVA-challenged mice treated with budesonide, the levels of total IgE, OVA-specific IgE, IL-4, IL-6, and IL-17A in the BALF of OVA-challenged mice treated with QF were significantly decreased (Figures 3A–E). Both QF and budesonide-treated mice showed increased levels of IL-10 to some extent (Figure 2F). In addition, QF was less effective than budesonide at reducing the levels of total IgE, OVA-specific IgE, and IL-4 ($p < 0.05$). QFM showed no significant difference in reducing IL-6 levels or increasing IL-10 levels when compared to budesonide ($p > 0.05$), but significantly improved in reducing IL-17A levels ($p < 0.05$). The effect of QF on reducing IL-6, IL-4, and IL-17A levels was especially significant in mice treated with intermediate doses of QF. These results suggest that QF regulates cytokine levels in the BALF of OVA-induced asthmatic mice.

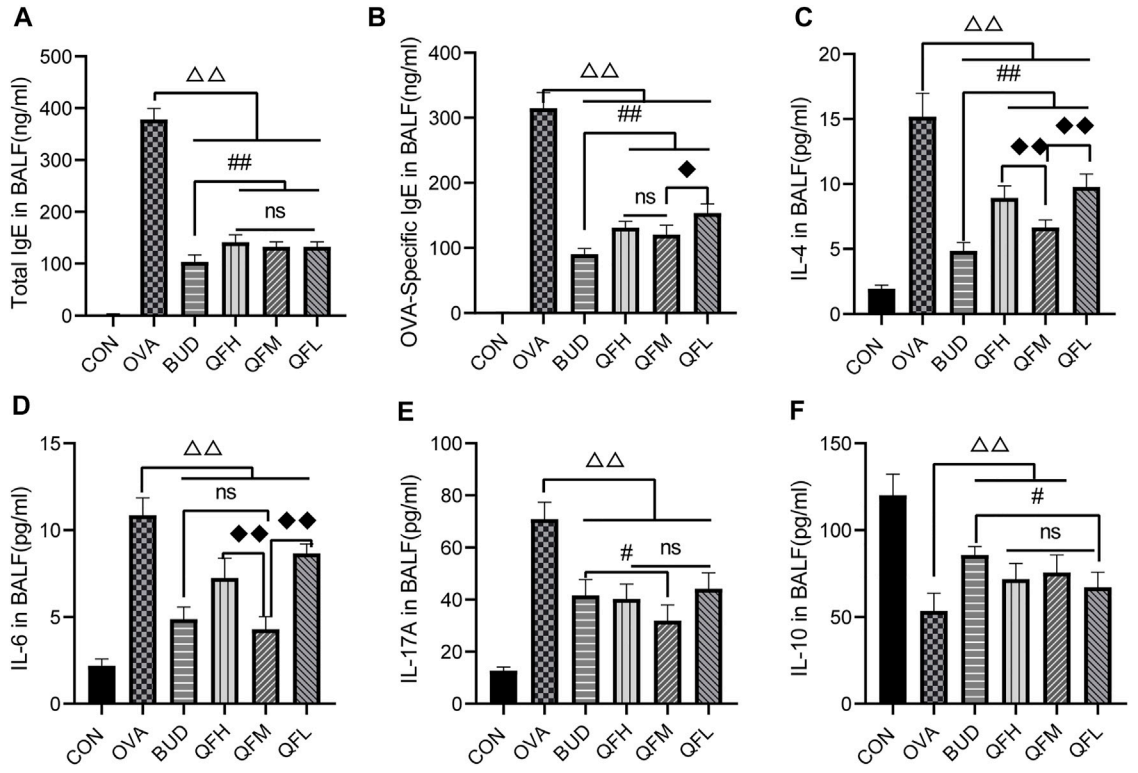


FIGURE 3
Effects of Qingfei oral liquid (QF) on IgE and inflammatory cytokines in bronchoalveolar lavage fluid (BALF) of asthmatic mice. BALF samples from different groups were collected to detect the levels of **(A)** total IgE, **(B)** Ovalbumin (OVA)-specific IgE, **(C)** IL-4, **(D)** IL-6, **(E)** IL-17A, and **(F)** IL-10 using ELISA. Data are expressed as mean ± standard deviation (SD) and were performed with one-way ANOVA followed by Tukey's tests. $n = 6$, $^{\triangle}p \leq 0.05$ vs. the OVA group, $^{\triangle\triangle}p \leq 0.01$ vs. the OVA group; $^{\#}p \leq 0.05$ vs. the budesonide (BUD) group, $^{\#\#}p \leq 0.01$ vs. the BUD group; $^{\blacklozenge}p \leq 0.05$ vs. the QFM group, $^{\blacklozenge\blacklozenge}p \leq 0.01$ vs. the QFM group; ns: no difference; line over bars indicates that all groups are include.

3.4 Qingfei oral liquid altered the diversity of the respiratory microbiota in mice with ovalbumin-induced asthma

Since the mid-dose QF had the best anti-airway inflammation effect, this study further compared the differences in respiratory microbiota among the control, OVA, OVA + QFM (QFM), and OVA + BUD (BUD) groups. The shape of the alpha rarefaction curve indicated that the sequencing depth was sufficient (Figures 4A,B). The α diversity represents the diversity of microbial groups in the model, and the β diversity analysis indicated the microbial diversity in different groups of mice. The upper respiratory tract (URT) microbiota (NLF samples) of the OVA-induced mice had a significantly higher α diversity ($p < 0.05$) than that of the control mice, as determined using Pielou's evenness (Figure 4C). The URT microbiota of OVA-challenged mice treated with QF or budesonide did not differ significantly from that of the untreated OVA-challenged mice. However, there was no significant difference in the Pielou's evenness indices of the LRT microbiota (BALF samples) among the control, OVA, QFM, and BUD groups ($p > 0.05$) (Figure 4D).

The weighted UniFrac distance was used to evaluate β-diversity among the different groups. The scatter plot based on the PCoA scores indicated a clear separation of community composition between the control and OVA groups. PCoA showed that the β-diversity of the respiratory microbiota of OVA-induced mice treated with QF was significantly different from that of untreated OVA-challenged mice (Figures 4E,F). These results indicate that QF can affect the diversity of the respiratory microbiota in OVA-induced asthmatic mice.

3.5 Qingfei oral liquid regulated the composition of the respiratory microbiota in mice with ovalbumin-induced asthma

To understand the effect of QF on the composition of the respiratory microbiota in OVA-induced asthmatic mice, we analyzed the microbial composition at the phylum and genus levels. At the phylum level, Actinobacteria was predominant in the control group, whereas Proteobacteria was the most

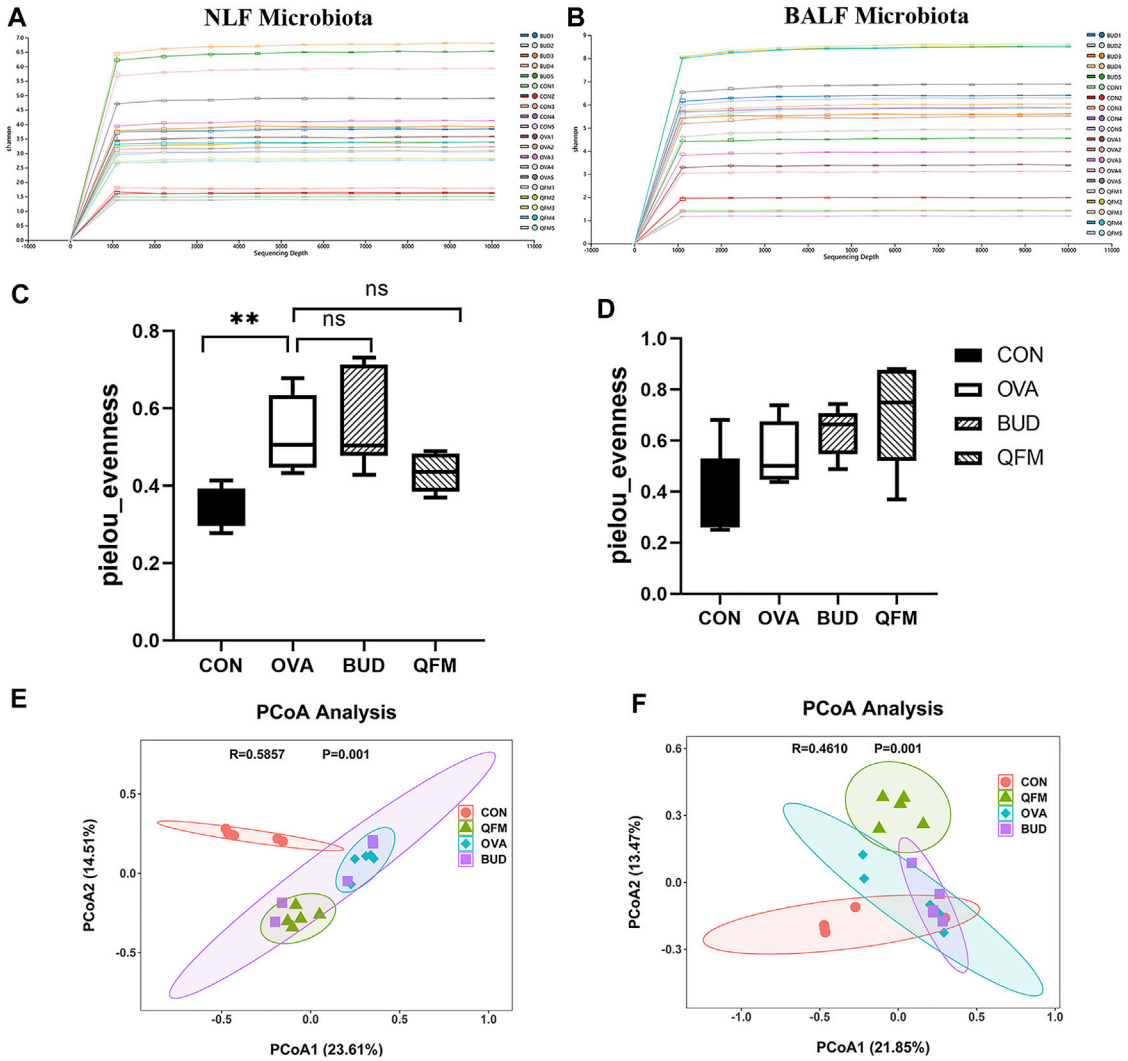


FIGURE 4
Effects of Qingfei oral liquid (QF) on the diversity of the respiratory microbiota in ovalbumin (OVA)-induced asthmatic mice. **(A)** The alpha rarefaction curve in nasal lavage fluid (NLF) microbiota. **(B)** The alpha rarefaction curve in bronchoalveolar lavage fluid (BALF) microbiota. **(C)** α diversity analysis (using the Pielou's evenness) of the NLF microbiota. **(D)** α diversity analysis (using the Pielou's evenness) of the BALF microbiota. **(E)** PCoA plot showing the β diversity of NLF microbiota ($p = 0.001$). **(F)** PCoA plot showing the β diversity of BALF microbiota ($p = 0.001$). PCoA of all samples using weighted UniFrac distance. PCoA, principal coordinates analysis. ($n = 5$ in each group).

abundant phylum in the OVA group (in both URT and LRT samples). Fortunately, Proteobacteria were significantly reduced in OVA-induced asthmatic mice treated with QF and budesonide, and Actinobacteria were significantly increased in OVA-induced asthmatic mice treated with QF (Figures 5A,B).

At the genus level, Micrococcaceae was most abundant in the control group, whereas *Pseudomonas* and *Cupriavidus* were more abundant in the OVA group (Figures 5C,D). In asthmatic mice treated with QF and budesonide, the proportion of *Pseudomonas* decreased significantly. *Cupriavidus* decreased significantly in asthmatic mice treated with budesonide, but there was no significant difference between QF and untreated asthmatic mice.

To further identify the effect of QF on the composition of the microbiota in OVA-induced asthmatic mice, we analyzed the different abundances of bacterial communities using LEfSe (Figure 6). Analysis of microbial composition at the genus level using LEfSe revealed that among the URT and LRT microbiota, *Pseudomonas*, *Cupriavidus* and many other genera from Proteobacteria were present in the OVA group, *Bacilli* from Firmicutes in the QFM group, and *Bacillus* from Firmicutes in the BUD group. These results indicate that QF can regulate the composition of respiratory microbiota in asthmatic mice to some extent.

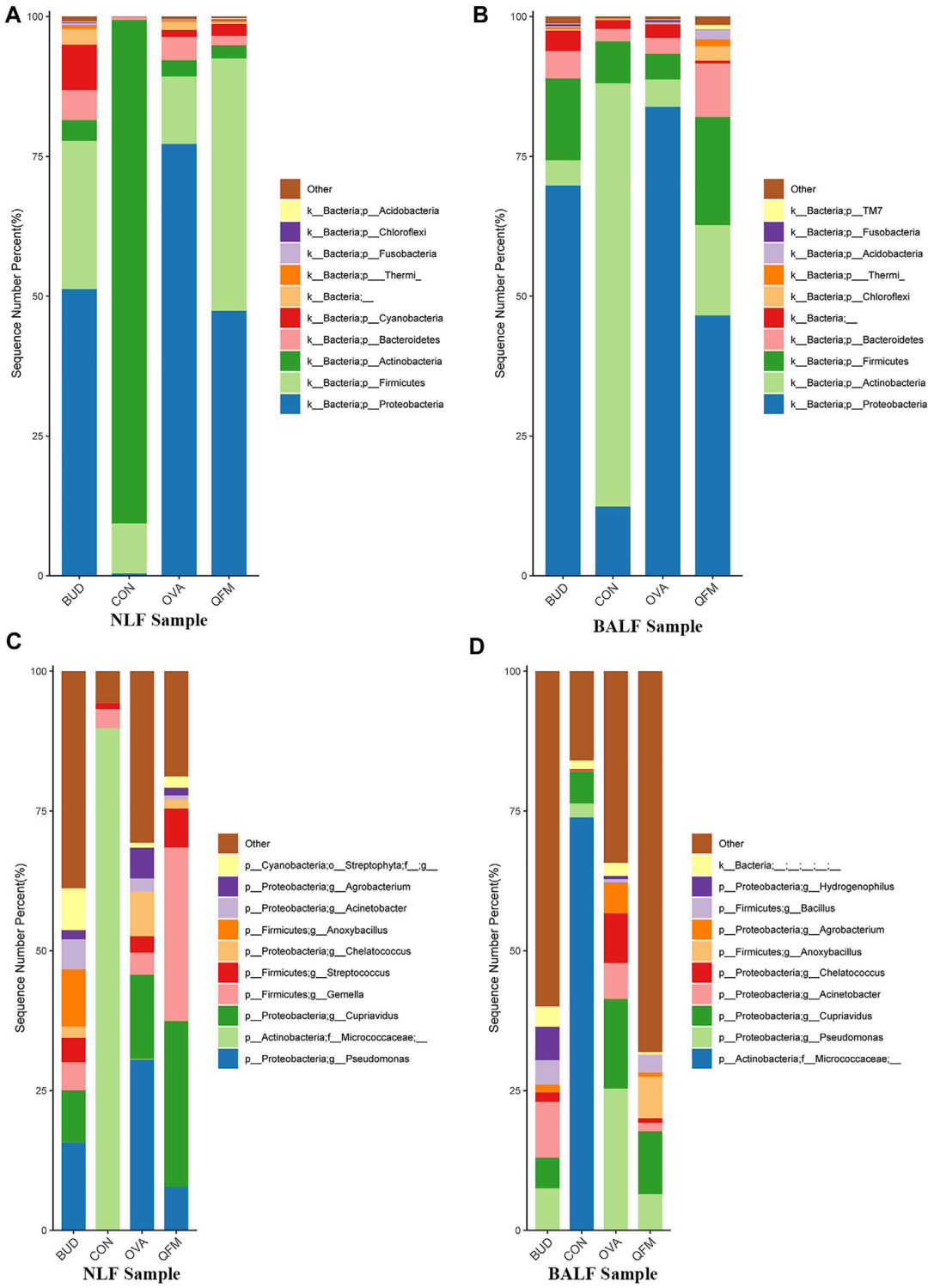


FIGURE 5
Effects of Qingfei oral liquid (QF) on the composition of respiratory microbiota in ovalbumin (OVA)-induced asthmatic mice. **(A)** The composition of nasal lavage fluid (NLF) microbiota at the phylum level. **(B)** The composition of bronchoalveolar lavage fluid (BALF) microbiota at the phylum level. **(C)** The composition of NLF microbiota at the genus level. **(D)** The composition of the BALF microbiota at the genus level. (n = 5 in each group; only the top 10 legends with high abundance are shown).

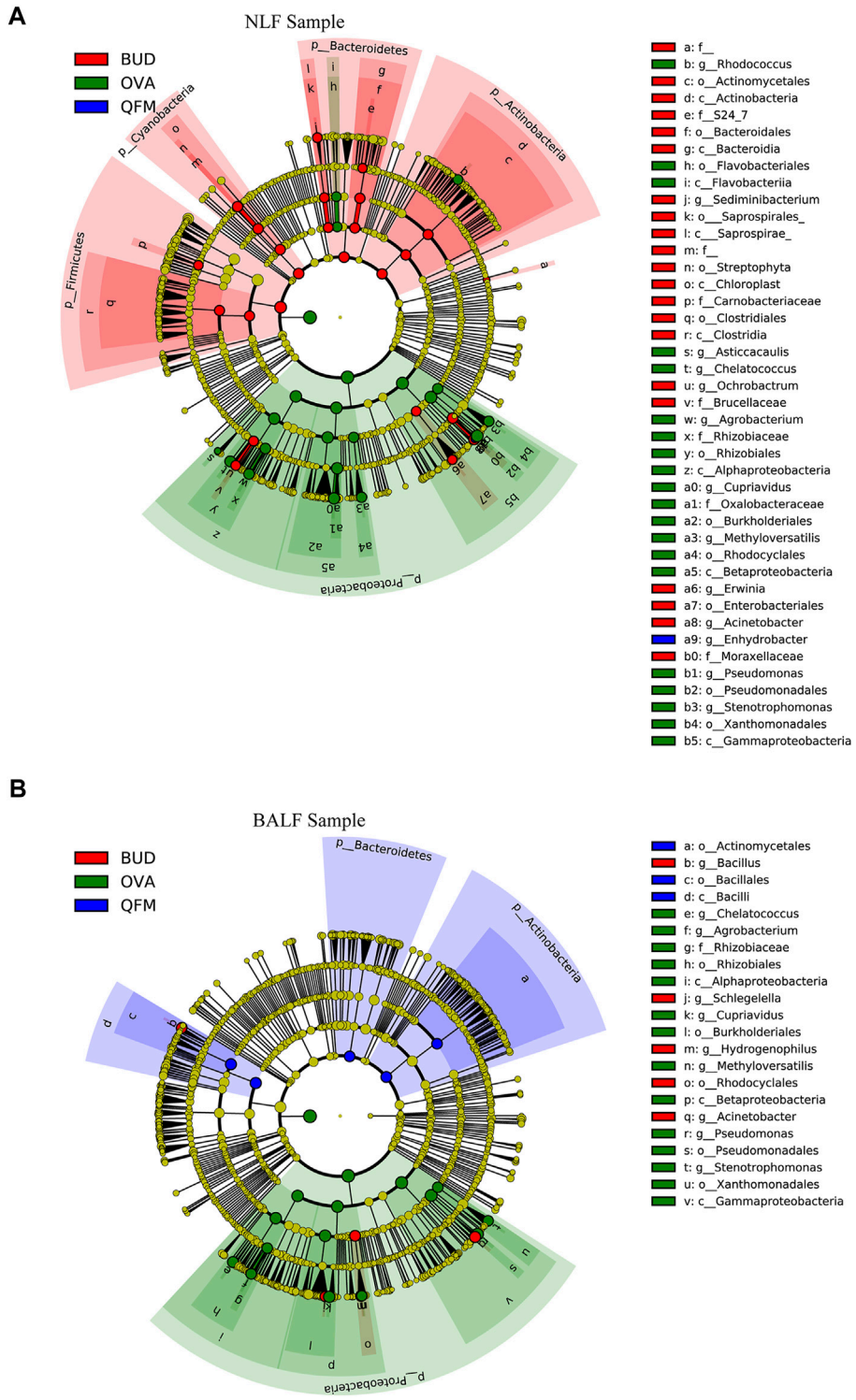


FIGURE 6
Different abundances of bacterial communities in the respiratory samples, as indicated in LEfSe analysis. The differences are indicated by the color of over-represented taxa: green indicating ovalbumin (OVA)-induced mice, blue indicating Qingfei oral liquid medium concentration (QFM) treated mice, and red indicating budesonide (BUD) treated mice. **(A)** Different abundances of bacterial communities in the upper respiratory tract (URT) (nasal lavage fluid (NLF) samples) with LDA scores >2.5. **(B)** Different abundances of bacterial communities in the lower respiratory tract (LRT) (bronchoalveolar lavage fluid (BALF) samples) with LDA scores >3.0. The circles represent phylogenetic levels from phylum (innermost circle) to genera (outermost circle). n = 5 in each group; adjusted p values ≤0.05.

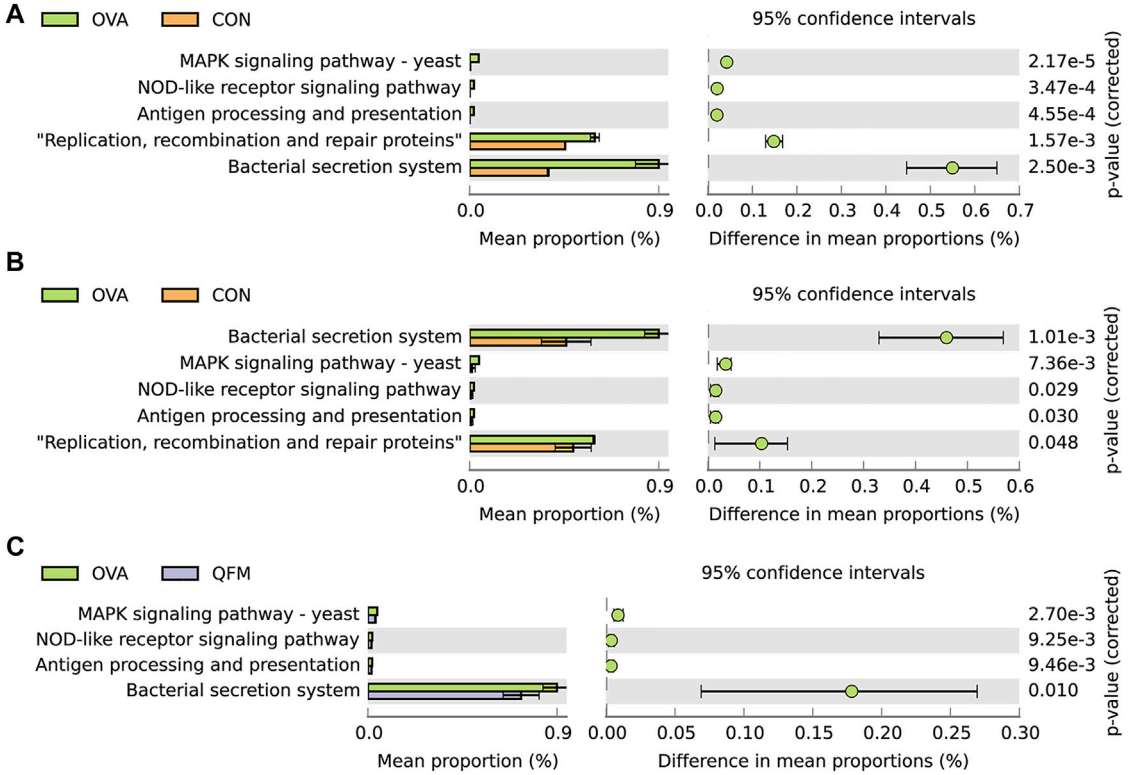


FIGURE 7
Effects of Qingfei oral liquid (QF) on metagenomic functional prediction of the respiratory microbiota in ovalbumin (OVA)-induced asthmatic mice. Bacterial metagenomic functional categories were derived from Level 3 KEGG pathways. **(A)** Control group vs. the OVA group for the nasal lavage fluid (NLF) microbiota. **(B)** Control group vs. the OVA group in bronchoalveolar lavage fluid (BALF) microbiota. **(C)** BALF microbiota of the OVA group vs. the QF medium (QFM) concentration group. Gene functions with significant differences (corrected p-value ≤ 0.05, White's non-parametric t test in STAMP) and parts of the pathways associated with asthma are shown.

3.6 Effects of Qingfei oral liquid on metagenomic functional prediction of the respiratory microbiota of mice with ovalbumin-induced asthma

To understand whether alterations in the respiratory microbiota contribute to airway inflammation in asthmatic mice, we performed bacterial metagenomic function prediction analyses using the PICRUSt program. We explored the differences in the bacterial metagenomic functional prediction among the different groups at KEGG Level 3 (Figure 7).

In both URT and LRT samples, the MAPK signaling pathway-yeast, NOD-like receptor signaling pathway, antigen processing and presentation, bacterial secretion system, and replication, recombination, and repair proteins were significantly upregulated in the OVA group compared to the control group (Figures 7A,B). Among these pathways, MAPK signaling pathway-yeast and NOD-like receptor signaling pathway have been shown to be closely associated with airway inflammation. In LRT samples, MAPK signaling pathway-yeast and Nod-like receptor signaling pathway were significantly downregulated in QF treated asthmatic mice, but no similar changes were found in URT samples (Figure 7C). Therefore, we speculated that the underlying mechanism of QF to improve airway inflammation in OVA-induced asthmatic mice by regulating the respiratory microbiota may be related to the down-regulation of MAPK signaling pathway-yeast and NOD-like receptor signaling pathway.

3.7 Qingfei oral liquid regulated the expression of key proteins related to MAPK and NOD-like receptor signal pathway in ovalbumin-induced asthmatic mice

Metagenomic functional prediction showed that QF could downregulate the MAPK and Nod-like receptor signaling pathways related to airway inflammation. MAPK is an important transmitter of signals from the cell surface to the

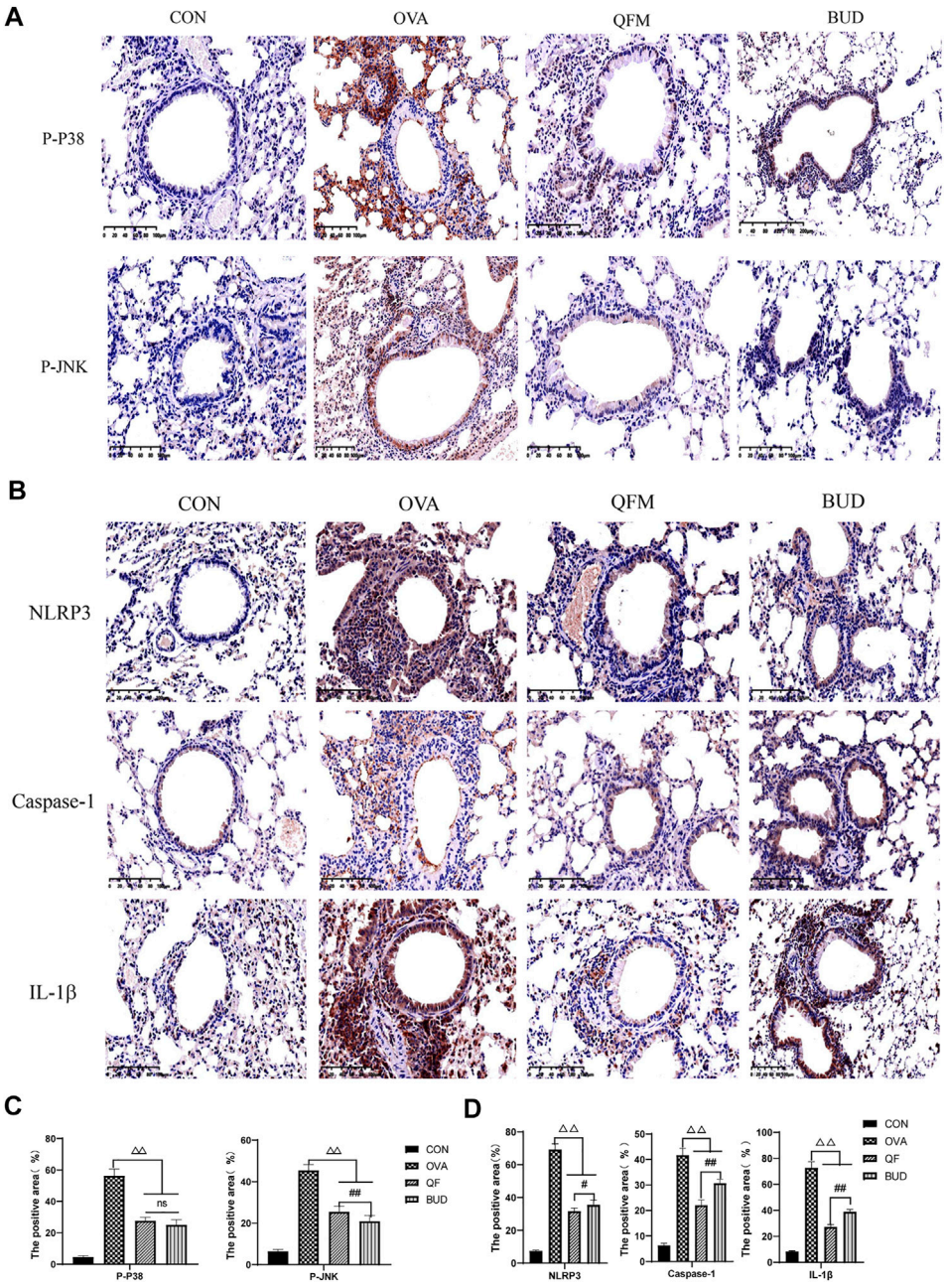


FIGURE 8
Effects of Qingfei oral liquid (QF) on the expression of key proteins related to MAPK and NOD-like receptor signal pathway. **(A)** Representative immunohistochemistry staining of p-P38 and p-JNK (200×, Brown particles represent positive protein expression). **(B)** Representative immunohistochemistry staining of NLRP3, caspase-1, and IL-1β (precursor IL-1 β and cleaved IL-1 β) (200×, Brown particles represent positive protein expression). **(C)** The positive area of p-P38 and p-JNK (%). **(D)** The positive area of NLRP3, caspase-1, and IL-1β (%). Each bar represents the mean ± standard deviation (SD). For all of these key proteins, there were significant differences between the control and the OVA groups ($p \leq 0.01$). ($n = 6$, $^{\triangle}p \leq 0.05$ vs. the OVA group, $^{\triangle\triangle}p \leq 0.01$ vs. the OVA group; $^{\#}p \leq 0.05$ vs. the budesonide (BUD) group, $^{\#\#}p \leq 0.01$ vs. the BUD group; ns: no difference; one-way ANOVA followed by Tukey's test).

nucleus. Three distinct MAPK pathways have been described: p38 MAPK pathway, c-Jun amino-terminal kinase (JNK) pathway, and extracellular signal-regulated kinases (ERKs or p42/44 MAPK pathway). The JNK and p38 MAPK pathways, known as stress-activated protein kinases, respond to inflammatory and environmental physical insults, whereas the ERK pathway is activated by mitogenic and proliferative stimuli (Khorasanizadeh et al., 2017; Manley et al., 2019). The Nod-like

receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome is an important component of innate immunity that can recognize intracellular pathogens or injury-related molecular patterns and mediate the processing, maturation, and release of a variety of cytokines, including Il-1β, are involved in the regulation of the occurrence and development of various inflammatory diseases. The NLRP3 inflammasome is mainly composed of NLRP3, apoptosis-associated speck-like protein containing CARD (ASC) and caspase-1 (Gritsenko et al., 2020; Louvrier et al., 2020).

Therefore, the expression levels of p-P38, p-JNK and NLRP3, Caspase-1, IL-1β in the lung tissues were measured using Immunohistochemistry to confirm the prediction results (Figure 8). Compared with the control mice, the expression levels of p-P38 and p-JNK were significantly increased in the lung tissues of OVA-induced asthmatic mice but significantly decreased in the lung tissues of asthmatic mice treated with QF (Figure 8A). The effect of QF on reducing p-JNK expression levels was less efficient than BUD treatment ($p < 0.01$), whereas the effect on reducing p-P38 expression levels was not significantly different from BUD ($p > 0.05$; Figure 8C). Similarly, compared to the control mice, mice with OVA-induced asthma showed a significantly higher expression of NLRP3, Caspase-1, IL-1β in the lung tissues, and the expression of these key proteins was significantly decreased in asthmatic mice treated with QF (Figure 8B). When compared to BUD treatment, QF had more effect on lowering NLRP3, Caspase-1 and IL-1β expression levels (Figure 8D). These results further confirmed the metagenomic functional prediction of QF in the respiratory microbiota.

4 Discussion

In the present study, we explored the anti-inflammatory activities of QF and its influence on respiratory microbiota in mice with OVA-induced asthma. We found that the downregulation of MAPK and Nod-like receptor signaling pathways may be the underlying mechanism of QF which alleviates airway inflammation in asthma by regulating respiratory microbiota. Furthermore, we also identified and analyzed bioactive compounds in QF, including baicalin, wogonoside, amygdalin, cryptotanshinone, polydatin, emodin, resveratrol, and baicalein, among others, using UHPLC-MS/MS. Previous studies have shown that these compounds have anti-inflammatory (He et al., 2020), anti-oxidation (Nagappan et al., 2019), anti-allergic, spasmolytic, immune response inhibition (Bui et al., 2017; Son et al., 2021) and other effects.

Our study found that QF can significantly reduce the aggregation of inflammatory cells around the small airway, thickening of the bronchial wall, and secretion of mucus in the lung tissue of OVA-induced asthmatic mice, and also reduce the levels of IgE, IL-4, IL-6, and IL-17A in BALF. The best effect was delivered by QF at a medium dose. QF has been widely used in the treatment of pediatric respiratory diseases in China and has been approved by the Chinese National Drug administration (Lin et al., 2021). Children aged about 6 years old should take one dose (crude drug 91 g) daily, which is equivalent to the medium dose of mice according to the conversion method of body surface area between mice and human. In our study, the high-dose and low-dose groups were set up respectively, that is, the dose of the medium-dose group was doubled, and the dose of the medium-dose group was halved. The high-dose group was set to see if increasing the dose would lead to better results. The experimental results showed that the high dose group could not improve the clinical efficacy in OVA-induced asthmatic mouse models. From the composition of Qingfei oral liquid, there are some herbs that affect the metabolism of bacteria. High dose of QF might affect some beneficial bacteria in respiratory tract, so the effect is reduced. It is suggested that there is no need to increase the dose of QF in clinical application to improve the efficacy. Previous research by other members of the research team found that QF alleviated asthma exacerbation by decreasing the levels of IL-4, IL-6, and IL-13 in the serum and inflammatory cells in the lung tissue of RSV-infected asthmatic mice (Zhou et al., 2018; Yu et al., 2021). In this study, QF was comparable to budesonide in reducing IL-6 and IL-17A and increasing IL-10 levels, but not as effective as budesonide in reducing IgE and IL-4 levels. Numerous previous studies (Kuo et al., 2017; Lambrecht et al., 2019; Hinks et al., 2021) have shown that eosinophilic asthma is mainly characterized by IgE and Th2 cytokines (IL-4, IL-5, etc.), whereas neutrophil asthma is mainly characterized by Th17 cytokines (IL-17, IL-6, etc.), which are involved in the development of steroid-resistant asthma (Nabe, 2020). In this study, a mouse model of OVA-induced asthma was dominated by eosinophil inflammatory infiltration. This is based on the increased the total IgE, OVA-specific IgE, and IL-4 levels as shown in Figure 3, and the highest proportion of eosinophils in BALF in Supplementary Figure S2. Therefore, the mouse model of OVA-induced asthma is mainly Th2-driven inflammation in this study. Interestingly, genetic predisposition and microbial metabolites appear to influence T cell differentiation plasticity. Several recent studies have linked the pathogenesis of asthma caused by IgE and IL-4 to a TH17-dependent mechanism. LPS stimulation promotes the transition from Th2-derived airway eosinophil inflammation to Th17-derived neutrophil inflammation in an ovalbumin allergy mouse model of asthma (Zhao et al., 2017), and IgE-related polymorphisms affect asthma TH 17 gene expression (Worth et al., 2018). Furthermore, IL4R variants linked to allergic asthma were discovered to exacerbate airway inflammation by promoting regulatory T cell conversion to TH17-like cells (Massoud et al., 2016). Numerous studies have shown that allergic asthma can progress to IL-17-mediated neutrophilic asthma. Even so, as the inflammation persisted, neutrophil inflammation mediated by Th17 cells and cytokines

began to play a role in the asthma process, due to the persistence of inflammation, neutrophil inflammation mediated by Th17 cell and cytokines also began to participate in the process of asthma. In our previous study (Zheng et al., 2021), dynamic observation of OVA-induced asthmatic mouse models showed that the 2-week phase of nebulization was the transition from eosinophilic inflammation to neutrophil inflammation and the initiation of small airway remodeling, so we selected the asthmatic mouse model during this critical transition period for our study. Many previous studies (Lönnkvist et al., 2001; Wenzel et al., 2016) have shown that Budesonide has a unique advantage for eosinophil asthma. In our asthmatic mouse model, it was found that Budesonide had a stronger inhibitory effect on eosinophilic inflammation than Qingfei oral Liquid (QF), but Traditional Chinese Medicine (TCM) compounds QF had a stronger inhibitory effect on IL-17 induced neutrophil inflammation than Budesonide. These results suggest that QF may have some advantages in the treatment of neutrophil asthma.

Our previous studies found that changes in respiratory microbiota are closely related to the pathophysiological processes of asthma, including airway inflammation (Zheng et al., 2021). Therefore, we proposed the following hypothesis: QF alleviates airway inflammation by regulating the respiratory microbiota of asthmatic mice. We found that QF and budesonide had no significant effect on the α-diversity of the respiratory microbiota in asthmatic mice, but both could affect the β-diversity of the respiratory microbiota to some extent. Since QF can alter the β-diversity of the respiratory microbiota composition in asthmatic mice, we further explored its effect on the composition of the respiratory microbiota. At the phylum level, compared to normal mice, the abundance of Proteobacteria in asthmatic mice was significantly increased, while that of Actinobacteria was significantly decreased. Previous studies have shown a significant increase in Proteobacteria in patients with asthma, especially in severe or steroid-resistant asthma (Li et al., 2017; Taylor et al., 2018). Actinobacteria were found to be more abundant in the normal population and in patients with eosinophilic asthma (Li et al., 2017; Hufnagl et al., 2020). Surprisingly, QF significantly reduced the abundance of Proteobacteria and slightly increased the abundance of Actinobacteria in the asthmatic mice. This may be one of the mechanisms through which QF reduces neutrophil inflammation. At the genus level, the abundance of *Pseudomonas* was significantly higher in asthmatic mice than in normal mice, while QF significantly reduced the abundance of *Pseudomonas*. Ferri et al. (2020) showed that the presence of *Pseudomonas* in sputum is an important risk factor for the persistent and frequent exacerbation of asthma. These results suggest that QF can regulate the composition of the respiratory microbiota in asthmatic mice.

Therefore, what effect does QF have on the host by regulating the dysbiosis of the respiratory microbiota in asthmatic mice? We explored the differences in bacterial metagenomic functional predictions. The results showed that the "MAPK signaling pathway-yeast" and "NOD-like receptor signaling pathway" related to airway inflammation were significantly upregulated in asthmatic mice, and QF significantly downregulated these two signaling pathways in the LRT. The three subfamilies of MAPKs have been involved in the pathogenesis of asthma, and JNK and P-38, in particular, are more closely associated with asthmatic airway inflammation, which can cause high expression of downstream factors such as IL-6, IL-1β, and IL-4 and IL-5, respectively (Dong et al., 2002; Pahl et al., 2002; Khorasanizadeh et al., 2017). The phosphorylation states and/or activities of all three MAPK members are upregulated in animal models of asthma (Dong et al., 2002). Inhibitors of JNK and P-38 both can significantly reduce airway inflammation in asthmatic patients (Bhavsar et al., 2010; Khorasanizadeh et al., 2017). NOD-like receptors (NLRs; nucleotide-binding oligomerization do-main-like receptors) represent a class of widespread, sophisticated signaling regulators, including more than 20 members have been reported (Liu et al., 2019). NLRP3 belongs to the NLRP subfamily, which is an inflammatory protein complex composed of the intracellular innate immune receptor NLRP3, adaptor protein ASC, and protease caspase-1 (Guo et al., 2015). They can help the body recognize endogenous and exogenous abnormal substances and release the inflammatory factors IL-1β and IL-18 (Tang et al., 2019). Previous studies have shown that elevated caspase-1 and IL-1β can be detected in mouse models of asthma, and increased IL-18 can also be detected in the sputum of asthmatic patients and is associated with asthma severity (Harada et al., 2009; Liao et al., 2015).

In order to verify the metagenomic function prediction results, this study further explored the expression of key proteins related to MAPK and NOD-like receptor signaling pathways in the lung tissues. The expression levels of p-P38 and p-JNK were significantly increased in the lung tissues of asthmatic mice but significantly decreased in the lung tissues of asthmatic mice treated with QF. Similarly, mice with OVA-induced asthma showed a significantly higher expression of NLRP3, Caspase-1, IL-1β in lung tissues, and QF can significantly downregulate the expression levels of these proteins. These results suggest that the potential mechanism of QF to alleviate airway inflammation in asthma by regulating respiratory microbiota may be related to downregulation of MAPK and NOD-like receptor signaling pathways. However, there were still many limitations to our research. For example, the *in vivo* sample size is relatively small. In addition, more studies are needed to confirm the strong link between QF in reducing airway inflammation and regulating respiratory microbiota.

In conclusion, we observed the anti-airway inflammation effect of QF in OVA-induced asthmatic mice, and for the first time, we explored the effect of QF on the composition of the

respiratory microbiota in asthmatic mice. Our study found that QF can regulate the composition of respiratory microbiota in asthmatic mice, and metagenomic function prediction suggests that QF can downregulate MAPK and NOD-like receptor signaling pathways that are significantly upregulated in asthmatic mice. Immunohistochemical results showed that QF could downregulate the expression of p-JNK, p-P38, NLRP3, Caspase-1, and IL-1β, which are the key proteins in the signaling pathways of lung tissue. This study provides a theoretical basis for effective use of QF in asthmatic airway inflammation.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA730096 and SRP320140.

Ethics statement

The animal study was reviewed and approved by the Center for Laboratory Animals, Shanghai Children's Hospital, School of Medicine. Shanghai Jiao Tong University, Shanghai, China.

Author contributions

LH and SG conceived and designed the study, critically revised the manuscript, and were responsible for the funding. JZ, QW, LZ, YZ, and MW completed the animal experiments, acquired and interpreted the data, and drafted and critically revised the manuscript. All the authors have read and approved the final manuscript.

Funding

This study was financially supported by two grants from the National Natural Science Foundation of China (Nos. 81774365 and 81973902).

Acknowledgments

We are grateful to Xiao-Yong Fan (TB Center, Shanghai Emerging and Reemerging Infectious Disease Institute, Shanghai) for his help with the experimental techniques.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.911667/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Total ion chromatography (TIC) of the blank samples. TIC of the blank samples in positive ion mode; **(B)** Total ion chromatography of the blank samples in negative ion mode.

SUPPLEMENTARY FIGURE S2
The leukocyte cell type counts in BALF samples. The BALF was centrifuged and the precipitated cells were counted after staining by Wright-Giemsa. Data are expressed as the mean ± standard deviation (SD) and were performed with one-way ANOVA followed by Tukey's tests. ($n = 6$, $^{\triangle}p \le 0.05$ vs. the OVA group, $^{\triangle\triangle}p \le 0.01$ vs. the OVA group; $^{\#}p \le 0.05$ vs. the budesonide (BUD) group, $^{\#\#}p \le 0.01$ vs. the BUD group; $^{\blacklozenge}p \le 0.05$ vs. the QFM group, $^{\blacklozenge\blacklozenge}p \le 0.01$ vs the QFM group; ns: no difference; line over bars indicates that all groups are include.).

References

Asher, M. I., García-Marcos, L., Pearce, N. E., and Strachan, D. P. (2020). Trends in worldwide asthma prevalence. *Eur. Respir. J.* 56, 2002094. doi:10.1183/13993003.02094-2020

Bhavsar, P., Khorasani, N., Hew, M., Johnson, M., and Chung, K. F. (2010). Effect of p38 MAPK inhibition on corticosteroid suppression of cytokine release in severe asthma. *Eur. Respir. J.* 35, 750–756. doi:10.1183/09031936.00071309

Bokulich, N. A., Dillon, M. R., Bolyen, E., Kaehler, B. D., Huttley, G. A., Caporaso, J. G., et al. (2018). q2-sample-classifier: machine-learning tools for microbiome classification and regression. *J. Open Res. Softw.* 3, 934. doi:10.21105/joss.00934

Budden, K. F., Shukla, S. D., Rehman, S. F., Bowerman, K. L., Keely, S., Hugenholtz, P., et al. (2019). Functional effects of the microbiota in chronic respiratory disease. *Lancet. Respir. Med.* 7, 907–920. doi:10.1016/s2213-2600(18)30510-1

Bui, T. T., Piao, C. H., Song, C. H., Lee, C. H., Shin, H. S., Chai, O. H., et al. (2017). Baicalein, wogonin, and Scutellaria baicalensis ethanol extract alleviate ovalbumin-induced allergic airway inflammation and mast cell-mediated anaphylactic shock by regulation of Th1/Th2 imbalance and histamine release. *Anat. Cell Biol.* 50, 124–134. doi:10.5115/acb.2017.50.2.124

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., Holmes, S. P., et al. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi:10.1038/nmeth.3869

Cui, F., Shi, C. L., Zhou, X. J., Wen, W., Gao, X. P., Wang, L. Y., et al. (2020). Lycium barbarum polysaccharide extracted from lycium barbarum leaves ameliorates asthma in mice by reducing inflammation and modulating gut microbiota. *J. Med. Food* 23, 699–710. doi:10.1089/jmf.2019.4544

Dong, C., Davis, R. J., and Flavell, R. A. (2002). MAP kinases in the immune response. *Annu. Rev. Immunol.* 20, 55–72. doi:10.1146/annurev.immunol.20.091301.131133

Ferri, S., Crimi, C., Campisi, R., Cacopardo, G., Paoletti, G., Puggioni, F., et al. (2020). Impact of asthma on bronchiectasis severity and risk of exacerbations. *J. Asthma* 59, 469–475. doi:10.1080/02770903.2020.1857395

Finkelman, F. D., Hogan, S. P., Hershey, G. K., Rothenberg, M. E., and Wills-Karp, M. (2010). Importance of cytokines in murine allergic airway disease and human asthma. *J. Immunol.* 184, 1663–1674. doi:10.4049/jimmunol.0902185

Gandhi, G. R., Leão, G. C. S., Calisto, V., Vasconcelos, A. B. S., Almeida, M. L. D., Quintans, J. S. S., et al. (2020). Modulation of interleukin expression by medicinal plants and their secondary metabolites: a systematic review on anti-asthmatic and immunopharmacological mechanisms. *Phytomedicine* 70, 153229. doi:10.1016/j.phymed.2020.153229

Gritsenko, A., Green, J. P., Brough, D., and Lopez-Castejon, G. (2020). Mechanisms of NLRP3 priming in inflammaging and age related diseases. *Cytokine Growth Factor Rev.* 55, 15–25. doi:10.1016/j.cytogfr.2020.08.003

Guo, H., Callaway, J. B., and Ting, J. P. (2015). Inflammasomes: mechanism of action, role in disease, and therapeutics. *Nat. Med.* 21, 677–687. doi:10.1038/nm.3893

Hall, M., and Beiko, R. G. (2018). 16S rRNA gene analysis with QIIME2. *Methods Mol. Biol.* 1849, 113–129. doi:10.1007/978-1-4939-8728-3_8

Harada, M., Obara, K., Hirota, T., Yoshimoto, T., Hitomi, Y., Sakashita, M., et al. (2009). A functional polymorphism in IL-18 is associated with severity of bronchial asthma. *Am. J. Respir. Crit. Care Med.* 180, 1048–1055. doi:10.1164/rccm.200905-0652OC

He, X. Y., Wu, L. J., Wang, W. X., Xie, P. J., Chen, Y. H., Wang, F., et al. (2020). Amygdalin - a pharmacological and toxicological review. *J. Ethnopharmacol.* 254, 112717. doi:10.1016/j.jep.2020.112717

Hinks, T. S. C., Levine, S. J., and Brusselle, G. G. (2021). Treatment options in type-2 low asthma. *Eur. Respir. J.* 57, 2000528. doi:10.1183/13993003.00528-2020

Huang, Y. J., Nariya, S., Harris, J. M., Lynch, S. V., Choy, D. F., Arron, J. R., et al. (2015). The airway microbiome in patients with severe asthma: associations with disease features and severity. *J. Allergy Clin. Immunol.* 136, 874–884. doi:10.1016/j.jaci.2015.05.044

Hufnagl, K., Pali-Schöll, I., Roth-Walter, F., and Jensen-Jarolim, E. (2020). Dysbiosis of the gut and lung microbiome has a role in asthma. *Semin. Immunopathol.* 42, 75–93. doi:10.1007/s00281-019-00775-y

Jing, X., Yan, W., Zeng, H., and Cheng, W. (2020). Qingfei oral liquid alleviates airway hyperresponsiveness and mucus hypersecretion via TRPV1 signaling in RSV-infected asthmatic mice. *Biomed. Pharmacother.* 128, 110340. doi:10.1016/j.biopha.2020.110340

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277–D280. doi:10.1093/nar/gkh063

Khorasanizadeh, M., Eskian, M., Gelfand, E. W., and Rezaei, N. (2017). Mitogen-activated protein kinases as therapeutic targets for asthma. *Pharmacol. Ther.* 174, 112–126. doi:10.1016/j.pharmthera.2017.02.024

Kubo, F., Ariestanti, D. M., Oki, S., Fukuzawa, T., Demizu, R., Sato, T., et al. (2019). Loss of the adhesion G-protein coupled receptor ADGRF5 in mice induces airway inflammation and the expression of CCL2 in lung endothelial cells. *Respir. Res.* 20, 11. doi:10.1186/s12931-019-0973-6

Kujur, W., Gurram, R. K., Haleem, N., Maurya, S. K., and Agrewala, J. N. (2015). Caerulomycin A inhibits Th2 cell activity: a possible role in the management of asthma. *Sci. Rep.* 5, 15396. doi:10.1038/srep15396

Kuo, C. S., Pavlidis, S., Loza, M., Baribaud, F., Rowe, A., Pandis, I., et al. (2017). T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. *Eur. Respir. J.* 49, 1602135. doi:10.1183/13993003.02135-2016

Lambrecht, B. N., Hammad, H., and Fahy, J. V. (2019). The cytokines of asthma. *Immunity* 50, 975–991. doi:10.1016/j.immuni.2019.03.018

Langille, M. G., Zaneveld, J., Caporaso, J. G., Mcdonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi:10.1038/nbt.2676

Larsen, J. M., Musavian, H. S., Butt, T. M., Ingvorsen, C., Thysen, A. H., Brix, S., et al. (2015). Chronic obstructive pulmonary disease and asthma-associated Proteobacteria, but not commensal Prevotella spp., promote Toll-like receptor 2-independent lung inflammation and pathology. *Immunology* 144, 333–342. doi:10.1111/imm.12376

Li, N., Qiu, R., Yang, Z., Li, J., Chung, K. F., Zhong, N., et al. (2017). Sputum microbiota in severe asthma patients: relationship to eosinophilic inflammation. *Respir. Med.* 131, 192–198. doi:10.1016/j.rmed.2017.08.016

Liao, Z., Xiao, H. T., Zhang, Y., Tong, R. S., Zhang, L. J., Bian, Y., et al. (2015). IL-1β: a key modulator in asthmatic airway smooth muscle hyper-reactivity. *Expert Rev. Respir. Med.* 9, 429–436. doi:10.1586/17476348.2015.1063422

Licari, A., Brambilla, I., Marseglia, A., De Filippo, M., Paganelli, V., Marseglia, G. L., et al. (2018). Difficult vs. Severe asthma: definition and limits of asthma control in the pediatric population. *Front. Pediatr.* 6, 170. doi:10.3389/fped.2018.00170

Lin, L., An, L., Chen, H., Feng, L., Lu, M., Liu, Y., et al. (2021). Integrated network Pharmacology and lipidomics to reveal the inhibitory effect of Qingfei oral liquid on excessive autophagy in RSV-induced lung inflammation. *Front. Pharmacol.* 12, 777689. doi:10.3389/fphar.2021.777689

Liu, P., Lu, Z., Liu, L., Li, R., Liang, Z., Shen, M., et al. (2019). NOD-like receptor signaling in inflammation-associated cancers: from functions to targeted therapies. *Phytomedicine* 64, 152925. doi:10.1016/j.phymed.2019.152925

Lönnkvist, K., Hellman, C., Lundahl, J., Halldén, G., and Hedlin, G. (2001). Eosinophil markers in blood, serum, and urine for monitoring the clinical course in childhood asthma: impact of budesonide treatment and withdrawal. *J. Allergy Clin. Immunol.* 107 (5), 812–817. doi:10.1067/mai.2001.114246

Louvrier, C., Assrawi, E., El Khouri, E., Melki, I., Copin, B., Bourrat, E., et al. (2020). NLRP3-associated autoinflammatory diseases: phenotypic and molecular characteristics of germline versus somatic mutations. *J. Allergy Clin. Immunol.* 145, 1254–1261. doi:10.1016/j.jaci.2019.11.035

Manley, G. C. A., Parker, L. C., and Zhang, Y. (2019). Emerging regulatory roles of dual-specificity phosphatases in inflammatory airway disease. *Int. J. Mol. Sci.* 20, E678. doi:10.3390/ijms20030678

Massoud, A. H., Charbonnier, L. M., Lopez, D., Pellegrini, M., Phipatanakul, W., Chatila, T. A., et al. (2016). An asthma-associated IL4R variant exacerbates airway inflammation by promoting conversion of regulatory T cells to TH17-like cells. *Nat. Med.* 22 (9), 1013–1022. doi:10.1038/nm.4147

Murphy, K. R., Hong, J. G., Wandalsen, G., Larenas-Linnemann, D., El Beleidy, A., Zaytseva, O. V., et al. (2020). Nebulized inhaled corticosteroids in asthma treatment in children 5 Years or younger: a systematic review and global expert analysis. *J. Allergy Clin. Immunol. Pract.* 8, 1815–1827. doi:10.1016/j.jaip.2020.01.042

Nabe, T. (2020). Steroid-resistant asthma and neutrophils. *Biol. Pharm. Bull.* 43, 31–35. doi:10.1248/bpb.b19-00095

Nagappan, A., Kim, J. H., Jung, D. Y., and Jung, M. H. (2019). Cryptotanshinone from the salvia miltiorrhiza bunge attenuates ethanol-induced liver injury by activation of AMPK/SIRT1 and Nrf2 signaling pathways. *Int. J. Mol. Sci.* 21, E265. doi:10.3390/ijms21010265

Pahl, A., Zhang, M., Kuss, H., Szelenyi, I., and Brune, K. (2002). Regulation of IL-13 synthesis in human lymphocytes: implications for asthma therapy. *Br. J. Pharmacol.* 135, 1915–1926. doi:10.1038/sj.bjp.0704656

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). Stamp: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi:10.1093/bioinformatics/btu494

Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi:10.1093/molbev/msp077

Ramakrishnan, R. K., Al Heialy, S., and Hamid, Q. (2019). Role of IL-17 in asthma pathogenesis and its implications for the clinic. *Expert Rev. Respir. Med.* 13, 1057–1068. doi:10.1080/17476348.2019.1666002

Robinson, D., Humbert, M., Buhl, R., Cruz, A. A., Inoue, H., Korom, S., et al. (2017). Revisiting type 2-high and type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. *Clin. Exp. Allergy* 47, 161–175. doi:10.1111/cea.12880

Segal, L. N., Clemente, J. C., Li, Y., Ruan, C., Cao, J., Danckers, M., et al. (2017a). Anaerobic bacterial fermentation products increase tuberculosis risk in antiretroviral-drug-treated HIV patients. *Cell Host Microbe* 21, 530. doi:10.1016/j.chom.2017.03.003

Segal, L. N., Clemente, J. C., Wu, B. G., Wikoff, W. R., Gao, Z., Li, Y., et al. (2017b). Randomised, double-blind, placebo-controlled trial with azithromycin selects for anti-inflammatory microbial metabolites in the emphysematous lung. *Thorax* 72, 13–22. doi:10.1136/thoraxjnl-2016-208599

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi:10.1186/gb-2011-12-6-r60

Son, S. H., Kang, J., Ahn, M., Nam, S., Jung, Y. W., Lee, K. Y., et al. (2021). Synthesis and biochemical evaluation of baicalein prodrugs. *Pharmaceutics* 13, 1516. doi:10.3390/pharmaceutics13091516

Tang, Y. S., Zhao, Y. H., Zhong, Y., Li, X. Z., Pu, J. X., Luo, Y. C., et al. (2019). Neferine inhibits LPS-ATP-induced endothelial cell pyroptosis via regulation of ROS/NLRP3/Caspase-1 signaling pathway. *Inflamm. Res.* 68, 727–738. doi:10.1007/s00011-019-01256-6

Taylor, S. L., Leong, L. E. X., Choo, J. M., Wesselingh, S., Yang, I. A., Upham, J. W., et al. (2018). Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J. Allergy Clin. Immunol.* 141, 94. doi:10.1016/j.jaci.2017.03.044

Temelkovski, J., Hogan, S. P., Shepherd, D. P., Foster, P. S., and Kumar, R. K. (1998). An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. *Thorax* 53, 849–856. doi:10.1136/thx.53.10.849

Teo, S. M., Tang, H. H. F., Mok, D., Judd, L. M., Watts, S. C., Pham, K., et al. (2018). Airway microbiota dynamics uncover a critical window for interplay of pathogenic bacteria and allergy in childhood respiratory disease. *Cell Host Microbe* 24, 341. doi:10.1016/j.chom.2018.08.005

Tsang, M. S., Cheng, S. W., Zhu, J., Atli, K., Chan, B. C., Liu, D., et al. (2018). Anti-inflammatory activities of pentaherbs formula and its influence on gut microbiota in allergic asthma. *Molecules* 23, E2776. doi:10.3390/molecules23112776

Tumes, D. J., Papadopoulos, M., Endo, Y., Onodera, A., Hirahara, K., Nakayama, T., et al. (2017). Epigenetic regulation of T-helper cell differentiation, memory, and plasticity in allergic asthma. *Immunol. Rev.* 278, 8–19. doi:10.1111/imr.12560

Wenzel, S., Castro, M., Corren, J., Maspero, J., Wang, L., Zhang, B., et al. (2016). Dupilumab efficacy and safety in adults with uncontrolled persistent asthma despite use of medium-to-high-dose inhaled corticosteroids plus a long-acting β2 agonist: a randomised double-blind placebo-controlled pivotal phase 2b dose-ranging trial. *Lancet (London, Engl.)* 388 (10039), 31–44. doi:10.1016/S0140-6736(16)30307-5

Worth, L., Michel, S., Gaertner, V. D., Kabesch, M., and Schieck, M. (2018). Asthma- and IgE-associated polymorphisms affect expression of TH 17 genes. *Allergy* 73 (6), 1342–1347. doi:10.1111/all.13422

Yu, L., Wang, J., Zou, Y., Zeng, H., Cheng, W., Jing, X., et al. (2021). Qingfei oral liquid inhibited autophagy to alleviate inflammation via mTOR signaling pathway in RSV-infected asthmatic mice. *Biomed. Pharmacother.* 138, 111449. doi:10.1016/j.biopha.2021.111449

Zhao, S., Jiang, Y., Yang, X., Guo, D., Wang, Y., Wang, J., et al. (2017). Lipopolysaccharides promote a shift from Th2-derived airway eosinophilic inflammation to Th17-derived neutrophilic inflammation in an ovalbumin-sensitized murine asthma model. *J. Asthma* 54 (5), 447–455. doi:10.1080/02770903.2016.1223687

Zheng, J., Wu, Q., Zou, Y., Wang, M., He, L., Guo, S., et al. (2021). Respiratory microbiota profiles associated with the progression from airway inflammation to remodeling in mice with OVA-induced asthma. *Front. Microbiol.* 12, 723152. doi:10.3389/fmicb.2021.723152

Zhou, L. H., Xu, J. Y., Dai, C., Fan, Y. M., and Yuan, B. (2018). Label-free quantitative proteomics reveals fibrinopeptide B and heparin cofactor II as potential serum biomarkers in respiratory syncytial virus-infected mice treated with Qingfei oral liquid formula. *Chin. J. Nat. Med.* 16 (4), 241–251. doi:10.1016/S1875-5364(18)30054-2

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 26 September 2022
DOI 10.3389/fphar.2022.907921

OPEN ACCESS

EDITED BY
Carlos L. Cespedes-Acuña,
University of Bio Bio Chillan Chile, Chile

REVIEWED BY
Guang Dong Tong,
The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, China
Yuehong Huang,
Fujian Medical University Union Hospital, China

*CORRESPONDENCE
Xudong Hu,
huxudongsh@126.com
Chenghai Liu,
Chenghai.liu@outlook.com

SPECIALTY SECTION
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

RECEIVED 30 March 2022
ACCEPTED 06 September 2022
PUBLISHED 26 September 2022

CITATION
Xing L, Zeng R, Huang K, Xue J, Liu H, Zhao Z, Peng Y, Hu X and Liu C (2022), Fuzheng Huayu Recipe and its active compounds inhibited HBeAg production by promoting TOMM34 gene expression in HBV-infected hepatocytes.
Front. Pharmacol. 13:907921.
doi: 10.3389/fphar.2022.907921

Fuzheng Huayu Recipe and its active compounds inhibited HBeAg production by promoting TOMM34 gene expression in HBV-infected hepatocytes

Lu Xing[1], Rui Zeng[2], Kai Huang[1], Jingbo Xue[1], Hongliang Liu[1], Zhimin Zhao[1], Yuan Peng[1], Xudong Hu[2]* and Chenghai Liu[1,3]*

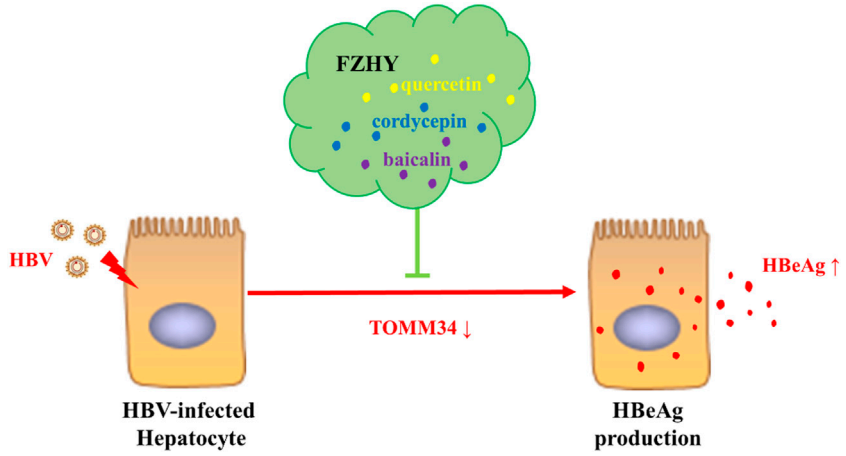
[1]Institute of Liver diseases, Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2]Department of Biology, School of Basic Medical Sciences, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [3]Shanghai Key Laboratory of Traditional Chinese Clinical Medicine, Shanghai, China

Background and aim: Fuzheng Huayu Recipe (FZHY) is a Chinese patent medicine (approval No. Z20020074) included in the national medical insurance catalogue, which is mainly used for anti-hepatic fibrosis treatment of hepatitis B virus (HBV) induced liver fibrosis and liver cirrhosis. In clinical practice, we discovered that FZHY might also have a direct anti-HBV effect on inhibiting HBeAg production, but the mechanism underlying was unclear. This study aimed to clarify the molecular mechanism of the inhibition effect of FZHY on HBeAg production.

Methods: The decrease degree of serum HBeAg titer in FZHY + entecavir (ETV) group patients were analyzed through clinical data. C57BL/6N-Tg (1.28HBV)/Vst HBV transgenic mice were used for *in vivo* experiments. HepG2. 2.15 cells (wild-type HBV replication cells) were used for *in vitro* experiments.

Results: The clinical study results showed that the decrease degree of serum HBeAg titer in FZHY+ETV group was significantly higher than that in ETV group after 48 weeks treatment. *In vivo* experiments results showed that FZHY could significantly reduce the serum HBeAg titer in HBV transgenic mice, and promote HBeAg seroconversion. *In vitro* experiments results showed that FZHY could reduce HBeAg titer dependently, but it did not significantly inhibit the expression of HBsAg and HBV-DNA. Further cell experiments *in vitro* discovered that TOMM34 might be the key target for FZHY to inhibit HBeAg production. The subsequent pharmacological screening experiment of 20 active compounds in FZHY showed that quercetin, baicalin and cordycepin could promote the expression of TOMM34 gene and reduce the production of HBeAg.

Conclusion: In conclusion, FZHY and its active compounds quercetin, baicalin and cordycepin could inhibit HBeAg production by promoting the expression of TOMM34 gene in HBV-infected hepatocytes.



GRAPHICAL ABSTRACT

KEYWORDS

Fuzheng Huayu Recipe, HBeAg, TOMM34, active compounds, HBV

Introduction

Hepatitis B virus (HBV) has brought a heavy burden on global health. According to WHO estimates, there were 296 million chronic HBV infections worldwide in 2019, with 1.5 million new infections each year. In 2019, hepatitis B caused 820,000 deaths, mainly due to liver cirrhosis and hepatocellular carcinoma (HCC) (WHO, 2021). Around 10% people with HBV infection will be the patients with HBV-related cirrhosis (Tan et al., 2021), at least 50% of liver cancer cases in the world come from chronic HBV infection (Xie, 2017).

Hepatitis B virus e antigen (HBeAg) is a major product of HBV replication in human body, which is encoded by pre-C and C genes. HBeAg is a soluble component of hepatitis B core antigen and a marker of HBV replication and infectivity. As an immunomodulatory factor, HBeAg can regulate the host immune response, inhibit the cytotoxic activity of host T cells, and form immune tolerance to HBV infection (Merkle et al., 2000; Barth et al., 2001; Wilson et al., 2011). HBeAg loss and serum transformation are very important for prognosis. The continuous positive HBeAg in patients with chronic hepatitis B indicates persistent HBV infection, which is a sign of active hepatitis, and the probability of developing cirrhosis is relatively high (Deng et al., 2009). High serum HBeAg levels are associated with the occurrence of liver cirrhosis and HCC (Lin et al., 2007; Liu et al., 2016). Therefore, it is a very important goal of hepatitis B treatment to promote serum HBeAg loss and serum HBeAg conversion.

In recent years, the practice of complementary medicine and alternative medicine including traditional Chinese medicine (TCM) in the treatment of chronic hepatitis B has increased significantly all over the world (Chan et al., 2010). The combination of traditional Chinese medicine and modern medicine has gradually become an important way to treat chronic hepatitis B and its related diseases. In China, clinicians use a variety of traditional Chinese medicine prescriptions to treat chronic hepatitis B liver fibrosis. Fuzheng Huayu Recipe is one of the first-line traditional Chinese medicine prescriptions recommended in the Chinese pharmacopoeia for chronic hepatitis B liver fibrosis. In clinical practice, we discovered that FZHY could inhibit HBeAg production, but the mechanism underlying was unknown. In this study, we aimed to discover the inhibitory mechanism of FZHY on HBeAg production (Figure 1).

Materials and methods

Patient acceptance criteria and grouping

The cases were from 5 hospitals including Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine, Zhongshan Hospital Affiliated to Fudan University, Beijing Ditan Hospital Affiliated to Capital Medical University, Beijing Youan Hospital Affiliated to Capital Medical University, and Shijiazhuang Fifth Hospital: clinical study of TCM combined with entecavir (ETV) in the treatment of hepatitis B cirrhosis (2015–445-73–02). All patients were approved by the Ethics Committee. Patient grouping with HBeAg-positive hepatitis B fibrosis which treated for 48 weeks: entecavir + Fuzheng Huayu tablet group (treatment group): 79 cases; entecavir + Fuzheng Huayu placebo group (control group): 77 cases.

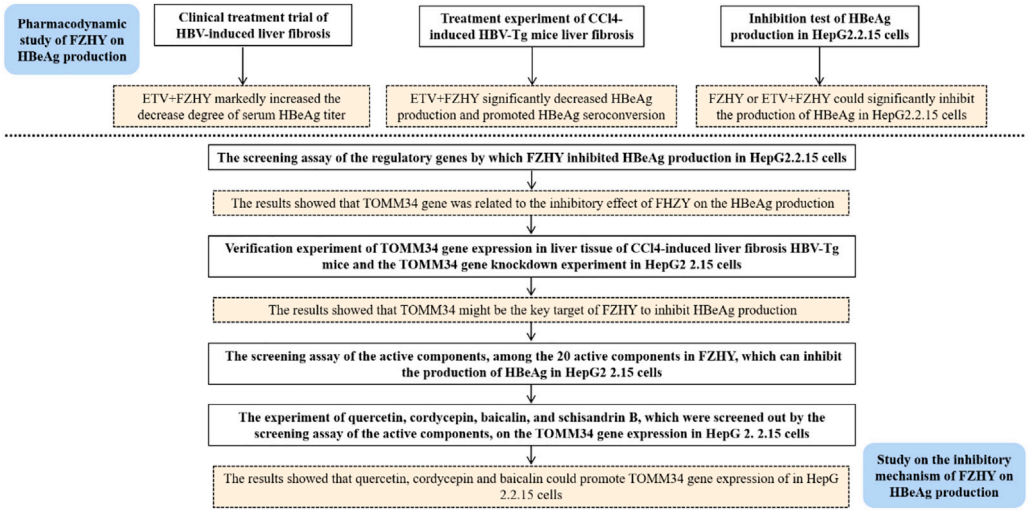


FIGURE 1
The flowchart of the study.

Drug preparation, animal grouping, modeling and administration

Fuzheng Huayu Recipe extract powder (batch No.180,206) was provided by Shanghai Huanghai Pharmaceutical Co., Ltd. Quality control standard: brown powder, bitter and astringent; For each gram of fermented grass fungus powder, adenosine shall not be less than 1000 g/batch, Salvia miltiorrhiza sodium shall not be less than 3000 g/batch, salvianolic acid B shall not be less than 5000 g/batch, water content shall be less than 8.0%, the total number of aerobic bacteria shall not exceed 1000 CFU/g, the total number of molds and yeasts shall not exceed 100 CFU, and *Escherichia coli* shall not be detected. Entecavir (ETV) (cat#21995) was purchased from MCE.

48 male C57BL/6N-Tg (1.28HBV)/Vst HBV transgenic mice (HBV-Tg mice) and 12 male C57BL/6N wild-type mice, weighing (20 ± 5) g, SPF grade, were purchased from and raised in Shanghai Branch of Beijing Weitonglihua Technology Co., Ltd. All mice were given free diet and drinking water.

After 1 week of adaptive feeding of all mice, 48 male HBV-Tg mice were randomly divided into 4 groups (12 mice/group): control group (HBV-Tg control), CCl4 liver fibrosis model group (HBV-Tg CCl4 model), ETV group (HBV-Tg CCl4 model + ETV), and ETV+FZHY group (HBV-Tg CCl4 model+ETV+FZHY). At the same time, 12 male C57BL/6N wild-type mice were used as the wild-type control group (WT control). Mice in HBV-Tg CCl4 model group, HBV-Tg CCl4 model+ETV group, and HBV-Tg CCl4 model+ETV+FZHY group were injected with 10% CCl4 olive oil solution intraperitoneally at the dose of 2 ml/kg mouse body weight, once every other day for 6 weeks. Mice in WT control group and HBV-Tg control group were given the same dose of olive oil at the same time.

After the 6th injection of CCl4, ETV (0.1 mg drug/kg mouse body weight) was administered to HBV-Tg CCl4 model+ETV group and HBV-Tg CCl4 model+ETV+FZHY group mice by gavage, which was dissolved in 0.1% sodium carboxymethyl cellulose solution, once a day for 28 days. FZHY (5.6 g crude drug/kg mouse body weight) was administered to HBV-Tg CCl4 model+ETV+FZHY group by gavage, which was dissolved in double distilled water, at least 4 h apart from ETV administered, once a day for 28 days. WT control group, HBV-Tg control group and HBV-Tg CCl4 model group were given the same amount of solvent by gavage. 48 h after the 18th injection of CCl4, the mice were sacrificed.

Determination of serum HBsAg, HBeAg, HBeAb and HBV DNA

Serum HBsAg, HBeAg and HBeAb were detected by ELISA according to the operation instructions of the kit. Serum HBV DNA was detected by fluorescence quantitative PCR (probe method), samples were prepared according to the instructions of the kit, and amplified by ABIViiA7 fluorescence quantitative PCR instrument. The diagnostic kits (ELISA) for Hepatitis B Virus Surface Antigen, Hepatitis B Virus e Antigen, Hepatitis B e Antibody and the quantification kit (PCR-Fluorescence Probing) for Hepatitis B Virus DNA were purchased from Shanghai Kehua Bio-Engingeering Co.,Ltd.

Liver histopathology

The liver tissue of mice was fixed with 10% neutral formaldehyde for 48 h, then dehydrated with gradient alcohol,

embedded in paraffin, 4 μM thick section, dewaxing with xylene to water. Finally, HE staining was used to observe the degree of liver inflammation and Sirius red staining was used to observe the collagen deposition in liver tissue.

Immunohistochemical staining of liver tissue

Mouse liver tissue sections were dewaxed to water, endogenous peroxidase was inactivated by 3% H_2O_2, antigen was thermally repaired by citrate buffer microblog boiling method, 5% BSA was blocked, primary antibody was incubated at 4 °C overnight, secondary antibody was incubated at 37°C, DAB color was developed, hematoxylin was lined, alcohol was gradually dehydrated, xylene was transparent, neutral gum was sealed, and observed and photographed under the microscope, Image-Pro Plus software was used for semi quantitative analysis.

Cell culture and cytotoxicity assay

Caffeic acid (cat#100317), entomolic acid (Lot#WH0834), ergosterol (cat#140627), adenosine (Lot#WH0506), protopanaxadiol (cat#110729), baicalin (cat#110830), rutin (cat#140108), ginsenoside F2 (cat#131229), uridine (cat#130311), tanshinone IIA (cat#200506), salvianolic acid B (cat#200316), cryptotanshinone (cat#140119), sodium tanshinol (Lot#WH0060), protopanaxatriol (cat#150429), quercetin (cat#140413), amygdalin (cat#191227), cordycepin (cat#141124), ginsenoside Rb1 (cat#141128), ginsenoside RB3 (cat#121216), and ginsenoside Rg3 (cat#121229) were purchased from Shanghai Ronghe pharmaceutical. schisandrin B (cat#15110531) was purchased from TAUTO BIOTECH.

Human liver cancer HepG2 2.15 cell line was purchased from BeNa Culture Collection (BNCC). HepG2 2.15 cells were cultured in DMEM medium containing 10% FBS +0.4% G418. Culture conditions: 37°C, 5% CO_2 and 95% humidity.

1×10^5/well HepG2.2.15 cells were seeded into 96 well plates, FZHY (6.25, 12.5, 25, 50, 100 μg/ml), ETV (3.125, 6.25, 12.5, 25, 50 μg/ml) or 20 monomers (1, 10, 100 μM caffeic acid, cordycepin acid, ergosterol, adenosine, protopanaxadiol, baicalin, rutin, ginsenoside F2, uridine, tanshinone IIA, salvianolic acid B, cryptotanshinone, Danshensu Sodium, protopanaxatriol, quercetin, amygdalin, cordycepin, schisandrin B, ginsenoside Rb1, ginsenoside Rb3 and ginsenoside Rg3) were administered, 6 duplicate wells were set for each drug. After incubation for 24 h, the supernatant was discarded, 100 μl 10% CCK8 was added to each well and continue to culture for 2 h. Then, the absorbance was measured at the wavelength of 450/630 nm and the cell survival rate was calculated, which was used to evaluate the cytotoxicity of FZHY and 20 monomers. The Cell Counting Kit (CCK8) was purchased from Thermo Fisher Scientific Inc.

TABLE 1 Gene primer sequence.

Gene	Sequence
Human/mouse-βactin-F	TGACGAGGCCCAGAGCAAGA
Human/mouse-βactin-R	ATGGGCACAGTGTGGGTGAC
Human-C11orf9-F	GACCCCAACTACCAGTCCATC
Human-C11orf9-R	TCGGGCGTCTTGACGTACT
Human-FJX1-F	CCGGCTCGTAAGCAACCTC
Human-FJX1-R	AGCGGCTCGTTATACTTGTCC
Human-ADCY5-F	TCTCCTGCACCAACATCGTG
Human-ADCY5-R	CATGGCAACATGACGGGGA
Human-SLA39A11-F	CAGCTCTCGTGTTCGTATTCTC
Human-SLA39A11-R	TCAGCCAAGTAGACAAAAGCC
Human-SETMAR-F	GAAGCGGCAAAGACGACAC
Human-SETMAR-R	GAGTGGGATCAATGTCTGCTC
Human-TNFAIP3-F	TCCTCAGGCTTTGTATTTGAGC
Human-TNFAIP3-R	TGTGTATCGGTGCATGGTTTTA
Human-SRA1-F	CTGAGGTCAGTCAGTGGATGG
Human-SRA1-R	AGCCTGGTATGGTATGGTTCT
Human-HLA-E-F	TTCCGAGTGAATCTGCGGAC
Human-HLA-E-R	GTCGTAGGCGAACTGTTCATAC
Human-HIGD1A-F	AAGAGGCACCATTCGTACCC
Human-HIGD1A-R	ACCAACAGTCATTGCTCCTACA
Human-FAM176A-F	AGCAACATCCTAGCGGCCTA
Human-FAM176A-R	TGTGTGGCAAGAGATCCTTATCA
Human-TOMM34-F	TGCATCAAAGATTGCACTTCAGC
Human-TOMM34-R	GCAGCACAGTCTTATAGTCAACA
Mouse-TOMM34-F	CCTGGAAGGCATCAACAGAAT
Mouse-TOMM34-R	GGCACTCTGCTCTTCGTAGC

siTOMM34 interference experiment

1.25 μl of 20 μM TOMM34 siRNA storage solution was diluted with 30 μl riboFECT™ CP Buffer and gently mixed. Then, 3 μl riboFECT™ CP Reagent was added, gently blown and mixed, incubated for 15 min at room temperature to prepare transfection complex. Next, transfection complex was added to appropriate amount of non-double antibody complete DMEM medium and gently mixed. 5×10^4/well HepG2.2.15 cells were seeded into 48-well plate and TOMM34 siRNA was transfected into 48-well plates at the final transfection concentration of 50 nM. Control group, FZHY group, NC group, positive group, siTOMM34 group, ETV group and FZHY+ETV group were set. The 48-well plate was cultured in 5% CO_2, 37°C for 96 h. Finally, the gene expression of TOMM34 was detected by qPCR, and the expression of HBeAg was detected by ELISA.

TABLE 2 The therapeutic outcomes of the two groups after 48 weeks treatment.

	FZHY + ETV n = 79	ETV n = 77	*p* Value
Age, year (mean±SD)	42.20±8.45	42.43±8.41	0.880
Male, n (%)	64 (81.01)	60 (77.92)	0.633
HBeAg, COI (median, IQR) 0–48 weeks	0.18 (-0.02–18.10)	0.01 (-0.04–1.28)	0.039
HBV DNA, Log10 IU/ml (median, IQR) 0–48 weeks	3.97 (3.18–5.67)	3.64 (2.21–5.45)	0.070
ALT, U/L (median, IQR) 0–48 weeks	15.00 (4.00–32.70)	12.00 (1.00–36.25)	0.389
AST, U/L (median, IQR) 0–48 weeks	10.00 (1.50–29.00)	9.00 (2.00–28.50)	0.697
TBIL, μmol/L (median, IQR) 0–48 weeks	0.83 (-3.20–6.10)	-0.50 (-3.05–4.30)	0.497

HBeAg: Hepatitis B e antigen; HBV-DNA: Hepatitis B virus DNA; ALT: alanine transaminase; AST: aspartate aminotransferase; TBIL: total bilirubin; 0–48, weeks: the value detected at 0 weeks minus the value detected at 48 weeks.

TOMM34 siRNA and transfection Kit were purchased from Guangzhou RiboBio Co., Ltd.

qRT-PCR

Cells and liver tissues were collected and lysed by Trizol, total RNA was extract, reverse transcription and amplification kit was used to synthesize and amplify cDNA. $2^{-\Delta\Delta CT}$ method was used for relative quantitative analysis of gene expression with β-actin internal reference gene. All primers were synthesized by Shanghai Sangong Bioengineering Co. Ltd. The primer sequences were shown in Table 1.

Western blot

Mice liver tissues, HepG2.2.15 cells were collected and lysed with RIPA containing 1% PMSF and 0.01% phosphatase inhibitor for 30min, centrifuged at 4°C and 12,000g for 15 min, and the supernatant was collected. Protein denaturation, electrophoresis, membrane transfer and blocking were performed after protein quantified by BCA method. 1: 250 diluted TOMM34 antibody was added and incubated overnight. The fluorescent secondary antibody was incubated in the dark for 60 min at room temperature, and the Odyssey infrared imaging system was used for scanning and reading the target band. The relative quantitative value of TOMM34 protein expression was measured with GAPDH internal reference protein. The Rabbit anti-TOMM34 Polyclonal Antibody was purchased from Absin Bioscience Inc.

Statistical analysis

The counting data was expressed by frequency. The measurement data with normal distribution were expressed by mean ± standard deviation (x ± s), and those with non-normal distribution were expressed by P50 (P25, P75) quartile. One-way ANOVA was used for pairwise comparison between groups. $P< 0.05$ means the difference is statistically significant.

Results

The serum HBeAg titer in ETV+FZHY group decreased significantly than that in ETV group after 48 weeks treatment

156 chronic hepatitis B patients with HBeAg positive were divided into 2 groups according to the treatment method. The baseline data of demographic data, biochemical virology and fibrosis grades were balanced before treatment. By analyzing and screening the clinical cohort data of patients with HBeAg (S/CO) > 1 in the experimental group (FZHY+ETV, $n = 79$) and the control group (placebo+ETV, n = 77), the result showed that the decrease degree of serum HBeAg titer in FZHY+ETV group was significantly higher than that in ETV group after 48 weeks treatment ($p < 0.05$) (Table 2). This result indicated that FZHY could decrease the HBeAg production in chronic hepatitis B patients with HBeAg positive.

Fuzheng Huayu Recipe could reduce the production of serum HBeAg in CCl4-induced liver fibrosis HBV-Tg mice and induce HBeAg seroconversion

To verify the pharmacological inhibitory effect of FZHY on HBeAg production, we induced CCl4 liver fibrosis HBV-Tg mice model and treated it with ETV or FZHY+ETV. The HE staining results showed that ETV and FZHY+ETV both could reduce hepatic lobular structure destruction, and significantly reduced pathological changes of hepatocytes (Figure 2A). The Sirius red staining results showed that ETV could reduce the extension of the fibrous septum in the portal area of liver tissue, and

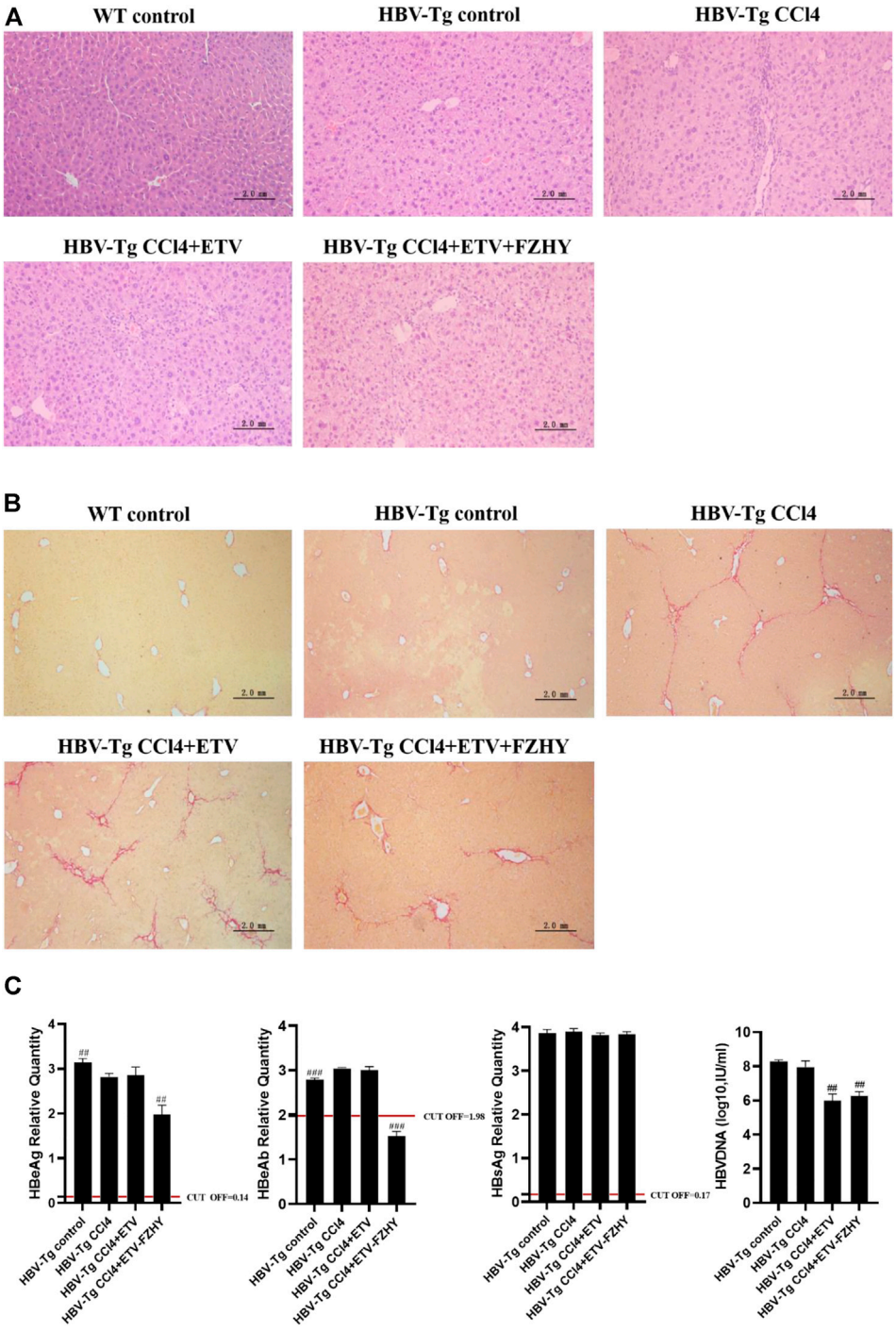


FIGURE 2
The pharmacological effect of FZHY on the expression of HBeAg, HBeAb, HBsAg, and HBV-DNA in CCl4-induced liver fibrosis HBV-Tg mice. **(A)**. HE staining. **(B)**. Sirius red staining. **(C)**. HBeAg, HBeAb, HBsAg, and HBV-DNA detection in HBV-Tg mice serum. Each group vs. CCl4-induced liver fibrosis group (HBV-Tg CCl4 group), ##$p < 0.01$, ###$p < 0.001$. Note: HBeAb cut-off value (COV) = 1.977, the result is positive if the sample value is less than COV value, and the result is negative if the sample value is greater than COV value.

FZHY+ETV could regress collagen fiber more obviously: the fiber septum in the portal area of liver tissue was alleviated, and some of the fibers became thinner and narrower, or even disappeared (Figure 2B). These results suggested that FZHY could improve the inflammation damage and fibrosis degree of liver tissue in CCl4-induced liver fibrosis HBV-Tg mice.

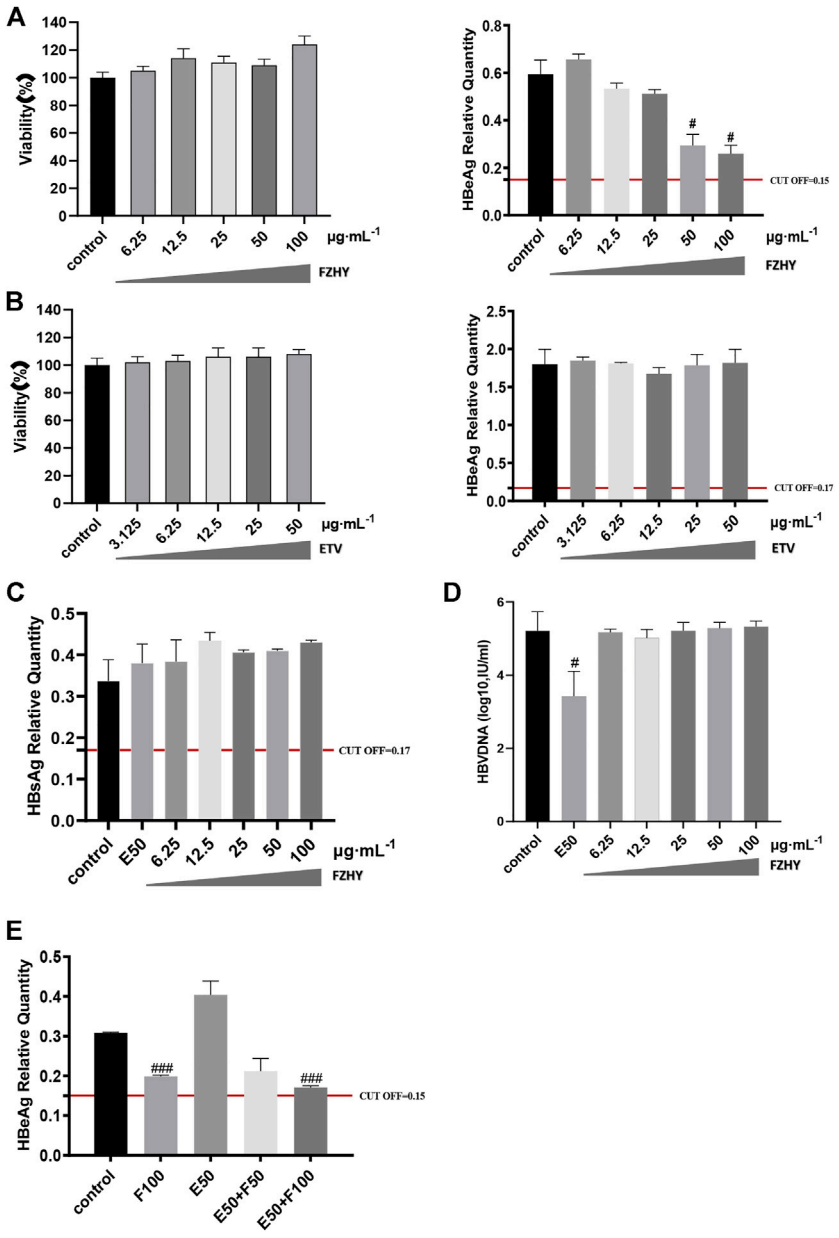


FIGURE 3
Cytotoxicity of FZHY on HepG2.2.15 cells and the effect of FZHY on the HBV expression in HepG2.2.15 cells. **(A)** Cytotoxicity and HBeAg production-inhibition effect of FZHY on HepG2.2.15 cells; **(B)** Cytotoxicity and HBeAg production-inhibition effect of ETV on HepG2.2.15 cells; **(C)** Effect of 50 μg/ml ETV (E50) and various concentrations of FZHY on HBsAg production; **(D)** Effects of 50 μg/ml ETV (E50) and various concentrations of FZHY on HBV-DNA production; **(E)** Effect of 50 μg/ml ETV (E50) combined with 50 or 100 μg/ml FZHY (F50 or F100) on HBeAg production. Each group vs. control group, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$.

Further serum detection results showed that, compared with CCl4-induced liver fibrosis HBV-Tg mice (HBV-Tg CCl4 group), HBeAg production and HBeAg seroconversion had no difference in ETV-administration mice (HBV-Tg CCl4+ETV group), but HBeAg production was significantly decreased and HBeAg seroconversion occurred in FZHY+ETV-administration mice (HBV-Tg CCl4+ETV+FZHY group). ETV-administration and FZHY+ETV-administration both had no effect on the production of HBsAg in CCl4-induced liver fibrosis group HBV-Tg mice. ETV-administration and FZHY+ETV-administration could both significantly decrease the production of HBV-DNA in CCl4-induced liver fibrosis group HBV-Tg mice, but there was no difference between ETV-administration and FZHY+ETV-administration. These results showed that FZHY could markedly decrease HBeAg production and promote HBeAg seroconversion, but had no effect on HBsAg and HBV-DNA production (Figure 2C).

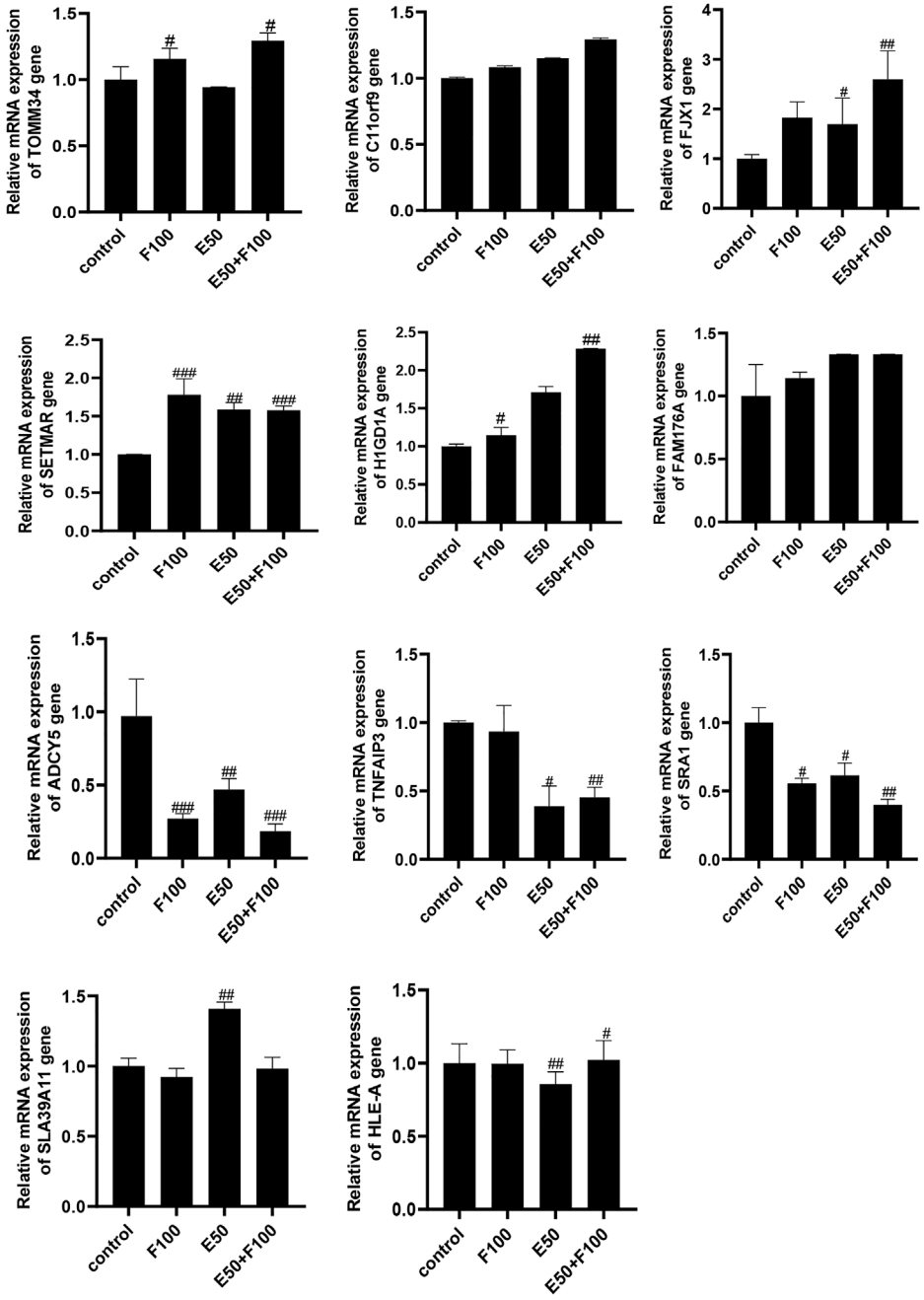


FIGURE 4
Screening of target genes for FZHY inhibiting HBeAg production. Each group vs. control group, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$. F100: 100 μg/ml FZHY, E50: 50 μg/ml ETV, E50 + F100: 50 μg/ml ETV +100 μg/ml FZHY

Fuzheng Huayu Recipe had no cytotoxicity on HepG2.2.15 cells and FZHY could significantly inhibit the production of HBeAg in HepG2.2.15 cells

To further verify the pharmacological inhibitory effect of FZHY on HBeAg production, we used HepG2.2.15 cell line, which is constructed by transfecting the recipient cell HepG2 with the recombinant plasmid of HBV-DNA whole gene, and treated it with FZHY or ETV or FZHY+ETV. CCK8 was used to detect the cytotoxicity of different concentrations of FZHY (6.25, 12.5, 25, 50, 100 μg/ml) and ETV (3.125, 6.25, 12.5, 25, 50 μg/ml) on HepG2.2.15 cells. Compared with control group, both FZHY and ETV had no

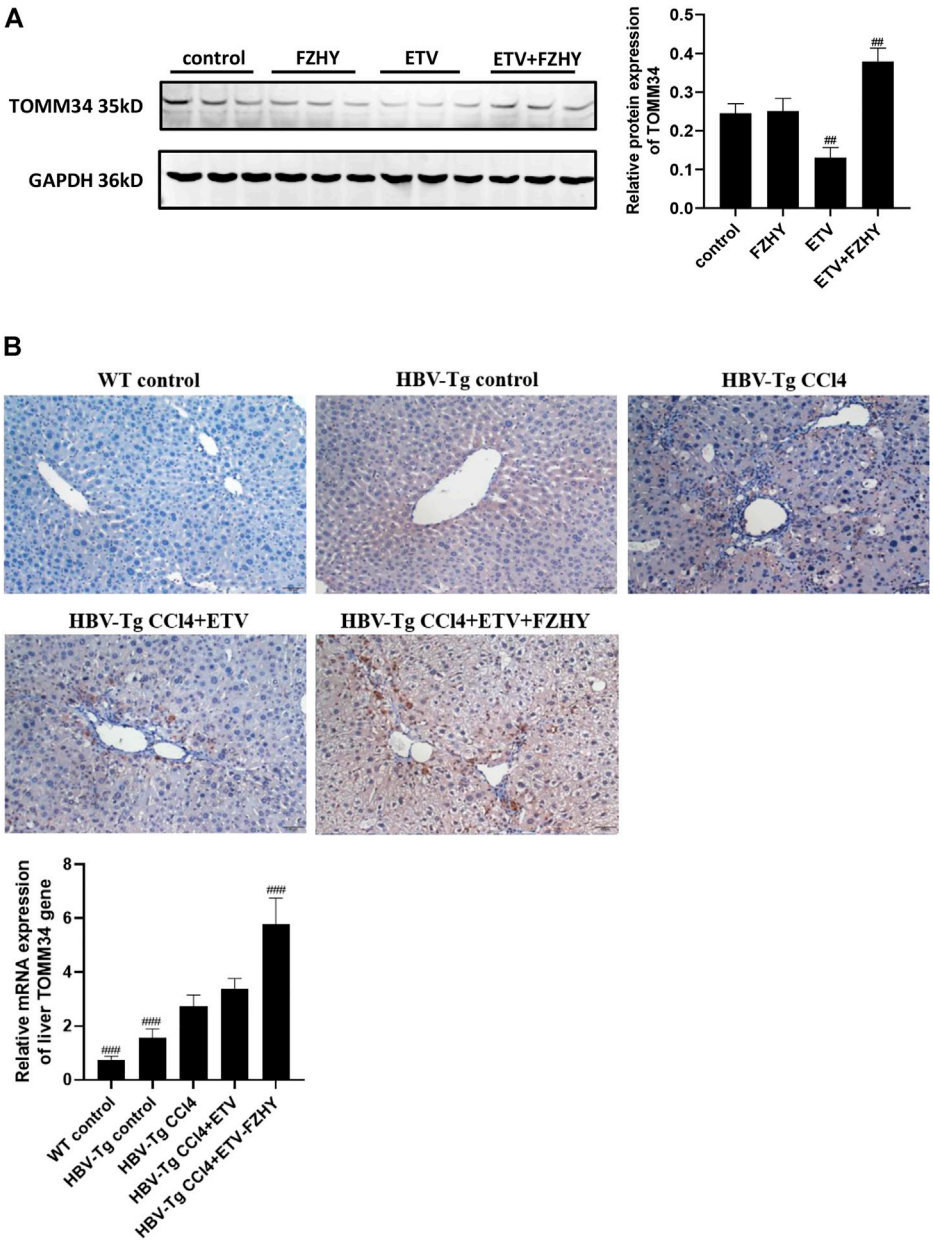


FIGURE 5
Effects of FZHY on the TOMM34 protein production in HepG2.2.15 cells and the TOMM34 gene expression in the liver tissues of CCl4-induced liver fibrosis HBV-Tg mice. **(A)** The TOMM34 protein production was detected by western blot assay. Each group vs. control group, ##$p < 0.01$. **(B)** The TOMM34 gene expression was detected by immunohistochemistry staining and qRT-PCR assay respectively. Each group vs. CCl4-induced liver fibrosis HBV-Tg mice group (HBV-Tg CCl4 group), ###$p < 0.001$.

obvious cytotoxicity on HepG2.2.15 cells (Figures 3A,B). Compared with the control group, FZHY had a dose-dependent inhibitory effect on the production of HBeAg in HepG2.2.15 cells and 50 or 100 μg/ml FZHY showed the marked inhibition effect (Figure 3A), but different concentrations of ETV had no inhibitory effect on the production of HBeAg (Figure 3B). In order to better compare the pharmacological effects of ETV and FZHY, we chose 50 μg/ml as the subsequent experimental concentration of ETV. The further experiments results showed that, neither FZHY nor ETV had inhibitory effect on the production of HBsAg in HepG2.2.15 cells (Figure 3C). ETV had a significant inhibitory effect on the expression of HBV-DNA, but FZHY had no effect on the expression of HBV-DNA (Figure 3D). When 50 μg/ml

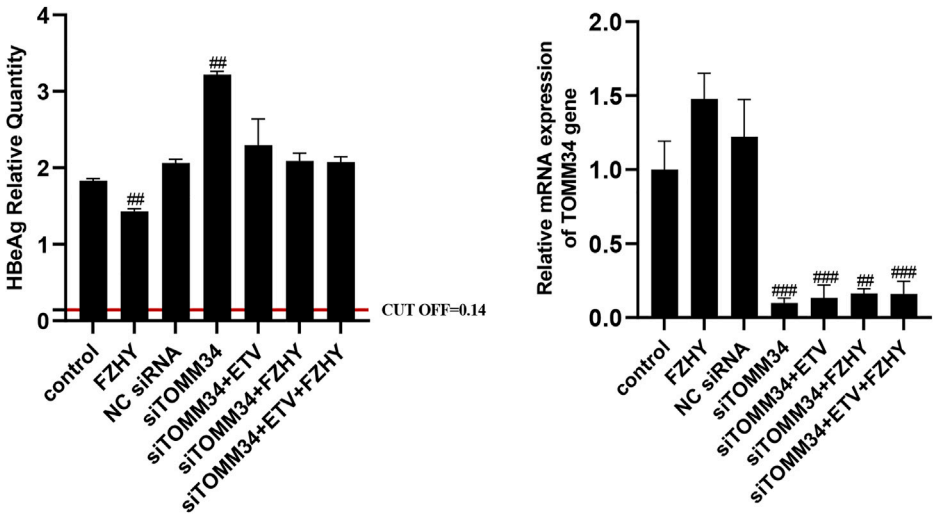


FIGURE 6
Effect of FZHY on HBeAg production in the TOMM34 gene-knocked down HepG2.2.15 cells. Each group vs. NC (negative control) siRNA group, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$.

ETV (E50) was combined with 50 or 100 μg/ml FZHY (F50 or F100), they both could significantly inhibit HBeAg production and E50 + F100 showed the better inhibition effect (Figure 3E). These results suggested that FZHY could specifically and markedly decrease the HBeAg production in HBV-infected hepatocytes. In the follow-up study on the mechanism of FZHY inhibiting HBeAg production, we selected 100ug/ml as the cell experimental concentration of FZHY.

TOMM34 might be the key target of Fuzheng Huayu Recipe to inhibit HBeAg production

Prof. Zhang Jiming's team conducted a systematic study on the regulatory genes of HBV replication and HBeAg expression in 2012 (Liu, 2012). They discovered that there were 109 significantly differentially expressed genes in liver tissue of patients in inactive immune control period compared with patients in immune tolerance period, of which 54 genes were significantly up-regulated and 55 genes were significantly down regulated. In order to find out the target of FZHY inhibiting HBeAg production, we selected the genes related to HBeAg expression regulation, which were C11ORF9, FJX1, SETMAR, ADCY5, HIGD1A, FAM176A, TOMM34, SLA39A11, TNFAIP3, SRA1, and HLA-E, to screen the target of FZHY. The results showed that the TOMM34 gene relative expression levels in Hep2.2.15 cells had no difference between ETV group and control group, however, the expression of TOMM34 gene in FZHY or FZHY+ETV group were significantly higher than that in ETV group or control group (Figure 4). These results suggested that TOMM34 gene was related to the inhibitory effect of FHZY on the HBeAg production.

We further detected the TOMM34 protein expression in Hep2.2.15 cells and HBV-Tg mice liver tissues. The western blot assay results showed that ETV decreased the TOMM34 protein expression in Hep2.2.15 cells, but FZHY+ETV significantly increased the TOMM34 protein expression (Figure 5A). The immunohistochemistry staining and qRT-PCR assay results also showed that ETV did not change TOMM34 protein and mRNA production in the liver tissues of CCl4-induced liver fibrosis HBV-Tg mice, but FZHY+ETV could markedly increase the production of TOMM34 protein and mRNA (Figure 5B). These results suggested that FZHY might inhibit HBeAg production by increasing TOMM34 gene expression.

To further demonstrate whether FZHY inhibited HBeAg production by regulating TOMM34 gene expression, we knocked down the TOMM34 gene expression in HepG2.2.15 cells and administrate FZHY simultaneously. The results showed that the HBeAg production was obviously increased in HepG2.2.15 cells after TOMM34 gene expression was knocked down, and there was no differential production of HBeAg between between FZHY+ETV group and ETV group (Figure 6). These results suggested that TOMM34 was a key regulatory gene for FZHY to reduce HBeAg production in HBV-infected hepatocytes.

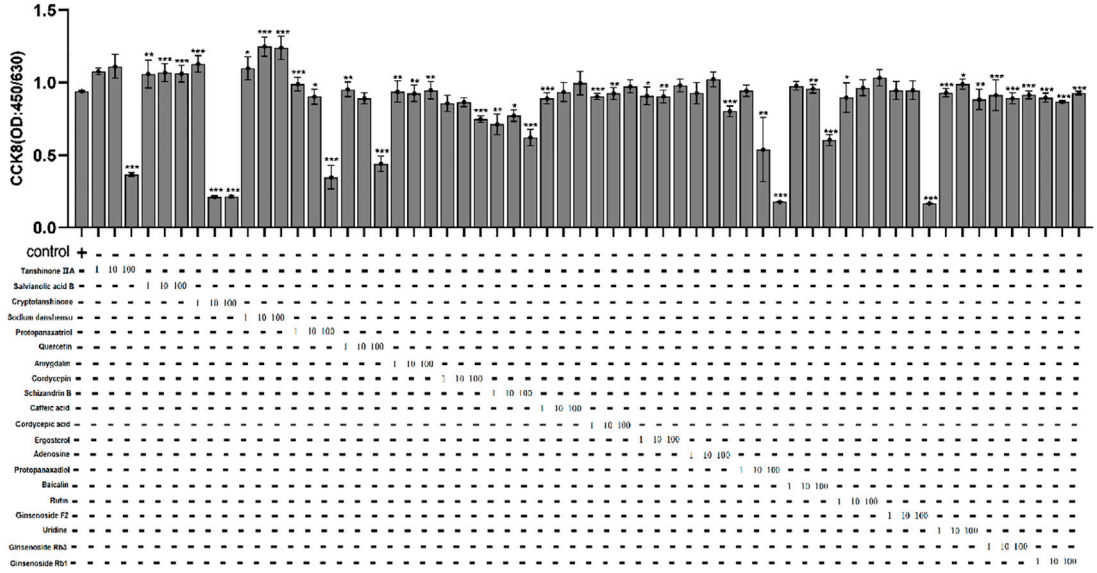


FIGURE 7
The cytotoxicity of 20 main active compounds of FZHY on HepG2.2.15 cells. Each group vs. control group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

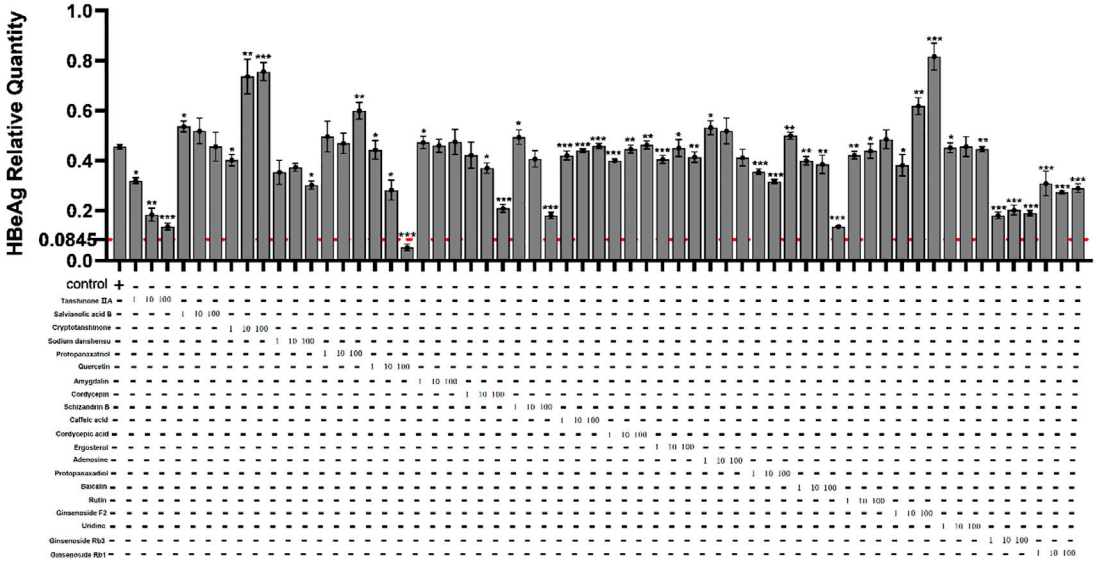


FIGURE 8
Effects of 20 main active compounds of FZHY on the HBeAg production in HepG2.2.15 cells. Each group vs. control group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

The cytotoxicity of active compounds in Fuzheng Huayu Recipe on HepG2.2.15 cells and the active compounds' effect on HBeAg production

In order to find out the main active compounds in FZHY that inhibit HBeAg production, the cytotoxicity of 20 active compounds in FZHY (caffeic acid, entomolic acid, ergosterol, adenosine, protopanaxadiol, baicalin, rutin, ginsenoside F2, uridine, tanshinone IIA, salvianolic acid B, cryptotanshinone, sodium tanshinol, protopanaxatriol, quercetin, amygdalin, cordycepin, ginsenoside Rb1, ginsenoside RB3, ginsenoside Rg3, and schisandrin B), was measured by CCK-8 assay (Figure 7) and the effect of these

20 active compounds in FZHY on the HBeAg production was detected by ELISA assay (Figure 8). The result showed that quercetin, cordycepin, baicalin, and schisandrin B could significantly inhibit HBeAg production at their low cytotoxic concentrations.

Effects of quercetin, cordycepin, baicalin, and schisandrin b on HBeAg production and TOMM34 gene expression in HepG2.2.15 cells

We conducted further pharmacological screening experiments on quercetin, baicalin and cordycepin selected from the above experiment. The results showed that quercetin, cordycepin, baicalin, and schisandrin B could significantly inhibit HBeAg production in HepG2.2.15 cells in a dose-dependent manner. The results also showed that quercetin, cordycepin, and baicalin could significantly increase TOMM34 expression, but schisandrin B seemed had no obvious effect on TOMM34 gene expression. The results showed that quercetin, cordycepin, and baicalin could inhibit HBeAg production by increasing TOMM34 gene expression, and schisandrin B might inhibit HBeAg production by another regulatory pathway still unknown (Figure 9).

Discussion

The degree of liver fibrosis in chronic hepatitis B affects the prognosis and management of patients. Antiviral treatment is related to the regression of liver fibrosis. Early HBeAg loss can reduce the risk of developing end-stage diseases, such as liver cirrhosis and liver cancer (Liaw et al., 2004). HBeAg may be used as an immune tolerance protein to help HBV virus escape the attack and clearance of the immune system. HBeAg clearance and HBeAg seroconversion after spontaneous or antiviral treatment show that HBV replication in patients is continuously controlled, patients' immunity to HBV is improved, continuous response can be obtained, the proportion of liver decompensation is reduced and the survival rate is improved (Yu et al., 1997; McMahon, 2005). The relief of spontaneous persistent chronic hepatitis B can not only slow down the progress of liver fibrosis, but also reduce the degree of liver fibrosis (Hui et al., 2007). The mutation in the pre-core region (Kim et al., 1993) or the basic core promoter (BCP) (Kramvis and Kew, 1999) of hepatitis B virus can lead to the reduction of hepatitis B e antigen (HBeAg) expression, which makes HBeAg unable to be used as a therapeutic marker for the whole population of chronic hepatitis B. However, HBeAg loss and seroconversion are still considered as the satisfactory end point for chronic hepatitis B treatment (Chinese Society of Hepatology et al., 2015).

According to current clinical guidelines, the goal of chronic hepatitis B treatment is to improve the quality of life and survival rate by preventing the disease from developing into cirrhosis, decompensated cirrhosis, end-stage liver disease, liver cancer and death. Pegylated interferon and nucleoside/nucleotide analogues are currently approved first-line treatments for chronic hepatitis B virus infection and have been used for many years (EASL, 2012). However, these therapies can only inhibit HBV replication and are difficult to cure most chronic hepatitis B patients. The treatment of chronic hepatitis B patients with antiviral drugs such as nucleoside/nucleotide analogues not only has a high recurrence rate after drug withdrawal, but also may promote the development of virus resistance, so as to accelerating the deterioration of the disease. Entecavir (ETV) is a guanine nucleotide analogue. Although it has good safety, some studies have reported that it might cause lactic acid poison (Lange et al., 2009). The efficacy of immunomodulators such as conventional or pegylated interferon is very limited, and some side effects may occur during the treatment, such as flu like symptoms (Yang and Bertoletti, 2016). Therefore, we still need to find a safer and more effective method to treat chronic hepatitis B.

Clinically, the treatment of integrated traditional Chinese and Western medicine has achieved good therapeutic results. Fuzheng Huayu Recipe is one of the first-line traditional Chinese medicine prescriptions for the treatment of chronic hepatitis B liver fibrosis. Combined with the current first-line drug ETV, FZHY can significantly improve the clinical efficacy. Previous studies have shown that FZHY could play an anti-hepatic fibrosis role by inhibiting the activation of hepatic stellate cells (Luo et al., 2013), regulating the phenotypic polarization of macrophages (Zhang et al., 2020), and so on. The combination of Fuzheng Huayu Recipe and ursodeoxycholic acid could play a synergistic role on the basis of antiviral therapy. It could better improve liver function, inhibit inflammatory reaction and prevent the process of liver fibrosis in patients with hepatitis B cirrhosis (Li et al., 2018). Fuzheng Huayu Recipe combined with ETV could significantly improve liver function and liver fibrosis in patients with chronic hepatitis B (Wang et al., 2018). ETV combined with Fuzheng Huayu Recipe was effective in the treatment of patients with HBeAg positive decompensated liver cirrhosis. It could significantly improve liver function and effectively inhibit HBV replication (Zhan et al., 2018).

In 2012, Dr. Liu Jihong screened 83 patients with chronic hepatitis B in the study of "Screening and action mechanism of HBV replication regulatory genes in hepatocytes of patients with chronic hepatitis B", including 22 patients in immune tolerance stage, 25 patients in immune clearance stage (positive chronic hepatitis B), 25 patients in immune activation stage (negative chronic hepatitis B) and 11 patients in inactive immune control stage. In addition, 6 healthy examinees were selected. The authors

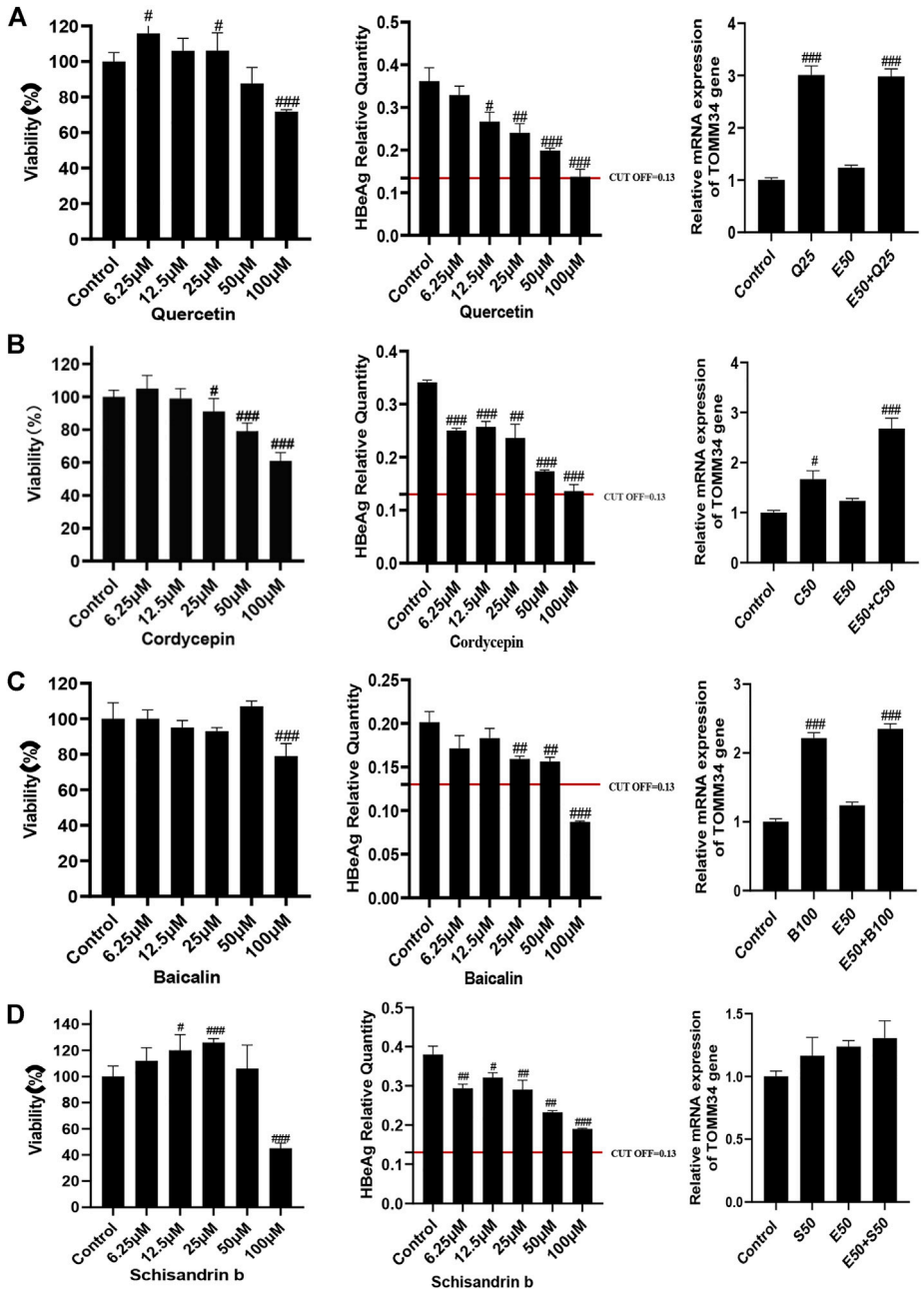


FIGURE 9
Effects of 4 active compounds of FZHY on the viability of HepG2.2.15 cells and the HBeAg production and TOMM34 gene expression in HepG2.2.15 cells. **(A)** Quercetin. E50: 50 μg/ml ETV, Q25: 25 μM Quercetin. **(B)** Cordycepin. E50: 50 μg/ml ETV, C50: 50 μM Cordycepin. **(C)** Baicalin. E50: 50 μg/ml ETV, B100: 100 μM Baicalin. **(D)** Schisandrin b. E50: 50 μg/ml ETV, S50: 50 μM Schisandrin b. Each group vs. control group, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$.

mainly observed the difference of gene expression in liver tissue under different chronic infection states in immune tolerance stage and immune control stage. Compared with patients in immune tolerance stage, there was significant difference in the expression of 109 genes in liver tissue of patients in inactive immune control stage (the expression of 54 genes was significantly up-regulated and 55 genes were significantly down regulated) (Liu, 2012). We conducted a pharmacological screening experiment of FZHY on the genes related to the

regulation of HBeAg production, the results showed that translocase of the outer mitochondrial membrane 34 (TOMM34) was the main target of FZHY inhibiting HBeAg production.

TOMM34 is a subunit of mitochondrial transporter, which can transfer mitochondrial proteins from cytoplasm to mitochondria (Nuttall et al., 1997). The imbalance of TOMM34 expression has been discovered to be associated with the growth of many cancers, such as rectal cancer (Shimokawa et al., 2006), breast cancer (Aleskandarany et al., 2012), lung cancer (Gimenez-Xavier et al., 2017), hepatic cell carcinoma (Toraih et al., 2019; Zhang et al., 2021). However, the relationship between TOMM34 and liver fibrosis and cirrhosis has not been reported. Silencing the expression of TOMM34 can up-regulate the production of HBsAg, HBeAg, and nucleocapsid (Liu, 2012). Our research showed that FZHY and its monomer compounds quercetin, cordycepin and baicalin could inhibit the expression of HBeAg through up-regulating TOMM34 expression. This might be an important reason that the curative effect of FZHY+ETV was better than that of ETV alone in the treatment of hepatitis B liver fibrosis. The molecular mechanism of TOMM34 reducing HBeAg production needs to be further studied. This will provide more convincing evidence for TOMM34 as a therapeutic target for hepatitis B liver fibrosis.

Conclusion

FZHY and its active compounds quercetin, baicalin and cordycepin could inhibit HBeAg production by promoting the expression of TOMM34 gene in HBV infected hepatocytes. FZHY+ETV combination therapy must be a recommended therapy for patients with HBeAg positive chronic hepatitis B and hepatitis B patients with liver fibrosis and cirrhosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Ethics statement

All experimental protocols of the animal study were reviewed and approved by the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine (Certificate of conformity: SCXK(Beijing)2019–0002; Approval Number: SZY201711007). All experimental protocols of the clinical study were reviewed and approved by the ethics committee of Shuguang Hospital Affiliated with Shanghai University of Traditional Chinese Medicine (Approval Number: 2015-445-73-02). Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

CL and XH conceived and designed the study. LX, RZ, KH, HL, JX and ZZ performed the experiments. LX and XH analyzed the data and wrote the manuscript, YP revised the manuscript, CL gave the final approval of the version to be published. All authors read and approved the final manuscript.

Funding

This work was supported by the Key Program of National Natural Science Foundation of China (grant numbers: 81730109) and the Shanghai Science and Technology Innovation Action Plan (grant numbers: 21S21900300).

Acknowledgments

The authors are grateful to Prof. Zhang Jiming of Huashan Hospital Affiliated to Fudan University for constructive advice.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aleskandarany, M. A., Negm, O. H., Rakha, E. A., Ahmed, M. A., Nolan, C. C., Ball, G. R., et al. (2012). TOMM34 expression in early invasive breast cancer: a biomarker associated with poor outcome. *Breast Cancer Res. Treat.* 136 (2), 419–427. doi:10.1007/s10549-012-2249-4

Barth, H., Klein, R., Berg, P. A., Wiedenmann, B., Hopf, U., and Berg, T. (2001). Induction of T helper cell type 1 response and elimination of HBeAg during treatment with IL-12 in a patient with therapy-refractory chronic Hepatitis B. *Hepatogastroenterology.* 48 (38), 553–555.

Chan, E., Tan, M., Xin, J., Sudarsanam, S., and Johnson, D. E. (2010). Interactions between traditional Chinese medicines and Western therapeutics. *Curr. Opin. Drug Discov. Devel.* 13 (1), 50–65.

Deng, J., Wang, J. H., Lu, Z. H., Qin, F., Huang, L. H., and Chen, W. (2009). Five-year follow-up study on Hepatitis B e antigen positive chronic Hepatitis B virus carriers. *Zhong. Hua. Chuan. Ran. Bing. Za. Zhi.* 27 (11), 677–679. Chinese. doi:10.3760/cma.j.issn.1000-6680.2009.11.010

EASL (2012). EASL clinical practice guidelines: Management of chronic Hepatitis B virus infection. *J. Hepatol.* 57 (1), 167–185. doi:10.1016/j.jhep.2012.02.010

Gimenez-Xavier, P., Pros, E., Bonastre, E., Moran, S., Aza, A., Graña, O., et al. (2017). Genomic and molecular screenings identify different mechanisms for acquired resistance to MET inhibitors in lung cancer cells. *Mol. Cancer Ther.* 16 (7), 1366–1376. doi:10.1158/1535-7163.MCT-17-0104

Chinese Society of Hepatology; Chinese Medical Association; Chinese Society of Infectious Diseases, Chinese Medical AssociationHou, J. L., and Wei, L. (2015). The guideline of prevention and treatment for chronic Hepatitis B: a 2015 update. *Zhonghua. Gan. Zang. Bing. Za. Zhi.* 23 (12), 888–905. Chinese. doi:10.3760/cma.j.issn.1007-3418.2015.12.002

Hui, C. K., Leung, N., Shek, T. W., Yao, H., Lee, W. K., Lai, J. Y., et al. (2007). Sustained disease remission after spontaneous HBeAg seroconversion is associated with reduction in fibrosis progression in chronic Hepatitis B Chinese patients. *Hepatology* 46 (3), 690–698. doi:10.1002/hep.21758

Kim, W. H., Kim, K. H., Chung, J. P., Kang, J. K., and Park, I. S. (1993). Mutations in the pre-core region of Hepatitis B virus DNA in patients with chronic liver diseases. *Yonsei Med. J.* 34 (2), 158–165. doi:10.3349/ymj.1993.34.2.158

Kramvis, A., and Kew, M. C. (1999). The core promoter of Hepatitis B virus. *J. Viral Hepat.* 6 (6), 415–427. doi:10.1046/j.1365-2893.1999.00189.x

Lange, C. M., Bojunga, J., Hofmann, W. P., Wunder, K., Mihm, U., Zeuzem, S., et al. (2009). Severe lactic acidosis during treatment of chronic Hepatitis B with entecavir in patients with impaired liver function. *Hepatology* 50 (6), 2001–2006. doi:10.1002/hep.23346

Li, Y. X., Zhang, F. Y., and Cui, Z. (2018). Effect of ursodeoxycholic acid combined with Fuzheng Huayu Capsule on HBeAg positive Hepatitis B cirrhosis patients with high viral load and its effects on liver fibrosis and inflammatory factors. *Xian. Dai. Zhong. Xi. Yi. Jie. He. Za. Zhi.* 27 (18), 2016–2019. Chinese. doi:10.3969/j.issn.1008-8849.2018.18.025

Liaw, Y. F., Sung, J. J., Chow, W. C., Farrell, G., Lee, C. Z., Yuen, H., et al. (2004). Lamivudine for patients with chronic Hepatitis B and advanced liver disease. *N. Engl. J. Med.* 351 (15), 1521–1531. doi:10.1056/NEJMoa033364

Lin, S. M., Yu, M. L., Lee, C. M., Chien, R. N., Sheen, I. S., Chu, C. M., et al. (2007). Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. *J. Hepatol.* 46 (1), 45–52. doi:10.1016/j.jhep.2006.08.021

Liu, D., Cui, L., Wang, Y., Yang, G., He, J., Hao, R., et al. (2016). Hepatitis B e antigen and its precursors promote the progress of hepatocellular carcinoma by interacting with NUMB and decreasing p53 activity. *Hepatology* 64 (2), 390–404. doi:10.1002/hep.28594

Liu, H. Y. (2012). *The study on the screening and mechanism of genes regulating HBV replication in hepatocytes of patients with chronic hepatitis B [D].* China: Fudan. University, 6–76. Chinese.

Luo, C., Chen, Z. X., Tan, X. H., Yi, W. H., Luo, L. S., Li, Y. L., et al. (2013). Therapeutic effects of Fuzhenghuayu decoction in a CCl4-induced liver cirrhosis rat model and on hepatic stellate cell activation. *Zhong. Hua. Gan. Zang. Bing. Za. Zhi.* 21 (9), 668–673. Chinese. doi:10.3760/cma.j.issn.1007-3418.2013.09.006

McMahon, B. J. (2005). Epidemiology and natural history of Hepatitis B. *Semin. Liver Dis.* 25, 3–8. doi:10.1055/s-2005-915644

Merkle, H., Deutschle, T., Gastrock-Balitsch, I., Nusser, P., Knehr, S., and Reifenberg, K. (2000). H-2(d) mice born to and reared by HBeAg-transgenic mothers do not develop T cell tolerance toward the Hepatitis B virus core gene products. *Virology* 273 (1), 149–159. doi:10.1006/viro.2000.0391

Nuttall, S. D., Hanson, B. J., Mori, M., and Hoogenraad, N. J. (1997). hTom34: a novel translocase for the import of proteins into human mitochondria. *DNA Cell Biol.* 16 (9), 1067–1074. doi:10.1089/dna.1997.16.1067

Shimokawa, T., Matsushima, S., Tsunoda, T., Tahara, H., Nakamura, Y., and Furukawa, Y. (2006). Identification of TOMM34, which shows elevated expression in the majority of human colon cancers, as a novel drug target. *Int. J. Oncol.* 29 (2), 381–386. doi:10.3892/ijo.29.2.381

Tan, M., Bhadoria, A. S., Cui, F., Tan, A., Van Holten, J., Easterbrook, P., et al. (2021). Estimating the proportion of people with chronic Hepatitis B virus infection eligible for Hepatitis B antiviral treatment worldwide: a systematic review and meta-analysis. *Lancet. Gastroenterol. Hepatol.* 6 (2), 106–119. doi:10.1016/S2468-1253(20)30307-1

Toraih, E. A., Alrefai, H. G., Hussein, M. H., Helal, G. M., Khashana, M. S., and Fawzy, M. S. (2019). Overexpression of heat shock protein HSP90AA1 and translocase of the outer mitochondrial membrane TOM34 in HCV-induced hepatocellular carcinoma: A pilot study. *Clin. Biochem.* 63, 10–17. doi:10.1016/j.clinbiochem.2018.12.001

Wang, T., Zhou, X., Liu, H., Wang, J., Zhang, P., Zhu, Y., et al. (2018). Fuzheng Huayu capsule as an adjuvant treatment for HBV-related cirrhosis: A systematic review and meta-analysis. *Phytother. Res.* 32 (5), 757–768. doi:10.1002/ptr.6009

WHO (2021). Available at: www.who.int/news-room/fact-sheets/detail/hepatitis-b (Last accessed July 27th, 2021).

Wilson, R., Warner, N., Ryan, K., Selleck, L., Colledge, D., Rodgers, S., et al. (2011). The Hepatitis B e antigen suppresses IL-1β-mediated NF-κB activation in hepatocytes. *J. Viral Hepat.* 18 (10), e499–507. doi:10.1111/j.1365-2893.2011.01484.x

Xie, Y. (2017). Hepatitis B virus-associated hepatocellular carcinoma. *Adv. Exp. Med. Biol.* 1018, 11–21. doi:10.1007/978-981-10-5765-6_2

Yang, N., and Bertoletti, A. (2016). Advances in therapeutics for chronic Hepatitis B. *Hepatol. Int.* 10 (2), 277–285. doi:10.1007/s12072-015-9661-x

Yu, M. W., Hsu, F. C., Sheen, I. S., Chu, C. M., Lin, D. Y., Chen, C. J., et al. (1997). Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic Hepatitis B virus carriers. *Am. J. Epidemiol.* 145 (11), 1039–1047. doi:10.1093/oxfordjournals.aje.a009060

Zhan, C. L., Wu, S. S., and Li, S. R. (2018). Analysis on the therapeutic effect of nucleoside antiviral drugs combined with Fuzheng Huayu capsule for treating HBeAg-positive HBV infection decompensated cirrhosis patients. *Zhong. Xi. Yi. Jie. He. Gan. Bing. Za. Zhi.* 28 (3), 148–150. Chinese. doi:10.3969/j.issn.1005-0264.2018.03.007

Zhang, M., Liu, H. L., Huang, K., Peng, Y., Tao, Y. Y., Zhao, C. Q., et al. (2020). Fuzheng Huayu Recipe prevented and treated CCl4-induced mice liver fibrosis through regulating polarization and chemotaxis of intrahepatic macrophages via CCL2 and CX3CL1. *Evid. Based. Complement. Altern. Med.* 2020, 8591892. doi:10.1155/2020/8591892

Zhang, T., Nie, Y., Gu, J., Cai, K., Chen, X., Li, H., et al. (2021). Identification of mitochondrial-related prognostic biomarkers associated with primary bile acid biosynthesis and tumor microenvironment of hepatocellular carcinoma. *Front. Oncol.* 11, 587479. doi:10.3389/fonc.2021.587479

frontiers | Frontiers in Pharmacology

TYPE Review
PUBLISHED 21 October 2022
DOI 10.3389/fphar.2022.909945

OPEN ACCESS

EDITED BY
Joan Villena García,
Universidad de Valparaíso, Chile

REVIEWED BY
Angela Corona,
University of Cagliari, Italy

*CORRESPONDENCE
Kevin Spelman,
drspelman@phytochemks.com

SPECIALTY SECTION
This article was submitted to Pharmacology of Infectious Diseases, a section of the journal Frontiers in Pharmacology

RECEIVED 31 March 2022
ACCEPTED 03 October 2022
PUBLISHED 21 October 2022

CITATION
Sheridan R and Spelman K (2022), Polyphenolic promiscuity, inflammation-coupled selectivity: Whether PAINs filters mask an antiviral asset.
Front. Pharmacol. 13:909945.
doi: 10.3389/fphar.2022.909945

Polyphenolic promiscuity, inflammation-coupled selectivity: Whether PAINs filters mask an antiviral asset

Rick Sheridan [1] and Kevin Spelman [2,3]*

[1]EMSKE Phytochem, Capitola, CA, United States, [2]Massachusetts College of Pharmacy and Health Sciences, Boston, MA, United States, [3]Health Education and Research, Driggs, ID, United States

The Covid-19 pandemic has elicited much laboratory and clinical research attention on vaccines, mAbs, and certain small-molecule antivirals against SARS-CoV-2 infection. By contrast, there has been comparatively little attention on plant-derived compounds, especially those that are understood to be safely ingested at common doses and are frequently consumed in the diet in herbs, spices, fruits and vegetables. Examining plant secondary metabolites, we review recent elucidations into the pharmacological activity of flavonoids and other polyphenolic compounds and also survey their putative frequent-hitter behavior. Polyphenols, like many drugs, are glucuronidated post-ingestion. In an inflammatory milieu such as infection, a reversion back to the active aglycone by the release of β-glucuronidase from neutrophils and macrophages allows cellular entry of the aglycone. In the context of viral infection, virions and intracellular virus particles may be exposed to promiscuous binding by the polyphenol aglycones resulting in viral inhibition. As the mechanism's scope would apply to the diverse range of virus species that elicit inflammation in infected hosts, we highlight pre-clinical studies of polyphenol aglycones, such as luteolin, isoginkgetin, quercetin, quercetagetin, baicalein, curcumin, fisetin and hesperetin that reduce virion replication spanning multiple distinct virus genera. It is hoped that greater awareness of the potential spatial selectivity of polyphenolic activation to sites of pathogenic infection will spur renewed research and clinical attention for natural products antiviral assaying and trialing over a wide array of infectious viral diseases.

KEYWORDS

polyphenols, polyphenolic antiviral mechanisms, antiviral MOAs, inflammation, deglucuronidation-through-inflammation mechanism, flavonoids

Introduction

Therapies with demonstrated efficacy for infection by SARS-CoV-2, the etiological agent of the COVID-19 pandemic, include small molecule antivirals such as molnupiravir (Dyer, 2021) and nirmatrelvir (Pfizer, 2021), monoclonal antibodies (U.S.Food and Drugs Administration, 2021), and repurposed drugs such as dexamethasone and fluvoxamine

(Reis et al., 2021). Monoclonal antibody therapy suffers from challenging logistics to administer. Dexamethasone has only modest effect on disease outcome (The RECOVERY Collaborative Group, 2021). Fluvoxamine remains prescribable but not yet mandated with agency approvals for COVID-19 (Leo and Erman, 2022). Researchers have called for mutagenicity studies of molnupiravir (Malone and Campbell, 2021; Masyeni et al., 2022). As SARS-CoV-2 variants continue to evolve, nirmatrelvir's future efficacy could be impacted, including under its own selection pressure on the main protease (Zhou et al., 2022).

Persistently low worldwide vaccination rates, the potential for breakthrough infections, and the ability for vaccinated individuals to achieve viral loads sufficient to infect others (Lipsitch et al., 2021), suggest that there remains ample scope for additional safe, replication-inhibiting antivirals in the panoply of pandemic-alleviating healthcare tools.

Natural products may present a potentially untapped source of antiviral activity. Plants must resist viruses whose constituent peptides are restricted to the same repertoire of proteinogenic amino acids as peptides in mammals. Plant virus proteins share similar fundamental constraints on protein secondary and tertiary structure as viruses with mammalian hosts. Plants' secondary metabolites are known particularly for plant-protection. Prevalent among the secondary metabolites are polyphenols. One of the three primary polyphenol classes are flavonoids (Quideau et al., 2011).

Flavonoids are a family of over eight thousand unique compounds that provide several advantages to plants (Pietta, 2000; Babu et al., 2009; Terahara, 2015). These compounds are responsible for some pigment and aroma of flowers and fruits, thereby attracting pollinators (Griesbach, 2010; Panche et al., 2016; Mathesius, 2018). Various flavonoids also protect plants from both biotic and abiotic stressors (Takahashi and Ohnishi, 2004; Kumar and Pandey, 2013; Panche et al., 2016), providing antimicrobial defenses (Treutter, 2005; Panche et al., 2016; Mathesius, 2018), acting as UV filters (Sisa et al., 2010; Panche et al., 2016; Mathesius, 2018), and serving as signaling molecules (Mierziak et al., 2014; Panche et al., 2016; Mathesius, 2018). Further, despite sparse literature on the topic, several flavonoids are also demonstrated to inhibit several plant viruses (French and Towers, 1992; Malhotra et al., 1996; Gutha et al., 2010; Likic et al., 2014; Honjo et al., 2020).

Recent research has demonstrated antiviral modes of activity for flavonoids by targeting neuraminidase (Ding et al., 2014; Sharma et al., 2021), proteases (Badshah et al., 2021; Jannat et al., 2021; Sharma et al., 2021), and DNA/RNA polymerases (Badshah et al., 2021). Several flavonoids such as quercetin, apigenin, and luteolin reduce HCV replication through inhibition of multiple viral non-structural proteins (Ninfali et al., 2020). A flavonoid, ladanein inhibited HCV passage into human hepatocytes (Haid et al., 2012). EGCG binds to HSV viral envelope glycoproteins gB and gD, inactivating the virions (Zakaryan et al., 2017). Lalani and Poh (2020)'s survey of flavonoid antiviral studies demonstrated that inhibition of viral enzymes and proteins is the most frequently identified mechanism of action against non-picornaviruses (Lalani and Poh, 2020). Flavonoid compounds from *Sambucus nigra* L. [Adoxaceae] extract were shown to inhibit H1N1 infection by binding to the viral envelope blocking entry into host cells (Roschek et al., 2009). Quercetin demonstrated anti-infective and anti-replicative activity in four different virus species (Middleton, 1998). Quercetin also blocks viral binding and penetration to the host cell in HSV (Zakaryan et al., 2017).

In a recent paper on the antiviral effects of flavonoids, Liskova et al. (2021) review the antiviral mechanisms of action for several flavonoids. For example, caflanone (from *Cannabis sativa* L.--Cannabaceae) pleiotropically inhibits viral entry factors such as ABL-2, cathepsin L, PI4Kiiiβ and AXL-2, which facilitate mother-to-fetus transmission of coronavirus (Ngwa et al., 2020). In addition, caflanone shows multi-modal anti-inflammatory activity through the inhibition of IL-1β, IL-6, IL-8, TNF-α and Mip-1α (Ngwa et al., 2020). Other flavonoids show anti-inflammatory activity through direct inhibition of NFκB (Rathee et al., 2009). Caflanone, and other flavonoids such as equivir, hesperetin, and myricetin also bind at high affinity to the helicase spike protein of SARS-CoV-2, as well as protease cleavage sites on the ACE2 receptor (Ngwa et al., 2020).

The antiviral effect of *Pelargonium sidoides* DC. [Geraniaceae], also known as umckaloaba, has been found to predominantly depend on the polyphenols, namely the flavonoids and oligomeric proanthocyanidins (Helfer et al., 2014). These compounds have been shown to directly interfere with the infectivity of HIV-1 particles before they interact with the host cell in a polyvalent manner. For instance, the flavonoid/anthocyanidin fraction of *P. sidoides* inhibited attachment of virus particles to cells by inhibiting the early viral proteins of Tat and Rev (positive regulators of gene expression) and inhibited the release of infectious virions. In addition, *P. sidoides* extracts demonstrated a strong reduction of input viral RNA levels in virus-exposed cells. In addition, the previously mentioned flavonoids target HIV-1 envelope proteins (X4 (LAI) and R5 (AD8 and JRFL), thereby inhibiting HIV-1 entry by interfering with the function of the envelope proteins (Helfer et al., 2014).

In *ex-vivo* investigations in rhinovirus-infected cells isolated from patients with severe asthma, moderate COPD, and disease-free controls, a *P. sidioides* extract (standardized to oligomeric prodelphinidins, a type of flavonoid) concentration-dependently demonstrated significantly increased human bronchial epithelial cell survival and decreased expression of inducible co-stimulator (ICOS) and its ligand ICOSL, as well as cell surface calreticulin. In both infected and uninfected, rhinovirus B-defensin-1 and suppressor of cytokine signaling-1 (SOCS1) were up-regulated suggesting a mode of activity for these flavonoid-rich extracts (Roth et al., 2019).

Clinical trials and *in vivo* models of *P. sidoides* flavonoid rich extracts have shown significant efficacy in treating uncomplicated upper respiratory tract infections (URIs) (Gökçe et al., 2021), URIs in asthmatic children (Patiroglu et al., 2012; Tahan and Yaman, 2013), acute bronchitis (Kamin et al., 2010; Kamin et al., 2012), and reduction in bacterial infection *via* immunomodulatory activity (Bao et al., 2015).

Besides the discussed anti-inflammatory activity, there is other immunomodulatory activity of flavonoids which has been reviewed elsewhere (Roshanravan et al., 2020; Liskova et al., 2021; Han et al., 2022). Besides the cytokine inhibition, cytokines have other roles that may significantly affect immune function. For example, the ubiquitous occurring quercetin and its glycoside rutin, have been found to facilitate the shift of macrophages from a proinflammatory to an anti-inflammatory phenotype (Bispo da Silva et al., 2017; Zaragozá et al., 2020). Additionally, apigenin, luteolin, and quercetin show significant immunomodulatory actions on natural killer cell cytotoxicity activity and granule secretion (Oo et al., 2021). Quercetin has also demonstrated a decreased in the expression levels of the major histocompatibility complex class two (MHC II) and costimulatory molecules resulting in a marked reduction of T cell activation (Huang et al., 2010). Finally, human peripheral blood mononuclear cells treated with quercetin preferentially induced interferon gamma (IFN-γ) expression and synthesis while inhibiting IL-4 production resulting in a differential activation of Th1 cells, suggesting potential anti-tumor activity (Nair et al., 2002).

A frequent target of coronavirus antivirals is the SARS-CoV-2 main protease, owing especially to the successful history of protease inhibitors on reducing HIV replication. Several polyphenols showed potent antiviral activity to SARS-CoV's main protease (Lin et al., 2005; Ryu et al., 2010; Nguyen et al., 2012; Park et al., 2013; Jo et al., 2020). Among these, the polyphenolic flavonoid hesperetin (**1**) was unique in potently inhibiting the action of the main protease in cell-based assay (Lin et al., 2005). Hesperetin dose-dependently inhibited cleavage activity of the 3CLpro in expressed in Vero E6 cells with an IC50 of 8.3 μM (Lin et al., 2005).

However, polyphenols like hesperetin are disfavored by industrial medicinal chemists for proceeding through the hit-to-lead (H2L) stage of the drug discovery pipeline (Lowe, 2020; Lowe, 2021). Polyphenols are categorized among the Pan-Assay INterference compounds (PAINs) (Lowe, 2012) [other terms are "frequent-hitters", "promiscuous inhibitors", "privileged structures/scaffolds", and "invalid metabolic panaceas" (Bisson et al., 2016)], and are suggested to obscure the results of various assays. They also bind broadly to assays' protein targets themselves. Selected examples of polyphenol aglycones are provided in Figure 1.

Due to the ongoing pressing need for further COVID treatment strategies, we review the pharmacokinetic and putative frequent-hitting behavior of polyphenols' as a class with an eye toward ascertaining 1) their potential as an antiviral 2) whether or not polyphenols simultaneously should pose risks to ordinary healthy cellular processes.

What defines a polyphenol?

While IUPAC has defined the term "phenols" (Gold, 2019), a definition of polyphenols remains yet to be formally accepted. Quideau (2011) explored definitions of polyphenols extensively, providing an applicable description:

> "*The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression.*" (Quideau et al., 2011)

Describing polyphenols in part based on their provenance provides excellent exclusivity. However, one could question whether it is helpful to exclude phenols such as acacetin which have only one phenolic ring. For large-scale cheminformatic purposes, which challenge the application of biosynthesis pathway criteria, an applicable definition may be to treat polyphenols as any molecule with more than one phenolic ring but lacking elements other than C, H, and O.

Poor polyphenol PK perception

The therapeutic efficacy of any antiviral whose purpose is to reduce viral replication requires maintaining an efficacious concentration of the ligand at its putative target for an extended period of time. Conservatively, this period should ideally be of long duration relative to a virus's replication time to reach peak viral load. An interval typically measured in days in the case of SARS-CoV-2 infection in humans (Kissler et al., 2021).

However, polyphenolic compounds' potential for efficacy for any particular pathology is criticized due to a prima facie poor pharmacokinetic ADMET profile. Consider, for example, diosmetin (**2**). A primary intermediate metabolite of the pharmaceutical formulation known as Daflon (comprised of 90% diosmin, and 10% other flavonoids expressed as hesperidin, diosmetin, linarin, and isorhoifolin), and similarly proportioned formulations are prescribed in many countries around the world for chronic venous insufficiency (CVI).

Ingested diosmin becomes diosmetin through Phase I metabolism through the intestinal wall, and then is either glucuronidated (primarily) to glucuronides (**4** and **5**), sulfated, or methylated through Phase II metabolism in the liver (Boutin

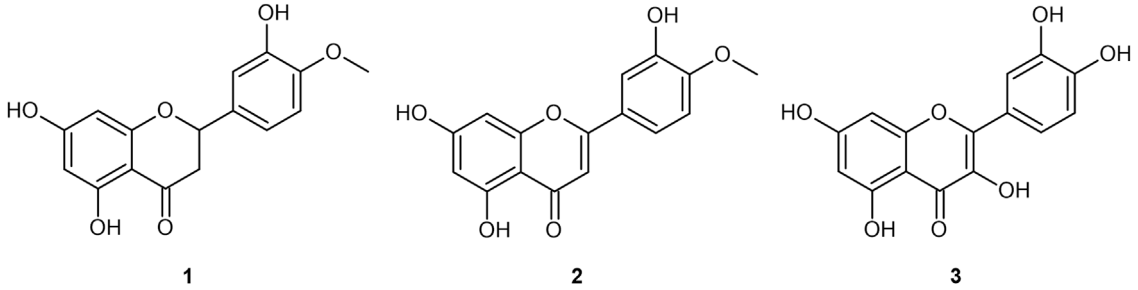


FIGURE 1
Four flavonoid aglycones referenced in the present work: hesperetin **(1)**; diosmetin **(2)**; quercetin **(3)**.

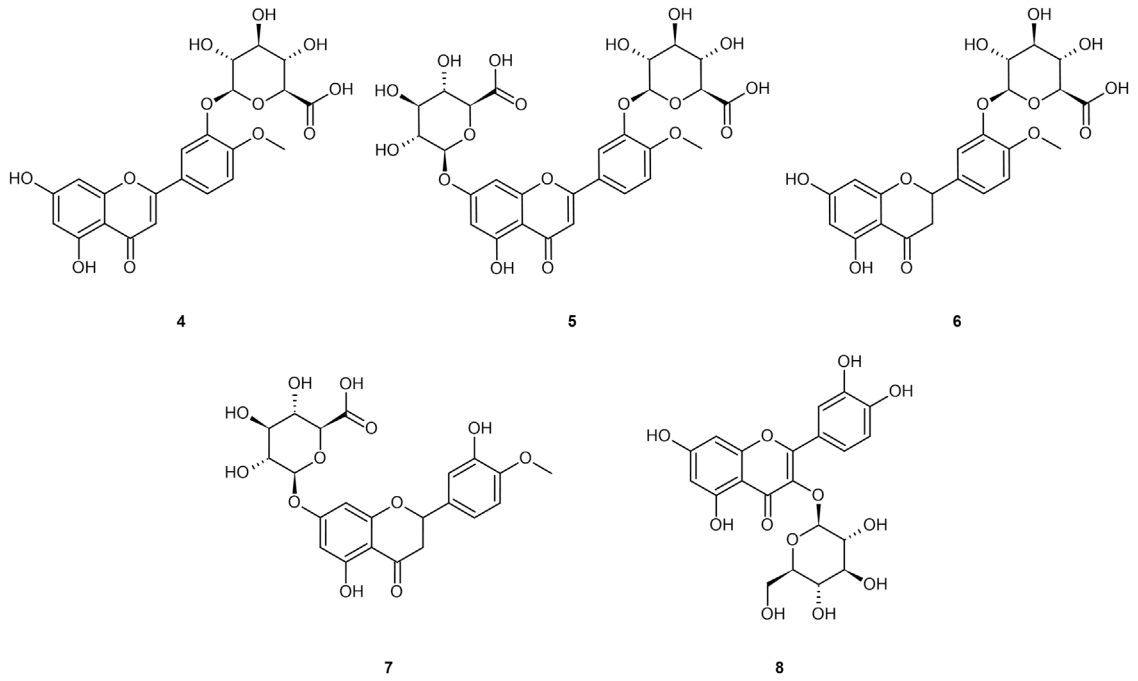


FIGURE 2
Common glucuronide metabolites of the referenced aglycones: diosmetin-3′-O-glucuronide **(4)**; diosmetin-3′-7-O-glucuronide **(5)**; hesperetin-3′-O-glucuronide **(6)**; hesperetin-7–O-glucuronide **(7)**; quercetin-3-O-glucuronide **(8)**.

et al., 1993; Meyer, 1994; Struckmann and Nicolaides, 1994; Spanakis et al., 2009; Campanero et al., 2010; Patel et al., 2013; Silvestro et al., 2013; Russo et al., 2015; Russo et al., 2018; Mandal et al., 2019; Bajraktari and Weiss, 2020). Serum analysis on healthy individuals demonstrates negligible presence of the aglycone in plasma, and low sustained levels of the diosmetin conjugates (primarily glucuronides) in plasma, with t_{max} of 2.3 h and $t_{1/2}$ ranging from 8–70 h (Boutin et al., 1993; Struckmann and Nicolaides, 1994; Spanakis et al., 2009; Campanero et al., 2010; Silvestro et al., 2013; Russo et al., 2015; Russo et al., 2018; Mandal et al., 2019; Bajraktari and Weiss, 2020). Stachulski and Meng (2013) and Tranoy-Opalinski et al. (2014) noted that most glucuronides are rapidly eliminated by the kidneys, posing an apparent limitation to their efficacy (Tranoy-Opalinski et al., 2014). Glucuronidation further reduces bioavailability to the intracellular compartment as the glucuronide moiety imparts a hydrophilicity that prevents cellular uptake (Tranoy-Opalinski et al., 2014). Glucuronides of the aglycones from Figure 1 are presented in Figure 2.

Note that a similar metabolic pathway can be described for other flavonoid aglycones. In the case of hesperidin, it is hydrolyzed to hesperetin, ultimately primarily becoming

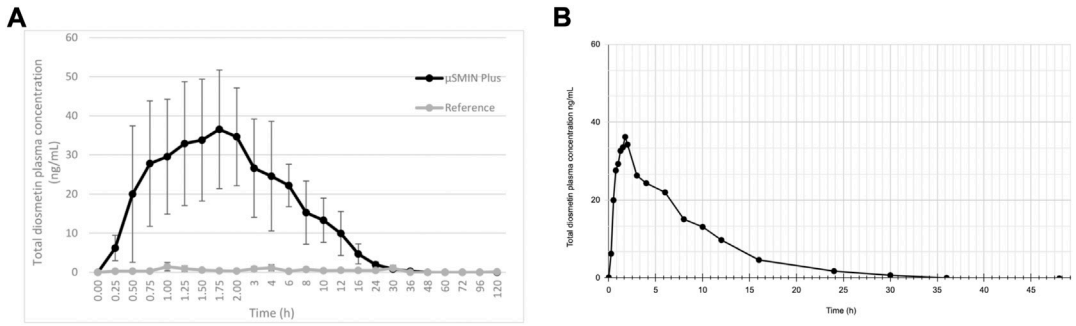


FIGURE 3
(A) Diosmetin plasma concentration (as ascertained following deglucuronidation), after administration of a 50 mg/kg micronized diosmin formulation to rats. Image licensed under CC by 4.0 from Russo et al. (2018) (Russo et al., 2018). **(B)** same plot with linearized axes.

glucuronides (**6** and **7**) or quercetin, primarily to glucuronide (**8**). Russo et al. (2018) provided a prototypical example of flavonoid plasma pharmacokinetics as demonstrated by diosmetin, which is reproduced and linearized in Figures 3A,B, respectively.

Yet to our knowledge 1) no quantitative bioavailability assays of diosmetin have taken place in non-plasma compartments such as extracellular fluid and tissue in humans; 2) tissue distribution studies of flavonoids in animal models are few. More *in vivo* distribution data to support therapeutic insights into polyphenols would be valuable.

Drawbacks of polyphenol PK analysis

On broader review of the polyphenol pharmacokinetic literature, five insights about pharmacological assays emerge:

1. The most commonly obtained pharmacological assay for concentration of polyphenols or their metabolites is blood plasma analysis, rather than interstitial fluid or intracellular fluid (Tozer, 1981; Ueno et al., 1983; Boutin et al., 1993; Meyer, 1994; Struckmann and Nicolaides, 1994; Walle et al., 2001; Spanakis et al., 2009; Campanero et al., 2010; Jin et al., 2010; Kaushik et al., 2012; Takumi et al., 2012; Patel et al., 2013; Silvestro et al., 2013; Russo et al., 2015; Yang et al., 2016; Nikiforov, 2017; HealthTech and de Zeneta, 2018; Russo et al., 2018; Mandal et al., 2019; Bajraktari and Weiss, 2020; Hai et al., 2020).
2. A polyphenol's plasma concentration profile alone provides no data on tissue distribution or biotransformation (Tozer, 1981; Walle et al., 2001; Ratain and Plunkett, 2003).
3. It is very difficult to sample intracellular fluid for drug/metabolite concentration profiling to the exclusion of extracellular and serum fluid (Lowe, 2018; Lowe, 2019).
4. Even radiolabeled assaying of all possible elimination routes fails to provide a complete accounting of polyphenol dosage intake (Walle et al., 2001).
5. Plasma samples of polyphenols are more frequently obtained from healthy individuals, rather than those suffering from a particular pathology (Ueno et al., 1983; Boutin et al., 1993; Meyer, 1994; Struckmann and Nicolaides, 1994; Walle et al., 2001; Spanakis et al., 2009; Campanero et al., 2010; Jin et al., 2010; Kaushik et al., 2012; Takumi et al., 2012; Patel et al., 2013; Silvestro et al., 2013; Russo et al., 2015; Yang et al., 2016; Nikiforov, 2017; HealthTech and de Zeneta, 2018; Russo et al., 2018; Mandal et al., 2019; Bajraktari and Weiss, 2020; Hai et al., 2020).

Therefore, if any particular pharmaceutical candidate's PK profile achieves significant distribution in organs other than those associated with either the GI tract or renal tract, it would be unascertainable from serum analysis alone. Further, if any particular pathology has an effect on a compound's tissue distribution (whether by causing sequestering in sanctuary sites, or adduct formation with the target in tissue both of which represent an increase in the volume of distribution), then plasma analysis alone remains poorly positioned to provide the relevant readout. Rather, tissue analysis in sacrificed animal models, or comprehensive radiolabeled elimination quantitation in humans, would be required.

Walle et al. (2001) demonstrated such a radiolabeled analysis. Notably, they found that carbon dioxide was a major metabolite of quercetin (**3**) in humans, (Walle et al., 2001) suggesting a rarer elimination pathway than typically encountered by pharmacological analysis. Even with this exotic elimination route taken into account, the full dose of quercetin was not always fully representative of the dose given. One can speculate that sequestration of quercetin products in tissue compartments was maintained past the 72-hr study period.

Moreover, while de Boer et al. (2005) and Bieger et al. (2008) demonstrated that quercetin reaches certain tissues other than those associated with GI and renal tracts in healthy animal

TABLE 1 Deglucuronidation-through-inflammation mechanism steps.

Stage B—Flavonoid aglycones are glucuronidated prior to arrival in the bloodstream
↓
Stage C—Neutrophils and macrophages are attracted to site(s) of inflammation
↓
Stage D—β-glucuronidase is expressed by neutrophils and macrophages
↓
Stage E—Serum flavonoid glucuronides are deglucuronidated ('deconjugated') by β-glucuronidase at site of inflammation
↓
Stage F—Flavonoid aglycones diffuse through cell membrane

models, these studies do not address any putative bioavailability of flavonoids uniquely to tissue affected by diseased circumstances.

Toward resolving the "flavonoid paradox"

To begin addressing these pharmacokinetic challenges, we examine them through the lens of a subtle but critically important feature of the pharmacokinetic profile of polyphenols, such as flavonoids, as elucidated by the literature.

Menendez et al. and Perez-Vicaino et al. frame flavonoids' pharmacokinetic challenges in the context of a "flavonoid paradox" (Menendez et al., 2011; Perez-Vizcaino et al., 2012). The paradox can be summarized as the observation that several flavonoid polyphenols have been shown to demonstrate therapeutic effects for various pathologies *in vivo*, and yet their pharmacological profiles suggest poor bioavailability, including the difficulty of glucuronide metabolites to pass through cell membranes, along with rapid plasma clearance.

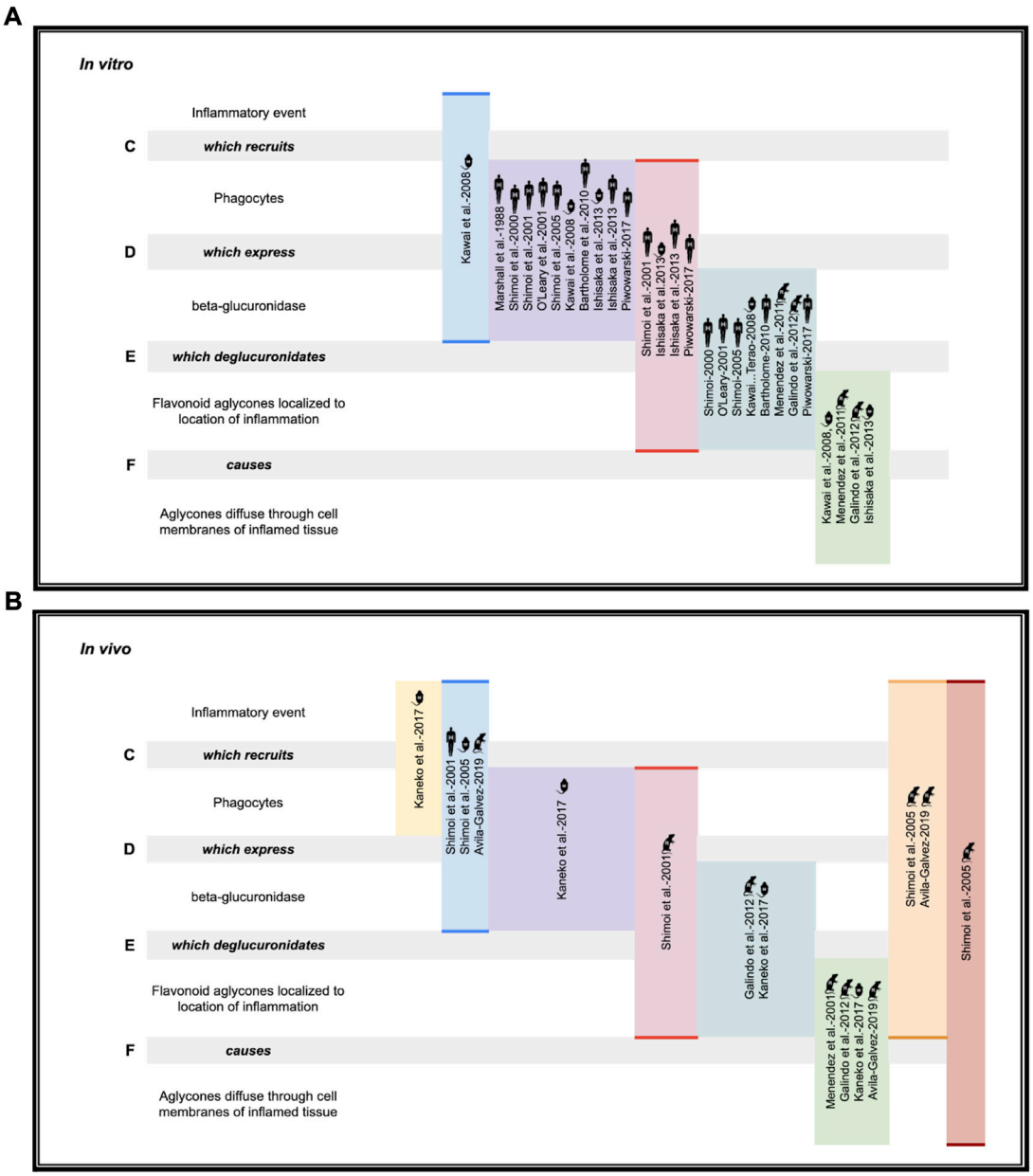
Investigators (Marshall et al., 1988; Shimoi et al., 2000; O'Leary et al., 2001; Shimoi et al., 2001; Shimoi and Nakayama, 2005; Kawai et al., 2008; Bartholomé et al., 2010; Menendez et al., 2011; Galindo et al., 2012; Ishisaka et al., 2013; Kaneko et al., 2017; Piwowarski et al., 2017; Ávila-Gálvez et al., 2019) of the following deconjugation mechanism offer a resolution to the flavonoid paradox: During inflammation (as happens during infection of several etiologies), phagocytes arrive at the extracellular fluid surrounding the sites of inflammation. The phagocytes express β-glucuronidase which accomplishes deglucuronidation (also known as deconjugation) of the flavonoid glucuronide into its aglycone form. The deconjugated flavonoid aglycone subsequently diffuses through the cell membrane where they can reach their target. The mechanism is summarized in Table 1. For purposes of this review, the mechanism steps are labeled stages B, C, D, E, and F.

Comparable pathology-specific activation of glucuronide drugs by β-glucuronidase has been examined and exploited in the context of anti-tumor agents (Tranoy-Opalinski et al., 2014). However, it is the "deconjugation in inflammation hypothesis" that was developed and supported progressively over the period 2000–2019 across several polyphenols *in vitro*, in animal models, and in humans that describes flavonoid conversion to cell-penetrating forms uniquely under inflammatory conditions (Marshall et al., 1988; Shimoi et al., 2000; O'Leary et al., 2001; Shimoi et al., 2001; Shimoi and Nakayama, 2005; Kawai et al., 2008; Bartholomé et al., 2010; Menendez et al., 2011; Galindo et al., 2012; Ishisaka et al., 2013; Kaneko et al., 2017; Piwowarski et al., 2017; Ávila-Gálvez et al., 2019).

While the deglucuronidation-through-inflammation hypothesis has been reviewed extensively by others, (Terao et al., 2011; Perez-Vizcaino et al., 2012; Kawai, 2014; Kawabata et al., 2015; Terao, 2017; Kawai, 2018) to our knowledge, this review is the first to unify the body of work into one cohesive, accessible evidentiary framework. Demonstration of the evidence generated through the deglucuronidation-through-inflammation body of work, (Marshall et al., 1988; Shimoi et al., 2000; O'Leary et al., 2001; Shimoi et al., 2001; Shimoi and Nakayama, 2005; Kawai et al., 2008; Bartholomé et al., 2010; Menendez et al., 2011; Galindo et al., 2012; Ishisaka et al., 2013; Kaneko et al., 2017; Piwowarski et al., 2017; Ávila-Gálvez et al., 2019) is provided against the model's labeled stages C-F in Figure 4.

Figure 4 illustrates the body of deglucuronidation-through-inflammation literature thusly: Original research investigators produced results demonstrating any one of the four stages of the mechanism (which we refer to here as steps C, D, E, F), the entire mechanism (CDEF), or consecutive combinations of steps (such as CD, CDE). We highlight whether evidence for an individual step has been produced, or instead over multiple consecutive steps end-to-end (without isolated verification of any intermediate step). Consecutive step verifications are illustrated by highlighting in bold the top and bottom of the relevant cells.

The figure annotates whether experiments have been performed *in vitro* (using mice, rat, or human cell lines) or *in vivo* in mice models, rat models, or humans. While steps of the pathway were verified across the polyphenols luteolin (Shimoi et al., 2000; Shimoi et al., 2001; Shimoi and Nakayama, 2005),



C - Phagocytes (whether neutrophils, macrophages or both) are recruited to the site of inflammation

D - Beta-glucuronidase is expressed by phagocytes (neutrophils, macrophages, or both)

CD - Beta-glucuronidase is expressed due to inflammation

E - Serum flavonoid glucuronides are deglucuronidated ('deconjugated') by beta-glucuronidase in situ at site of beta-glucuronidase expression

DE - Phagocytes deglucuronidate flavonoid glucuronides

CDE - Inflammation causes deglucuronidation of flavonoid glucuronides

F - Flavonoid aglycones diffuse through any cell membrane / are uptaken by tissue / exhibit an effect that can only be mediated in-cell

Bold endcap cells designate end-to-end verification and exclude intermediary verification

mouse; rat; human;

FIGURE 4
Deconjugation-through-inflammation literature basis **(A)** *in vitro* support **(B)** *in vivo* support.

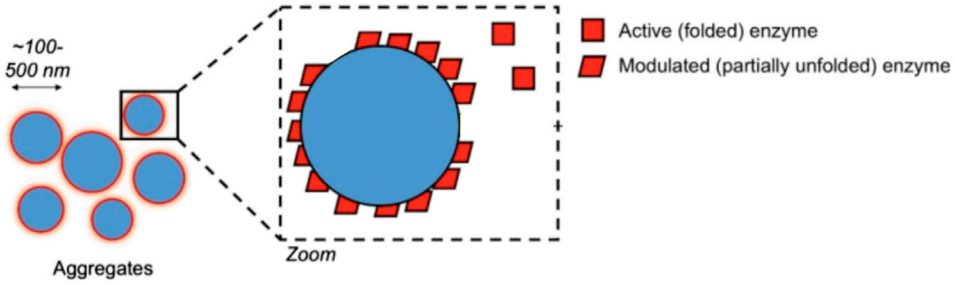


FIGURE 5
Non-selective aggregation inhibition model. Figure modified from Auld et al. (2017) for clarity under CC BY-NC-SA 3.0.

quercetin (O'Leary et al., 2001; Bartholomé et al., 2010; Menendez et al., 2011; Galindo et al., 2012; Ishisaka et al., 2013; Kawai, 2014), daidzein (O'Leary et al., 2001), and kaempferol (O'Leary et al., 2001), as well as the ellagic acid metabolites urolithin A (Piwowarski et al., 2017; Ávila-Gálvez et al., 2019; Bobowska et al., 2021), iso-urolithin A (Piwowarski et al., 2017; Bobowska et al., 2021), and the single-phenol urolithin B (Piwowarski et al., 2017; Bobowska et al., 2021), these are not annotated in the figure for brevity.

We believe the figure also adds value as it makes clear which steps are already demonstrated so that they can undergo simpler replication studies, as well as identifying which steps, such as *in vivo* human verification work at stages DEF, could be better elucidated with fresh original research.

We propose standardizing the mechanism's naming to the technical term "deglucuronidation-through-inflammation" or DTI. The term 'Shimoi pathway' further serves as a convenient shorthand that recognizes the lead researcher to first propose and study this mechanism with specific attention to inflammation with polyphenols.

The promiscuous inhibition of polyphenols

Promiscuous inhibition poses two primary implications for medicinal chemistry assaying. The first is the non-specific binding of protein/enzyme targets themselves. The second is the disruption of assay integrity by inhibiting non-target enzymes used for assay readout. As it can be difficult to distinguish between these two, orthogonal assays are sometimes performed to verify a target binding interaction.

Promiscuity could take any of several forms. A promiscuous ligand could simply be highly conforming to a protein surface's geometry, with a high number of hydrogen donors & acceptors to more likely "stick" nonspecifically to any given protein site's own set of H-donors and H-acceptors. Another mechanism sees promiscuous inhibition take the form of colloidal aggregations (Shoichet, 2006). In this mechanism, upon reaching a certain concentration, the ligand forms tightly-packed spherical aggregates with itself, even inside the cell (Ganesh et al., 2017; Shoichet, 2021) as illustrated in Figure 5. Proteins and enzymes non-specifically bind to the surface of the aggregation and are inhibited in the process (Auld et al., 2017). Often seen as a nuisance originally, it is now also seen as a source of opportunity in drug discovery as well (Ganesh et al., 2017; Ganesh et al., 2018; Ganesh et al., 2019). Deliberate study of aggregation in cell-based assays is a nascent sub-field, (Owen et al., 2012) thus cataloguing of non-specific aggregation among polyphenols in cells merits further investigation.

Quercetin has earned a reputation as a promiscuous inhibitor (Pohjala and Tammela, 2012; Jasial et al., 2016a; Gilberg et al., 2019; Lowe, 2020) as well as having served as one of the first aggregators identified. (McGovern et al., 2002; McGovern and Shoichet, 2003; Shoichet, 2006). Luteolin (Jasial et al., 2016a), curcumin (Jasial et al., 2016a), myricetin (Pohjala and Tammela, 2012; Jasial et al., 2016a; Jasial et al., 2016b; Gilberg et al., 2018; O'Donnell et al., 2021), and tannic acid (Pohjala and Tammela, 2012; Jasial et al., 2016a) are also promiscuous inhibitors, where myricetin and tannic acid have been further identified as aggregators (Pohjala and Tammela, 2012).

Of 123,844 assay records hosted by Pubchem and compiled by Gilberg et al. (2016) (Gilberg et al., 2016), their isolation of the most promiscuous 466 of them (99.6% percentile) contains 13 polyphenols based on our cheminformatic-oriented definition.

The catechol functional group, while not the exclusive province of polyphenols (and nor do polyphenols all contain catechol), certainly correlates with polyphenols. Bael and Holloway (2010), highlights catechol as a prominent PAINS functional group (Baell and Holloway, 2010) even as Capuzzi et al. (2017) cautions against blind application of PAINS filters. (Capuzzi et al., 2017). And yet Jasial et al. (2017) demonstrates that the catechol functional group is in the top ten percentile (9.5) of primary activity assays in Pubchem, and in the top seven percentile (6.9) of functional groups in Pubchem confirmatory assay activity (Jasial et al., 2017).

TABLE 2 Proposed deglucuronidation-based antiviral mechanism.

Stage A—Infection by any of several virus species induces inflammation
↓
Stage B—Flavonoid aglycones are glucuronidated prior to arrival in the bloodstream
↓
Stage C—Neutrophils and macrophages are attracted to site(s) of inflammation
↓
Stage D—β-glucuronidase is expressed by neutrophils and macrophages
↓
Stage E—Serum flavonoid glucuronides are deglucuronidated ("deconjugated") by β-glucuronidase at site of inflammation
↓
Stage F—Flavonoid aglycones diffuse through cell membrane
↓
Stage G—Flavonoid aglycones cause non-selective (and non-specific) inhibition within the cell—interfering with both ordinary cellular processes and the etiological source of inflammation (such as viral replication)

TABLE 3 *in vitro* evidence of in-cell viral inhibition (reported IC50) by phenols and polyphenols.

	DENV	FMDV	Influenza-A	JEV	CHIKV	ZIKV	SARS-CoV-2
Luteolin (9)		**9.7–10.0 μM** Natural phytochemicals (2002)	**6.9–7.2 μM** Yan et al. (2019)	**15.9 μM** Fan et al. (2016)			
Isoginkgetin (10)		**1.9–2.0 μM** Natural phytochemicals (2002)					
Quercetin (3)	**95.6–118 μM** Zandi et al. (2011b)		**8.9–25.8 μM** Wu et al. (2015)			**2.3 μM** Zou et al. (2020)	**18.2 μM** Kandeil et al. (2021)
Baicalein[a] (11)	**5.7–23.9 μM** Zandi et al. (2012)			**12.1–52.8 μM** Johari et al. (2012)	**7.0 μM** Lani et al. (2016)		**2.9 μM** Liu et al. (2021)
Curcumin (12)	**14.0 μM** Balasubramanian et al. (2019)		**0.5–3.8 μM** Kim et al., (2021), Chen et al. (2010)	**<30 μM** Chen et al. (2010)	**3.9 μM** Mounce et al. (2017)	**<1.9 μM** Mounce et al. (2017)	**0.4–38 μM** Bormann et al. (2021), Kandeil et al. (2021), Marín-Palma et al. (2021)
Fisetin (13)	**150 μM** Zandi et al. (2011a)				**29.5 μM** Lani et al. (2016)		
Quercetagetin (14)					**43.5 μM** Lani et al. (2016)		
Hesperetin (1)					**8.5 μM** Ahmadi et al. (2016)		
Naringenin (15)	**18–180 μM** Zandi et al. (2011a)				**6.8 μM** Ahmadi et al. (2016)		**<35 μM** Clementi et al. (2021)

[a]One phenol only.

Therapeutic role of a promiscuous binder?

The final step of a putative polyphenol deglucuronidation-based antiviral mechanism requires that a promiscuous-binding compound once inside a virus-infected human cell will arrest viable virion production. The complete proposed mechanism is presented in Table 2.

Table 2 is illustrated graphically in Figure 6—by way of one of the most studied flavonoids in the pharmacokinetic literature, quercetin. Inhibitory mechanisms of viral replication could be due to direct inhibition of viral proteins and enzymes, or by slowing ordinary cellular metabolic mechanisms such as respiration, translation,

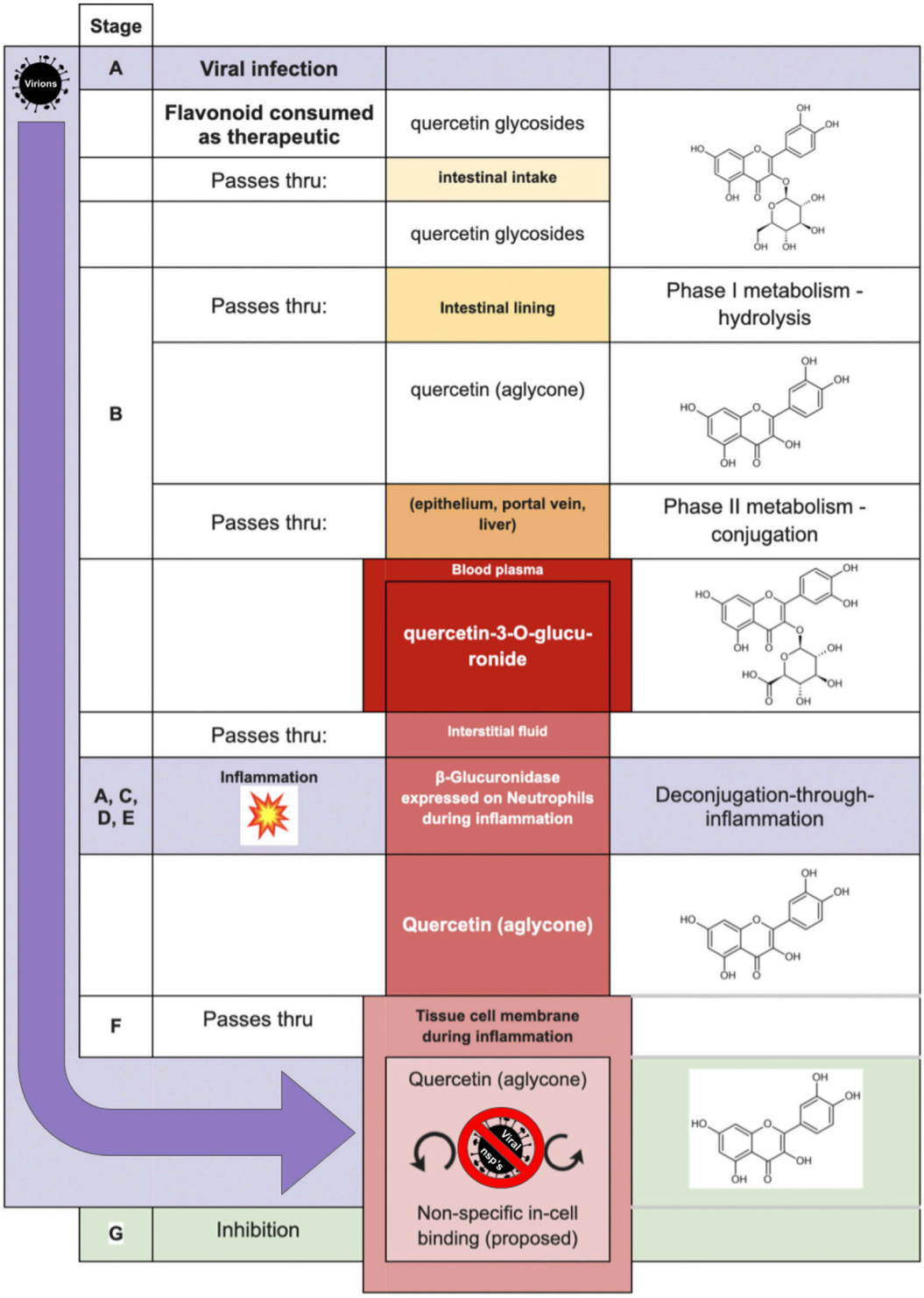


FIGURE 6
Proposed model of Shimoi mechanism for entry into intracellular compartment during viral infection, with quercetin serving in the role of the aglycone, and quercetin-3-O-glucuronide as the glucuronide [adapted from Perez-Vizcaino et al. (2012)].

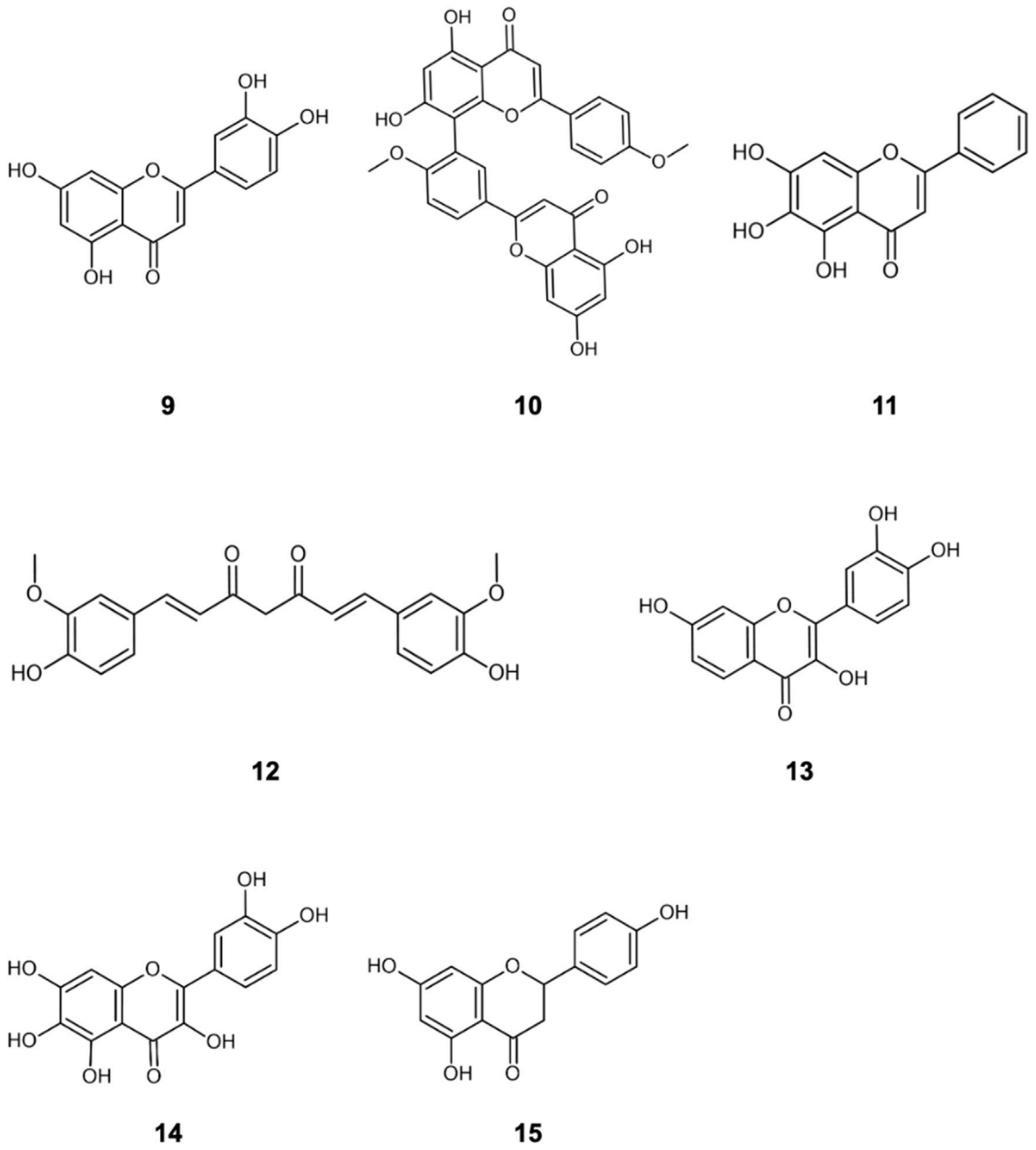


FIGURE 7
Additionally referenced polyphenols: luteolin **(9)**; Isoginkgetin **(10)**; baicalein **(11)**; curcumin **(12)**; fisetin **(13)**; quercetagetin **(14);** naringenin **(15)**.

transcription as co-opted by the infecting virus. In one case, that of fisetin applied to Dengue fever, (Zandi et al., 2011a) fisetin showed no direct activity against DENV virions outside the cell yet effectively inhibited replication in-cell. The study's authors suggest it could be due to forming complexes with RNA or inhibition of RNA polymerases. While inhibition of the dengue RdRp would represent a virus-specific inhibition, it remains intriguing to consider that the replication inhibition could also be due to general non-selective inhibition in a weakened cell.

Application of deglucuronidation-through-inflammation to antiviral assaying and clinical trialing

Given that many forms of viral infections are known to induce inflammation, it would be a logical extension to study whether consumption of certain flavonoids of sufficient quantity and in bioavailable forms could serve to reduce the rate of viral replication in the early stages of viral infection. The mechanism

of action could be by inhibition of viral entry to cells, direct inhibitory action on viral enzymes in-cell, or non-specific promiscuous disruption of the co-opted metabolism of infected cells.

The literature provides early *in vitro* evidence of achievable inhibition by phenolic flavonoids spanning across Dengue virus, Influenza-A virus (IAV), Chikungunya virus, Foot-and-mouth disease virus (FMDV), Japaneses Encephalitus Virus (JEV) and SARS-CoV-2, presented in Table 3. Corresponding ligand structures are available in Figure 1 and Figure 7.

Much care must be applied in interpreting *in vitro* viral replication inhibition results. Where an IC50 value is defined against a measure of viral RNA copies/mL, then qRT-PCR will show a difference of a single unit of Ct. For comparison, SARS-CoV-2 infection typically presents a Ct range between 10 and 40 for acute infection vs. non-detectable viral load, respectively (Kissler et al., 2021). However, *in vitro* and *in vivo* Ct values are not directly comparable, as *in vitro* reduction of viral replication may exhibit nonlinear effects at the *in vivo* scale, especially when the effects of the innate and specific immune system are considered. Where due analysis of toxicity allows, a higher IC value can be targeted, such as IC90 or even IC99 (Rusconi et al., 1994).

Viral inhibitory assays typically report the Selectivity Index (SI), defined as the ratio of the cytotoxicity (CC50) to the inhibitory concentration (IC50). A SI < 1 means that the ligand's cytotoxicity to cells occurs at a lower concentration than its inhibition of the target. Selectivity indices of 5 or greater are preferred. Ligand candidates suffering from lower selectivity indices may be excluded from further investigation. However, the deglucuronidation-through-inflammation mechanism would suggest that dismissing polyphenol ligands with a low selectivity index could be overly conservative. Given that polyphenols circulate in plasma primarily as glucuronides, (Boutin et al., 1993; Shimoi et al., 1998; Kuhnle et al., 2000; Shimoi et al., 2000; O'Leary et al., 2001; Manach et al., 2003; Campanero et al., 2010; Silvestro et al., 2013; Russo et al., 2015; Russo et al., 2018; Bajraktari and Weiss, 2020; Hai et al., 2020) a low selectivity index for the aglycone may not only be acceptable but may even be preferable. This of course will depend on how efficiently the deglucuronidation process discriminates between the localities of healthy and infected cells that induce the inflammatory process.

Given the selectivity that deglucuronidation-through-inflammation affords, and the putative validity of aggregation-based non-selective binding mechanisms, (Shoichet, 2021) the standard practice of applying aggregate-dissociating detergents such as Triton X-100 is called into question for antiviral assays of phenols that are known or expected to act through the DTI mechanism. A revisiting of relevant results of *in vitro* assays in the literature where such a detergent was applied would be appropriate. However, such a modification to laboratory practice should be considered carefully as the tendency for an aggregation to bind assay-specific enzymes could still benefit from detergent application.

Non-selective inhibition can be a double-edged sword. Dong (2014) demonstrates that the aglycone kaempferol increased IAV viral titers by log-2 compared with untreated mice, hastening their loss (Dong et al., 2014). This was attributed to attenuation of antiviral host-defense factor expression such as IFNα, IFNβ, IFNγ. By contrast, hesperidin was protective of the mice. Further laboratory and clinical investigation of demonstrably promiscuous-binding polyphenols utilizing *in vitro* viral infection culture and *in vivo* models will continue to be valuable. Attention would be particularly appropriate against those viruses that are known to induce inflammatory responses such as influenza A (IAV-A), dengue (DENV), chikungunya (CHIKV), and coronavirus (SARS-CoV-2).

Additional observations on antivirals trialing of polyphenols

While it is important to maintain ligand concentration at the target for a period sufficient to exert the relevant mechanism of action, it is worth noting that this period can be extremely short. Although not a polyphenol, artemisinin enjoys enormous efficacy against the malaria parasite *Plasmodium falciparum* with a T_{max} at less than 2 h and a half-life of 2–5 h (Benakis et al., 1997; de Vries and Dien, 2021). Also, the dosage is of utmost importance. Following due analysis of toxicity, protease inhibitors can target a C_{min} dose (minimum concentration between consecutive doses) of many multiples of the IC50 value (Boras et al., 2021) to achieve faster viral clearance. Indirect antiviral effects of certain polyphenols may also be possible, such as non-specific upregulation of immunosurveillance, as well as modulation of specific immune cells (Liu et al., 2016; Kang et al., 2021; Syafni et al., 2021).

Also, as promiscuous binders, due attention should be applied to inhibition of liver enzymes for drug-drug interactions (Bajraktari and Weiss, 2020), especially of drugs that study subjects might concomitantly consume for the same or unrelated conditions. For example, among the polyphenols studied are those known to bind to CYP1A2 (Bajraktari and Weiss, 2020), CYP3A4 (Bajraktari and Weiss, 2020) and OATP1A2, the latter giving rise to the famous "grapefruit effect" (Bailey et al., 2007). Conversely, this P450 or other liver enzyme inhibition may be advantageous to increase serum concentrations of verified pharmaceuticals, such as fluvoxamine, in context of combination therapy (James Duke, personal communication, 28 November 2009) for superior joint bioavailability.

Finally, as a given polyphenol can demonstrate differing bioavailabilities between different dosage forms, (Kaushik et al., 2012) consideration should also be given to oral delivery type, such as aqueous, softgel, dry tablet form, and degree of micronization. Further, owing to strong

bioavailability and/or release rate, delivery in the form of original plant matter while controlling for phytochemical content should also be considered (Kawai et al., 2008; Inoue et al., 2015; Hu et al., 2017).

Evolutionary role

A BLAST search demonstrates that the gene coding for β-glucuronidase [GUSB, and uidA in bacteria (Martins et al., 1993)] is extensively common across the animal kingdom (data not shown). Its homolog β-galactosidase (40% identity) is also commonly expressed in bacteria (data not shown). β-glucuronidase has several documented purposes (Martins et al., 1993). It targets glucuronic acid in the gut, (Wallace et al., 2015; Dashnyam et al., 2018) and is associated with the degradation of glucuronate-containing glycosaminoglycan (Naz et al., 2013). But its extensive expression on, and release from, neutrophils attracted in response to inflammatory signals is a mechanism whose genetic etiology and species prevalence will require further work to elucidate.

Given the long history of herbivory in animals (and associated polyphenolic compound ingestion), and the high prevalence frequency of the β-glucuronidase-coding gene GUSB across vertebrates, this mechanism could be a long-ago evolved broad response under selection pressure of viral pathologies in ancestral species. It would be worthwhile for future investigators to probe the genetic basis for neutrophilic β-glucuronidase expression and its orthology across vertebrate species in order to better localize how this response evolved.

Conclusion

In this paper, we add to the body of evidence in the literature that polyphenols are a frequent-binding class of chemicals produced by plants. We show that the pharmacology of polyphenols may allow for viral infection-fighting potential due to the human body's inflammatory response and provide conjecture as to the evolutionary basis for a putative inflammation-induced antiviral function. Future work could include quantifying the effect of *in vitro* antiviral studies under inflammation with neutrophils present for such viral targets as SARS-CoV-2, CHIKV, DENV, and IAV/IBV.

Author contributions

RS and KS contributed to conception of the review. RS wrote the first draft of the manuscript. KS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by EMSKE Phytochem.

Acknowledgments

The authors would like to thank Stéphane Quideau PhD, Jurgen Bajorath PhD, Pamela Weathers PhD, Brian Shoichet PhD, Pierre Laurin PhD, and Scott Ferguson PhD for helpful tips in the course of the preparation of this manuscript; and Yu Wai Chen PhD, Francisco Perez-Vizcaino PhD, and Erik de Clercq PhD for encouragement, and would especially like to recognize and appreciate Stephen Molnar PhD for insights and reviewing drafts of this manuscript. We are indebted to Fabiola de Marchi PhD for expert chemical structure graphic drafting. Finally, we would like to thank Priyanshu Jain for unflagging support and encouragement in the course of preparation.

Conflict of interest

Author RS was employed by EMSKE Phytochem.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Advanced chemistry development (2016). *Inc. ACD/ChemSketch*. Toronto, Canada.

Ahmadi, A., Hassandarvish, P., Lani, R., Yadollahi, P., Jokar, A., Bakar, S. A., et al. (2016). Inhibition of chikungunya virus replication by hesperetin and naringenin. *RSC Adv.* 6 (73), 69421–69430. doi:10.1039/C6RA16640G

Auld, D. S., Inglese, J., and Dahlin, J. L. (2017). "Assay interference by aggregation," in *Assay guidance manual*. Editors S. Markossian, A. Grossman, K. Brimacombe, M. Arkin, D. Auld, C. P. Austin, et al. (Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences).

Ávila-Gálvez, M. A., Giménez-Bastida, J. A., González-Sarrías, A., and Espín, J. C. (2019). Tissue deconjugation of urolithin A glucuronide to free urolithin A in systemic inflammation. *Food Funct.* 10 (6), 3135–3141. doi:10.1039/C9FO00298G

Babu, P. V. A., and Liu, D. (2009). "Chapter 18 - flavonoids and cardiovascular Health," in *Complementary and alternative therapies and the aging population.*

Editor R. R. Watson (San Diego: Academic Press), 371–392. doi:10.1016/B978-0-12-374228-5.00018-4

Badshah, S. L., Faisal, S., Muhammad, A., Poulson, B. G., Emwas, A. H., and Jaremko, M. (2021). Antiviral activities of flavonoids. *Biomed. Pharmacother. Biomedecine Pharmacother.* 140, 111596. doi:10.1016/j.biopha.2021.111596

Baell, J. B., and Holloway, G. A. (2010). New substructure filters for removal of Pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* 53 (7), 2719–2740. doi:10.1021/jm901137j

Bailey, D. G., Dresser, G. K., Leake, B. F., and Kim, R. B. (2007). Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clin. Pharmacol. Ther.* 81 (4), 495–502. doi:10.1038/sj.clpt.6100104

Bajraktari, G., and Weiss, J. (2020). The aglycone diosmetin has the higher perpetrator drug-drug interaction potential compared to the parent flavone diosmin. *J. Funct. Foods* 67, 103842. doi:10.1016/j.jff.2020.103842

Balasubramanian, A., Pilankatta, R., Teramoto, T., Sajith, A. M., Nwulia, E., Kulkarni, A., et al. (2019). Inhibition of dengue virus by curcuminoids. *Antivir. Res.* 162, 71–78. doi:10.1016/j.antiviral.2018.12.002

Bao, Y., Gao, Y., Koch, E., Pan, X., Jin, Y., and Cui, X. (2015). Evaluation of pharmacodynamic activities of EPs 7630, a special extract from roots of Pelargonium sidoides, in animals models of cough, secretolytic activity and acute bronchitis. *Phytomedicine* 22 (4), 504–509. doi:10.1016/j.phymed.2015.03.004

Bartholomé, R., Haenen, G., Hollman, P. C. H., Bast, A., Dagnelie, P. C., Roos, D., et al. (2010). Deconjugation kinetics of glucuronidated Phase II flavonoid metabolites by β-glucuronidase from neutrophils. *Drug Metab. Pharmacokinet.* 25 (4), 379–387. doi:10.2133/dmpk.DMPK-10-RG-002

Benakis, A., Paris, M., Loutan, L., Plessas, C. T., and Plessas, S. T. (1997). Pharmacokinetics of artemisinin and artesunate after oral administration in healthy volunteers. *Am. J. Trop. Med. Hyg.* 56 (1), 17–23. doi:10.4269/ajtmh.1997.56.17

Bieger, J., Cermak, R., Blank, R., de Boer, V. C. J., Hollman, P. C. H., et al. (2008). Tissue distribution of quercetin in pigs after long-term dietary supplementation. *J. Nutr.* 138 (8), 1417–1420. doi:10.1093/jn/138.8.1417

Bispo da Silva, A., Cerqueira Coelho, P. L., Alves Oliveira Amparo, J., Alves de Almeida Carneiro, M. M., Pereira Borges, J. M., Dos Santos Souza, C., et al. (2017). The flavonoid rutin modulates microglial/macrophage activation to a CD150/CD206 M2 phenotype. *Chem. Biol. Interact.* 274, 89–99. doi:10.1016/j.cbi.2017.07.004

Bisson, J., McAlpine, J. B., Friesen, J. B., Chen, S.-N., Graham, J., and Pauli, G. F. (2016). Can invalid bioactives undermine natural product-based drug discovery? *J. Med. Chem.* 59 (5), 1671–1690. doi:10.1021/acs.jmedchem.5b01009

Bobowska, A., Granica, S., Filipek, A., Melzig, M. F., Moeslinger, T., Zentek, J., et al. (2021). Comparative studies of urolithins and their Phase II metabolites on macrophage and neutrophil functions. *Eur. J. Nutr.* 60 (4), 1957–1972. doi:10.1007/s00394-020-02386-y

Boras, B., Jones, R. M., Anson, B. J., Arenson, D., Aschenbrenner, L., Bakowski, M. A., et al. (2021). Discovery of a novel inhibitor of coronavirus 3CL protease for the potential treatment of COVID-19. *bioRxiv.* 6055, 293498. doi:10.1101/2020.09.12.293498

Bormann, M., Alt, M., Schipper, L., van de Sand, L., Le-Trilling, V. T. K., Rink, L., et al. (2021). Turmeric root and its bioactive ingredient curcumin effectively neutralize SARS-CoV-2 *in vitro*. *Viruses* 13 (10), 1914. doi:10.3390/v13101914

Boutin, J. A., Meunier, F., Lambert, P. H., Hennig, P., Bertin, D., Serkiz, B., et al. (1993). *In vivo* and *in vitro* glucuronidation of the flavonoid diosmetin in rats. *Drug Metab. Dispos.* 21 (6), 1157–1166.

Campanero, M. A., Escolar, M., Perez, G., Garcia-Quetglas, E., Sadaba, B., and Azanza, J. R. (2010). Simultaneous determination of diosmin and diosmetin in human plasma by ion trap liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry: Application to a clinical pharmacokinetic study. *J. Pharm. Biomed. Anal.* 51 (4), 875–881. doi:10.1016/j.jpba.2009.09.012

Capuzzi, S. J., Muratov, E. N., and Tropsha, A. (2017). Phantom PAINS: Problems with the utility of alerts for pan-assay INterference CompoundS. *J. Chem. Inf. Model.* 57 (3), 417–427. doi:10.1021/acs.jcim.6b00465

Chen, D.-Y., Shien, J.-H., Tiley, L., Chiou, S.-S., Wang, S.-Y., Chang, T.-J., et al. (2010). Curcumin inhibits influenza virus infection and haemagglutination activity. *Food Chem. x.* 119 (4), 1346–1351. doi:10.1016/j.foodchem.2009.09.011

Clementi, N., Scagnolari, C., D'Amore, A., Palombi, F., Criscuolo, E., Frasca, F., et al. (2021). Naringenin is a powerful inhibitor of SARS-CoV-2 infection *in vitro*. *Pharmacol. Res.* 163, 105255. doi:10.1016/j.phrs.2020.105255

Dashnyam, P., Mudududdla, R., Hsieh, T.-J., Lin, T.-C., Lin, H.-Y., Chen, P.-Y., et al. (2018). β-Glucuronidases of opportunistic bacteria are the major contributors to xenobiotic-induced toxicity in the gut. *Sci. Rep.* 8 (1), 16372. doi:10.1038/s41598-018-34678-z

de Boer, V. C. J., Dihal, A. A., van der Woude, H., Arts, I. C. W., Wolffram, S., Alink, G. M., et al. (2005). Tissue distribution of quercetin in rats and pigs. *J. Nutr.* 135 (7), 1718–1725. doi:10.1093/jn/135.7.1718

de Vries, P., and Dien, T. (2021). Clinical pharmacology and therapeutic potential of artemisinin and its derivatives in the treatment of malaria. Available at: https://pubmed.ncbi.nlm.nih.gov/8957153/(Accessed 12 07, 2021).

Ding, Y., Dou, J., Teng, Z., Yu, J., Wang, T., Lu, N., et al. (2014). Antiviral activity of baicalin against influenza A (H1N1/H3N2) virus in cell culture and in mice and its inhibition of neuraminidase. *Arch. Virol.* 159 (12), 3269–3278. doi:10.1007/s00705-014-2192-2

Dong, W., Wei, X., Zhang, F., Hao, J., Huang, F., Zhang, C., et al. (2014). A dual character of flavonoids in influenza A virus replication and spread through modulating cell-autonomous immunity by mapk signaling pathways. *Sci. Rep.* 4 (1), 7237. doi:10.1038/srep07237

Dyer, O. (2021). Covid-19: FDA expert panel recommends authorising molnupiravir but also voices concerns. *BMJ* 375, n2984. doi:10.1136/bmj.n2984

Fan, W., Qian, S., Qian, P., and Li, X. (2016). Antiviral activity of luteolin against Japanese encephalitis virus. *Virus Res.* 220, 112–116. doi:10.1016/j.virusres.2016.04.021

French, C. J., and Towers, G. H. N. (1992). Inhibition of infectivity of potato virus X by flavonoids. *Phytochemistry* 31 (9), 3017–3020. doi:10.1016/0031-9422(92)83438-5

Galindo, P., Rodriguez-Gómez, I., González-Manzano, S., Dueñas, M., Jiménez, R., Menéndez, C., et al. (2012). Glucuronidated quercetin lowers blood pressure in spontaneously hypertensive rats via deconjugation. *PLoS ONE* 7 (3), e32673. doi:10.1371/journal.pone.0032673

Ganesh, A., Aman, A., Logie, J., Barthel, B., Cogan, P., Al-awar, R., et al. (2019). Colloidal drug aggregate stability in high serum conditions and pharmacokinetic consequence. *ACS Chem. Biol.* 14, 751–757. doi:10.1021/acschembio.9b00032

Ganesh, A., Donders, E., Shoichet, B., and Shoichet, M. (2018). Colloidal aggregation: From screening nuisance to formulation nuance. *Nano Today* 19, 188–200. doi:10.1016/j.nantod.2018.02.011

Ganesh, A., McLaughlin, C., Duan, D., Shoichet, B., and Shoichet, M. (2017). A new spin on antibody-drug conjugates: Trastuzumab-fulvestrant colloidal drug aggregates target HER2-positive cells. *ACS Appl. Mater. Interfaces* 9, 12195–12202. doi:10.1021/acsami.6b15987

Gilberg, E., Jasial, S., Stumpfe, D., Dimova, D., and Bajorath, J. (2016). Highly promiscuous small molecules from biological screening assays include many pan-assay interference compounds but also candidates for polypharmacology. *J. Med. Chem.* 59 (22), 10285–10290. doi:10.1021/acs.jmedchem.6b01314

Gilberg, E., Gütschow, M., and Bajorath, J. (2019). Promiscuous ligands from experimentally determined structures, binding conformations, and protein family-dependent interaction hotspots. *ACS Omega* 4 (1), 1729–1737. doi:10.1021/acsomega.8b03481

Gilberg, E., Stumpfe, D., and Bajorath, J. (2018). X-Ray-Structure-Based identification of compounds with activity against targets from different families and generation of templates for multitarget ligand design. *ACS Omega* 3 (1), 106–111. doi:10.1021/acsomega.7b01849

Gökçe, Ş., Dörtkardeşler, B. E., Yurtseven, A., and Kurugöl, Z. (2021). Effectiveness of Pelargonium sidoides in pediatric patients diagnosed with uncomplicated upper respiratory tract infection: A single-blind, randomized, placebo-controlled study. *Eur. J. Pediatr.* 180 (9), 3019–3028. doi:10.1007/s00431-021-04211-y

Gold, V. (Editor) (2019). *The IUPAC compendium of chemical terminology: The gold book*. 4th ed. (Research Triangle Park, NC: International Union of Pure and Applied Chemistry). doi:10.1351/goldbook

Griesbach, R. (2010). *Biochemistry and genetics of flower color*. New Jersey, United States: Wiley Online Library. doi:10.1002/9780470650301.CH4

Gutha, L. R., Casassa, L. F., Harbertson, J. F., and Naidu, R. A. (2010). Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (vitis ViniferaL.) leaves. *BMC Plant Biol.* 10 (1), 187. doi:10.1186/1471-2229-10-187

Hai, Y., Zhang, Y., Liang, Y., Ma, X., Qi, X., Xiao, J., et al. (2020). Advance on the absorption, metabolism, and efficacy exertion of quercetin and its important derivatives. *Food Front.* 1 (4), 420–434. doi:10.1002/fft2.50

Haid, S., Novodomská, A., Gentzsch, J., Grethe, C., Geuenich, S., Bankwitz, D., et al. (2012). A plant-derived flavonoid inhibits entry of all HCV genotypes into

human hepatocytes. *Gastroenterology* 143 (1), 213–222. e5. doi:10.1053/j.gastro.2012.03.036

Han, L., Fu, Q., Deng, C., Luo, L., Xiang, T., and Zhao, H. (2022). Immunomodulatory potential of flavonoids for the treatment of autoimmune diseases and tumour. *Scand. J. Immunol.* 95 (1), e13106. doi:10.1111/sji.13106

HealthTech, F., and de Zeneta, C. (2018). *Notice to US Food and Drug Administration of the Conclusion that the Intended Use of Orange Extract is Generally Recognized as Safe*. Silver Spring, MD: Food and Drug Administration, 2–77.

Helfer, M., Koppensteiner, H., Schneider, M., Rebensburg, S., Forcisi, S., Müller, C., et al. (2014). The root extract of the medicinal plant Pelargonium sidoides is a potent HIV-1 attachment inhibitor. *PLOS ONE* 9 (1), e87487. doi:10.1371/journal.pone.0087487

Honjo, M. N., Emura, N., Kawagoe, T., Sugisaka, J., Kamitani, M., Nagano, A. J., et al. (2020). Seasonality of interactions between a plant virus and its host during persistent infection in a natural environment. *ISME J.* 14 (2), 506–518. doi:10.1038/s41396-019-0519-4

Hu, Y., Zhang, W., Ke, Z., Li, Y., and Zhou, Z. (2017). *In vitro* release and antioxidant activity of Satsuma mandarin (citrus reticulata blanco cv. unshiu) peel flavonoids encapsulated by pectin nanoparticles. *Int. J. Food Sci. Technol.* 52, 2362–2373. doi:10.1111/ijfs.13520

Huang, R.-Y., Yu, Y.-L., Cheng, W.-C., OuYang, C.-N., Fu, E., and Chu, C.-L. (2010). Immunosuppressive effect of quercetin on dendritic cell activation and function. *J. Immunol.* 184 (12), 6815–6821. doi:10.4049/jimmunol.0903991

Inoue, T., Yoshinaga, A., Takabe, K., Yoshioka, T., Ogawa, K., Sakamoto, M., et al. (2015). *In situ* detection and identification of hesperidin crystals in satsuma Mandarin (citrus unshiu) peel cells. *Phytochem. Anal.* 26, 105–110. doi:10.1002/pca.2541

Ishisaka, A., Kawabata, K., Miki, S., Shiba, Y., Minekawa, S., Nishikawa, T., et al. (2013). Mitochondrial dysfunction leads to deconjugation of quercetin glucuronides in inflammatory macrophages. *PLoS ONE* 8 (11), e80843. doi:10.1371/journal.pone.0080843

Jannat, K., Paul, A. K., Bondhon, T. A., Hasan, A., Nawaz, M., Jahan, R., et al. (2021). Nanotechnology applications of flavonoids for viral diseases. *Pharmaceutics* 13 (11), 1895. doi:10.3390/pharmaceutics13111895

Jasial, S., Hu, Y., and Bajorath, J. (2016). Determining the degree of promiscuity of extensively assayed compounds. *PLOS ONE* 11 (4), e0153873. doi:10.1371/journal.pone.0153873

Jasial, S., Hu, Y., and Bajorath, J. (2017). How frequently are pan-assay interference compounds active? Large-scale Analysis of screening data reveals diverse activity profiles, low global hit frequency, and many consistently inactive compounds. *J. Med. Chem.* 60 (9), 3879–3886. doi:10.1021/acs.jmedchem.7b00154

Jasial, S., Hu, Y., and Bajorath, J. (2016). *PubChem compounds tested in primary and confirmatory assays*. Geneva, Switzerland: Zenodo. doi:10.5281/zenodo.44593

Jin, M. J., Kim, U., Kim, I. S., Kim, Y., Kim, D.-H., Han, S. B., et al. (2010). Effects of gut microflora on pharmacokinetics of hesperidin: A study on non-antibiotic and pseudo-germ-free rats. *J. Toxicol. Environ. Health. A* 73 (21–22), 1441–1450. doi:10.1080/15287394.2010.511549

Jo, S., Kim, S., Shin, D. H., and Kim, M.-S. (2020). Inhibition of SARS-CoV 3CL protease by flavonoids. *J. Enzyme Inhib. Med. Chem.* 35 (1), 145–151. doi:10.1080/14756366.2019.1690480

Johari, J., Kianmehr, A., Mustafa, M. R., Abubakar, S., and Zandi, K. (2012). Antiviral activity of baicalein and quercetin against the Japanese encephalitis virus. *Int. J. Mol. Sci.* 13 (12), 16785–16795. doi:10.3390/ijms131216785

Kamin, W., Ilyenko, L. I., Malek, F. A., and Kieser, M. (2012). Treatment of acute bronchitis with EPs 7630: Randomized, controlled trial in children and adolescents. *Pediatr. Int.* 54 (2), 219–226. doi:10.1111/j.1442-200X.2012.03598.x

Kamin, W., Maydannik, V., Malek, F. A., and Kieser, M. (2010). Efficacy and tolerability of EPs 7630 in children and adolescents with acute bronchitis - a randomized, double-blind, placebo-controlled multicenter trial with a herbal drug preparation from Pelargonium sidoides roots. *Int. J. Clin. Pharmacol. Ther.* 48 (3), 184–191. doi:10.5414/cpp48184

Kandeil, A., Mostafa, A., Kutkat, O., Moatasim, Y., Al-Karmalawy, A. A., Rashad, A. A., et al. (2021). Bioactive polyphenolic compounds showing strong antiviral activities against severe acute respiratory syndrome coronavirus 2. *Pathogens* 10 (6), 758. doi:10.3390/pathogens10060758

Kaneko, A., Matsumoto, T., Matsubara, Y., Sekiguchi, K., Koseki, J., Yakabe, R., et al. (2017). Glucuronides of phytoestrogen flavonoid enhance macrophage function via conversion to aglycones by β-glucuronidase in macrophages: Flavonoid glucuronides activate macrophage. *Immun. Inflamm. Dis.* 5 (3), 265–279. doi:10.1002/iid3.163

Kang, L., Miao, M.-S., Song, Y.-G., Fang, X.-Y., Zhang, J., Zhang, Y.-N., et al. (2021). Total flavonoids of Taraxacum mongolicum inhibit non-small cell lung cancer by regulating immune function. *J. Ethnopharmacol.* 281, 114514. doi:10.1016/j.jep.2021.114514

Kaushik, D., O'Fallon, K., Clarkson, P. M., Patrick Dunne, C., Conca, K. R., and Michniak-Kohn, B. (2012). Comparison of quercetin pharmacokinetics following oral supplementation in humans. *J. Food Sci.* 77 (11), H231–H238. doi:10.1111/j.1750-3841.2012.02934.x

Kawabata, K., Mukai, R., and Ishisaka, A. (2015). Quercetin and related polyphenols: New insights and implications for their bioactivity and bioavailability. *Food Funct.* 6 (5), 1399–1417. doi:10.1039/C4FO01178C

Kawai, Y. (2014). β-Glucuronidase activity and mitochondrial dysfunction: The sites where flavonoid glucuronides act as anti-inflammatory agents. *J. Clin. Biochem. Nutr.* 54 (3), 145–150. doi:10.3164/jcbn.14-9

Kawai, Y., Nishikawa, T., Shiba, Y., Saito, S., Murota, K., Shibata, N., et al. (2008). Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: Implication in the anti-atherosclerotic mechanism of dietary flavonoids. *J. Biol. Chem.* 283 (14), 9424–9434. doi:10.1074/jbc.M706571200

Kawai, Y. (2018). Understanding metabolic conversions and molecular actions of flavonoids *in vivo*:toward new strategies for effective utilization of natural polyphenols in human Health. *J. Med. Invest.* 65 (34), 162–165. doi:10.2152/jmi.65.162

Kim, M., Choi, H., Kim, S., Kang, L. W., and Kim, Y. B. (2021). Elucidating the effects of curcumin against influenza using in silico and *in vitro* approaches. *Pharmaceuticals* 14 (9), 880. doi:10.3390/ph14090880

Kissler, S. M., Fauver, J. R., Mack, C., Tai, C. G., Breban, M. I., Watkins, A. E., et al. (2021). *Viral dynamics of SARS-CoV-2 variants in vaccinated and unvaccinated individuals*. Cold Spring Harbor, NY: medRxiv, 21251535. doi:10.1101/2021.02.16.21251535

Kuhnle, G., Spencer, J. P. E., Chowrimootoo, G., Schroeter, H., Debnam, E. S., Srai, S. K. S., et al. (2000). Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem. Biophys. Res. Commun.* 272 (1), 212–217. doi:10.1006/bbrc.2000.2750

Kumar, S., and Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *ScientificWorldJournal.* 2013, 162750. doi:10.1155/2013/162750

Lalani, S., and Poh, C. L. (2020). Flavonoids as antiviral agents for enterovirus A71 (EV-A71). *Viruses* 12 (2), 184. doi:10.3390/v12020184

Lani, R., Hassandarvish, P., Shu, M.-H., Phoon, W. H., Chu, J. J. H., Higgs, S., et al. (2016). Antiviral activity of selected flavonoids against chikungunya virus. *Antivir. Res.* 133, 50–61. doi:10.1016/j.antiviral.2016.07.009

Leo, L., and Erman, M. (2022). FDA declines to authorize common antidepressant as COVID treatment. *Reuters.* 16, 2022.

Likic, S., Šola, I., Ludwig-Müller, J., and Rusak, G. (2014). Involvement of kaempferol in the defence response of virus infected arabidopsis thaliana. *Eur. J. Plant Pathol.* 138, 257–271. doi:10.1007/s10658-013-0326-0

Lin, C.-W., Tsai, F.-J., Tsai, C.-H., Lai, C.-C., Wan, L., Ho, T.-Y., et al. (2005). Anti-SARS coronavirus 3C-like protease effects of isatis indigotica root and plant-derived phenolic compounds. *Antivir. Res.* 68 (1), 36–42. doi:10.1016/j.antiviral.2005.07.002

Lipsitch, M., Krammer, F., Regev-Yochay, G., Lustig, Y., and Balicer, R. D. (2021). SARS-CoV-2 breakthrough infections in vaccinated individuals: Measurement, causes and impact. *Nat. Rev. Immunol.* 22, 57–65. doi:10.1038/s41577-021-00662-4

Liskova, A., Samec, M., Koklesova, L., Samuel, S. M., Zhai, K., Al-Ishaq, R. K., et al. (2021). Flavonoids against the SARS-CoV-2 induced inflammatory storm. *Biomed. Pharmacother.* 138, 111430. doi:10.1016/j.biopha.2021.111430

Liu, D.-D., Cao, G., Han, L.-K., Ye, Y.-L., Zhang, Q., Sima, Y.-H., et al. (2016). Flavonoids from radix tetrastigmae improve LPS-induced acute lung injury via the TLR4/MD-2-mediated pathway. *Mol. Med. Rep.* 14 (2), 1733–1741. doi:10.3892/mmr.2016.5412

Liu, H., Ye, F., Sun, Q., Liang, H., Li, C., Li, S., et al. (2021). Scutellaria baicalensis extract and baicalein inhibit replication of SARS-CoV-2 and its 3C-like protease *in vitro*. *J. Enzyme Inhib. Med. Chem.* 36 (1), 497–503. doi:10.1080/14756366.2021.1873977

Lowe, D. (2018). "Looking way down into the cells," in *The pipeline*. Washington D.C: the Pipeline. Available at: https://www.science.org/content/blog-post/looking-way-down-into-cells (Accessed December 13, 2021).

Lowe, D., (2019). "Drugs inside cells: How hard can it Be, right?," in *The pipeline* (Washington D.C: the Pipeline). Available at: https://www.science.org/content/blog-post/drugs-inside-cells-hard-can-right (Accessed December 13, 2021).

Lowe, D. (2020). "More on screening For coronavirus therapies," in *Science mgazine's in the pipeline* (Washington DC). Available at: https://www.science.org/content/blog-post/more-screening-coronavirus-therapies (Accessed December 5, 2021)

Lowe, D. (2021). "Too many papers," in *Science magazine's in the Pipeline* (Washington D.C). Available at: https://www.science.org/content/blog-post/too-many-papers (Accessed December 5, 2021)

Lowe, W. (2012). "Screen shots," in *Chemistry world*. Available at: https://www.chemistryworld.com/opinion/screen-shots/5266.article (Accessed December, 9 2021).

Malhotra, B., Onyilagha, J. C., Bohm, B. A., Towers, G. H. N., James, D., Harborne, J. B., et al. (1996). Inhibition of tomato ringspot virus by flavonoids. *Phytochemistry* 43 (6), 1271–1276. doi:10.1016/S0031-9422(95)00522-6

Malone, B., and Campbell, E. A. (2021). Molnupiravir: Coding for catastrophe. *Nat. Struct. Mol. Biol.* 28 (9), 706–708. doi:10.1038/s41594-021-00657-8

Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C., and Rémésy, C. (2003). Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr.* 57 (2), 235–242. doi:10.1038/sj.ejcn.1601547

Mandal, P., Dan, S., Chakraborty, S., Ghosh, B., Saha, C., Khanam, J., et al. (2019). Simultaneous determination and quantitation of diosmetin and hesperetin in human plasma by liquid chromatographic mass spectrometry with an application to pharmacokinetic studies. *J. Chromatogr. Sci.* 57 (5), 451–461. doi:10.1093/chromsci/bmz015

Marín-Palma, D., Tabares-Guevara, J. H., Zapata-Cardona, M. I., Flórez-Álvarez, L., Yepes, L. M., Rugeles, M. T., et al. (2021). Curcumin inhibits *in vitro* SARS-CoV-2 infection in Vero E6 cells through multiple antiviral mechanisms. *Molecules* 26 (22), 6900. doi:10.3390/molecules26226900

Marshall, T., Shult, P., and Busse, W. W. (1988). Release of lysosomal enzyme beta-glucuronidase from isolated human eosinophils. *J. Allergy Clin. Immunol.* 82 (4), 550–555. doi:10.1016/0091-6749(88)90964-5

Martins, M. T., Rivera, I. G., Clark, D. L., Stewart, M. H., Wolfe, R. L., and Olson, B. H. (1993). Distribution of UidA gene sequences in Escherichia coli isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbelliferyl-beta-D-glucuronide media. *Appl. Environ. Microbiol.* 59 (7), 2271–2276. doi:10.1128/AEM.59.7.2271-2276.1993

Masyeni, S., Iqhrammullah, M., Frediansyah, A., Nainu, F., Tallei, T., Emran, T. B., et al. (2022). Molnupiravir: A lethal mutagenic drug against rapidly mutating severe acute respiratory syndrome coronavirus 2-A narrative review. *J. Med. Virol.* 94 (7), 3006–3016. doi:10.1002/jmv.27730

Mathesius, U. (2018). Flavonoid functions in plants and their interactions with other organisms. *Plants* 7 (2), E30. doi:10.3390/plants7020030

McGovern, S. L., Caselli, E., Grigorieff, N., and Shoichet, B. K. (2002). A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem.* 45 (8), 1712–1722. doi:10.1021/jm010533y

McGovern, S. L., and Shoichet, B. K. (2003). Kinase inhibitors: Not just for kinases anymore. *J. Med. Chem.* 46 (8), 1478–1483. doi:10.1021/jm020427b

Menendez, C., Dueñas, M., Galindo, P., González-Manzano, S., Jimenez, R., Moreno, L., et al. (2011). Vascular deconjugation of quercetin glucuronide: The flavonoid paradox revealed? *Mol. Nutr. Food Res.* 55 (12), 1780–1790. doi:10.1002/mnfr.201100378

Meyer, O. C. (1994). Safety and security of Daflon 500 Mg in venous insufficiency and in hemorrhoidal disease. *Angiology* 45 (6), 579–584. doi:10.1177/000331979404500614

Middleton, E. (1998). Effect of plant flavonoids on immune and inflammatory cell function. *Adv. Exp. Med. Biol.* 439, 175–182. doi:10.1007/978-1-4615-5335-9_13

Mierziak, J., Kostyn, K., and Kulma, A. (2014). Flavonoids as important molecules of plant interactions with the environment. *Molecules* 19 (10), 16240–16265. doi:10.3390/molecules191016240

Mounce, B. C., Cesaro, T., Carrau, L., Vallet, T., and Vignuzzi, M. (2017). Curcumin inhibits zika and chikungunya virus infection by inhibiting cell binding. *Antivir. Res.* 142, 148–157. doi:10.1016/j.antiviral.2017.03.014

Nair, M. P. N., Kandaswami, C., Mahajan, S., Chadha, K. C., Chawda, R., Nair, H., et al. (2002). The flavonoid, quercetin, differentially regulates Th-1 (IFNgamma) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochim. Biophys. Acta* 1593 (1), 29–36. doi:10.1016/s0167-4889(02)00328-2

Natural phytochemicals (2002). luteolin and isoginkgetin, inhibit 3C protease and infection of FMDV, in silico and *in vitro*. Available at: https://pubmed.ncbi.nlm.nih.gov/34834926/(Accessed 2021-12-10).

Naz, H., Islam, A., Waheed, A., Sly, W. S., Ahmad, F., and Hassan, I. (2013). Human β-glucuronidase: Structure, function, and application in enzyme replacement therapy. *Rejuvenation Res.* 16 (5), 352–363. doi:10.1089/rej.2013.1407

Nguyen, T. T. H., Woo, H.-J., Kang, H.-K., Nguyen, V. D., Kim, Y.-M., Kim, D.-W., et al. (2012). Flavonoid-mediated inhibition of SARS coronavirus 3C-like protease expressed in pichia pastoris. *Biotechnol. Lett.* 34 (5), 831–838. doi:10.1007/s10529-011-0845-8

Ngwa, W., Kumar, R., Thompson, D., Lyerly, W., Moore, R., Reid, T.-E., et al. (2020). Potential of flavonoid-inspired phytomedicines against COVID-19. *Molecules* 25 (11), 2707. doi:10.3390/molecules25112707

Nikiforov, A. (2017). *FDA GRAS 719-pepsico-orange pomace-safety evaluation dossier supporting A generally recognized as safe (GRAS) conclusion for orange pomace*. Silver Spring, MD: Food and Drug Administration, 1-135.

Ninfali, P., Antonelli, A., Magnani, M., and Scarpa, E. S. (2020). Antiviral properties of flavonoids and delivery strategies. *Nutrients* 12 (9), 2534. doi:10.3390/nu12092534

O'Donnell, H. R., Tummino, T. A., Bardine, C., Craik, C. S., and Shoichet, B. K. (2021). Colloidal aggregators in biochemical SARS-CoV-2 repurposing screens. *J. Med. Chem.* 64, 17530–17539. doi:10.1021/acs.jmedchem.1c01547

O'Leary, K. A., Day, A. J., Needs, P. W., Sly, W. S., O'Brien, N. M., and Williamson, G. (2001). Flavonoid glucuronides are substrates for human liver β-glucuronidase. *FEBS Lett.* 503 (1), 103–106. doi:10.1016/S0014-5793(01)02684-9

Oo, A. M., Mohd Adnan, L. H., Nor, N. M., Simbak, N., Ahmad, N. Z., and Lwin, O. M. (2021). Immunomodulatory effects of flavonoids: An experimental study on natural-killer-cell-mediated cytotoxicity against lung cancer and cytotoxic granule secretion profile. *Proc. Singap. Healthc.* 30 (4), 279–285. doi:10.1177/2010105820979006

Owen, S. C., Doak, A. K., Wassam, P., Shoichet, M. S., and Shoichet, B. K. (2012). Colloidal aggregation affects the efficacy of anticancer drugs in cell culture. *ACS Chem. Biol.* 7 (8), 1429–1435. doi:10.1021/cb300189b

Panche, A. N., Diwan, A. D., and Chandra, S. R. (2016). Flavonoids: An overview. *J. Nutr. Sci.* 5, e47. doi:10.1017/jns.2016.41

Park, J.-Y., Kim, J. H., Kwon, J. M., Kwon, H.-J., Jeong, H. J., Kim, Y. M., et al. (2013). Dieckol, a SARS-CoV 3CLpro inhibitor, isolated from the edible Brown algae ecklonia cava. *Bioorg. Med. Chem.* 21 (13), 3730–3737. doi:10.1016/j.bmc.2013.04.026

Patel, K., Gadewar, M., Tahilyani, V., and Patel, D. K. (2013). A review on pharmacological and analytical aspects of diosmetin: A concise report. *Chin. J. Integr. Med.* 19 (10), 792–800. doi:10.1007/s11655-013-1595-3

Patiroglu, T., Tunc, A., Eke Gungor, H., and Unal, E. (2012). The efficacy of Pelargonium sidoides in the treatment of upper respiratory tract infections in children with transient hypogammaglobulinemia of infancy. *Phytomedicine* 19 (11), 958–961. doi:10.1016/j.phymed.2012.06.004

Perez-Vizcaino, F., Duarte, J., and Santos-Buelga, C. (2012). The flavonoid paradox: Conjugation and deconjugation as key steps for the biological activity of flavonoids. *J. Sci. Food Agric.* 92 (9), 1822–1825. doi:10.1002/jsfa.5697

Pfizer (2021). Pfizer's novel COVID-19 oral antiviral treatment candidate reduced risk of hospitalization or death by 89% in interim analysis of Phase 2/3 EPIC-HR study. Available at: https://www.pfizer.com/news/press-release/press-release-detail/pfizers-novel-covid-19-oral-antiviral-treatment-candidate (Accessed 12 05, 2021).

Pietta, P. G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.* 63 (7), 1035–1042. doi:10.1021/np9904509

Piwowarski, J. P., Stanisławska, I., Granica, S., Stefańska, J., and Kiss, A. K. (2017). Phase II conjugates of urolithins isolated from human urine and potential role of β-glucuronidases in their disposition. *Drug Metab. Dispos.* 45 (6), 657–665. doi:10.1124/dmd.117.075200

Pohjala, L., and Tammela, P. (2012). Aggregating behavior of phenolic compounds — a source of false bioassay results? *Molecules* 17 (9), 10774–10790. doi:10.3390/molecules170910774

Quideau, S., Deffieux, D., Douat-Casassus, C., and Pouységu, L. (2011). Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angew. Chem. Int. Ed. Engl.* 50 (3), 586–621. doi:10.1002/anie.201000044

Ratain, M. J., and Plunkett, William K. (2003). "J. Principles of pharmacokinetics," in *Holl.-Frei cancer med*. 6th Ed. (New Jersey, United States: Wiley Online Library).

Rathee, P., Chaudhary, H., Rathee, S., Rathee, D., Kumar, V., and Kohli, K. (2009). Mechanism of action of flavonoids as anti-inflammatory agents: A review. *Inflamm. Allergy Drug Targets* 8 (3), 229–235. doi:10.2174/187152809788681029

Reis, G., Moreira-Silva, E. A., dos, S., Silva, D. C. M., Thabane, L., Milagres, A. C., et al. (2021). Effect of early treatment with fluvoxamine on risk of emergency care and hospitalisation among patients with COVID-19: The TOGETHER randomised, platform clinical trial. *Lancet Glob. Health* 0 (0), e42–e51. doi:10.1016/S2214-109X(21)00448-4

Roschek, B., Fink, R. C., McMichael, M. D., Li, D., and Alberte, R. S. (2009). Elderberry flavonoids bind to and prevent H1N1 infection *in vitro*. *Phytochemistry* 70 (10), 1255–1261. doi:10.1016/j.phytochem.2009.06.003

Roshanravan, N., Seif, F., Ostadrahimi, A., Pouraghaei, M., and Ghaffari, S. (2020). Targeting cytokine storm to manage patients with COVID-19: A mini-review. *Arch. Med. Res.* 51 (7), 608–612. doi:10.1016/j.arcmed.2020.06.012

Roth, M., Fang, L., Stolz, D., and Tamm, M. (2019). Pelargonium sidoides radix extract EPs 7630 reduces rhinovirus infection through modulation of viral binding proteins on human bronchial epithelial cells. *PloS One* 14 (2), e0210702. doi:10.1371/journal.pone.0210702

Rusconi, S., Merrill, D. P., and Hirsch, M. S. (1994). Inhibition of human immunodeficiency virus type 1 replication in cytokine-stimulated monocytes/macrophages by combination therapy. *J. Infect. Dis.* 170 (6), 1361–1366. doi:10.1093/infdis/170.6.1361

Russo, R., Chandradhara, D., and De Tommasi, N. (2018). Comparative bioavailability of two diosmin formulations after oral administration to healthy volunteers. *Molecules* 23 (9), E2174. doi:10.3390/molecules23092174

Russo, R., Mancinelli, A., Ciccone, M., Terruzzi, F., Pisano, C., and Severino, L. (2015). Pharmacokinetic profile of μsmin Plus™, a new micronized diosmin formulation, after oral administration in rats. *Nat. Prod. Commun.* 10 (9), 1569–1572.

Ryu, Y. B., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, J.-Y., Kim, D., et al. (2010). Biflavonoids from torreya nucifera displaying SARS-CoV 3CLpro inhibition. *Bioorg. Med. Chem.* 18 (22), 7940–7947. doi:10.1016/j.bmc.2010.09.035

Sharma, V., Sehrawat, N., Sharma, A., Yadav, M., Verma, P., and Sharma, A. K. (2021). Multifaceted antiviral therapeutic potential of dietary flavonoids: Emerging trends and future perspectives. *Biotechnol. Appl. Biochem* 1–18. doi:10.1002/bab.2265

Sheridan, R., and Spelman, K. (2021). *Polyphenolic promiscuity, inflammation-coupled specificity: Whether PAINs filters mask an antiviral asset*. Geneva, Switzerland: Zenodo. [Preprint]. doi:10.5281/zenodo.5791777

Sheridan, R., and Spelman, K. (2022). *Polyphenolic promiscuity, inflammation-coupled specificity: Whether PAINs filters mask an antiviral asset*. Washington D. C.: ChemRxiv. [Preprint] doi:10.26434/chemrxiv-2022-qvd9n

Shimoi, K., and Nakayama, T. (2005). Glucuronidase deconjugation in inflammation. *Methods Enzymol.* 400, 263–272. Elsevier. doi:10.1016/S0076-6879(05)00015-7

Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., et al. (1998). Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. *FEBS Lett.* 5, 220–224. doi:10.1016/s0014-5793(98)01304-0

Shimoi, K., Saka, N., Kaji, K., Kinae, R. N., and KiNae, N. (2000). Metabolic fate of luteolin and its functional activity at focal site. *BioFactors* 12 (1–4), 181–186. doi:10.1002/biof.5520120129

Shimoi, K., Saka, N., Nozawa, R., Sato, M., Amano, I., Nakayama, T., et al. (2001). Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *Drug Metab. Dispos.* 29, 1521–1524.

Shoichet, B. K. (2006). Screening in a spirit haunted world. *Drug Discov. Today* 11 (13–14), 607–615. doi:10.1016/j.drudis.2006.05.014

Shoichet, Brian (2021). @Nairobih3 aggregates can enter cells, and as @MollyShoichet group and ours have show, that can Be a cool delivery technique. But the aggregates themselves would remain non-selective. *So in most cases not a good option*. San Francisco: @BShoichet. Available at: https://twitter.com/BShoichet/status/1464048456343851011 (Accessed December 4, 2021).

Silvestro, L., Tarcomnicu, I., Dulea, C., Attili, N. R. B. N., Ciuca, V., Peru, D., et al. (2013). Confirmation of diosmetin 3-O-glucuronide as major metabolite of diosmin in humans, using micro-liquid-chromatography-mass spectrometry and ion mobility mass spectrometry. *Anal. Bioanal. Chem.* 405 (25), 8295–8310. doi:10.1007/s00216-013-7237-y

Sisa, M., Bonnet, S. L., Ferreira, D., and Van der Westhuizen, J. H. (2010). Photochemistry of flavonoids. *Molecules* 15 (8), 5196–5245. doi:10.3390/molecules15085196

Spanakis, M., Kasmas, S., and Niopas, I. (2009). Simultaneous determination of the flavonoid aglycones diosmetin and hesperetin in human plasma and urine by a validated GC/MS method: *In vivo* metabolic reduction of diosmetin to hesperetin. *Biomed. Chromatogr.* 23 (2), 124–131. doi:10.1002/bmc.1092

Stachulski, A. V., and Meng, X. (2013). Glucuronides from metabolites to medicines: A survey of the *in vivo* generation, chemical synthesis and properties of glucuronides. *Nat. Prod. Rep.* 30 (6), 806–848. doi:10.1039/c3np70003h

Struckmann, J. R., and Nicolaides, A. N. (1994). Flavonoids: A review of the pharmacology and therapeutic efficacy of Daflon 500 Mg in patients with chronic venous insufficiency and related disorders. *Angiology* 45 (6), 419–428. doi:10.1177/000331979404500602

Syafni, N., Devi, S., Zimmermann-Klemd, A. M., Reinhardt, J. K., Danton, O., Gründemann, C., et al. (2021). Immunosuppressant flavonoids from scutellaria baicalensis. *Biomed. Pharmacother. Biomedecine Pharmacother.* 144, 112326. doi:10.1016/j.biopha.2021.112326

Tahan, F., and Yaman, M. (2013). Can the Pelargonium sidoides root extract EPs 7630 prevent asthma attacks during viral infections of the upper respiratory tract in children? *Phytomedicine* 20 (2), 148–150. doi:10.1016/j.phymed.2012.09.022

Takahashi, A., and Ohnishi, T. (2004). The significance of the study about the biological effects of solar ultraviolet radiation using the exposed facility on the international space station. *Biol. Sci. Space.* 18 (4), 255–260. doi:10.2187/bss.18.255

Takumi, H., Nakamura, H., Simizu, T., Harada, R., Kometani, T., Nadamoto, T., et al. (2012). Bioavailability of orally administered water-dispersible hesperetin and its effect on peripheral vasodilatation in human subjects: Implication of endothelial functions of plasma conjugated metabolites. *Food Funct.* 3 (4), 389–398. doi:10.1039/c2fo10224b

Terahara, N. (2015). Flavonoids in foods: A review. *Nat. Prod. Commun.* 10 (3), 1934578X1501000–528. doi:10.1177/1934578x1501000334

Terao, J. (2017). Factors modulating bioavailability of quercetin-related flavonoids and the consequences of their vascular function. *Biochem. Pharmacol.* 139, 15–23. doi:10.1016/j.bcp.2017.03.021

Terao, J., Murota, K., and Kawai, Y. (2011). Conjugated quercetin glucuronides as bioactive metabolites and precursors of aglycone*in vivo*. *Food Funct.* 2 (1), 11–17. doi:10.1039/C0FO00106F

The RECOVERY Collaborative Group (2021). Dexamethasone in hospitalized patients with covid-19. *N. Engl. J. Med.* 384 (8), 693–704. doi:10.1056/NEJMoa2021436

Tozer, T. N. (1981). Concepts basic to pharmacokinetics. *Pharmacol. Ther.* 12 (1), 109–131. doi:10.1016/0163-7258(81)90077-2

Tranoy-Opalinski, I., Legigan, T., Barat, R., Clarhaut, J., Thomas, M., Renoux, B., et al. (2014). β-Glucuronidase-Responsive prodrugs for selective cancer chemotherapy: An update. *Eur. J. Med. Chem.* 74, 302–313. doi:10.1016/j.ejmech.2013.12.045

Treutter, D. (2005). Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biol.* 7 (6), 581–591. doi:10.1055/s-2005-873009

Ueno, I., Nakano, N., and Hirono, I. (1983). Metabolic fate of [14C] quercetin in the ACI rat. *Jpn. J. Exp. Med.* 53 (1), 41–50.

U.S.Food and Drugs Administration (2021). *Research, C. For D. E. And. FDA authorizes, REGEN-COV monoclonal antibody therapy for post-exposure prophylaxis (prevention) for COVID-19. FDA* 2021.

Wallace, B. D., Roberts, A. B., Pollet, R. M., Ingle, J. D., Biernat, K. A., Pellock, S. J., et al. (2015). Structure and inhibition of microbiome β-glucuronidases essential to the alleviation of cancer drug toxicity. *Chem. Biol.* 22 (9), 1238–1249. doi:10.1016/j.chembiol.2015.08.005

Walle, T., Walle, U. K., and Halushka, P. V. (2001). Carbon dioxide is the major metabolite of quercetin in humans. *J. Nutr.* 131 (10), 2648–2652. doi:10.1093/jn/131.10.2648

Wu, W., Li, R., Li, X., He, J., Jiang, S., Liu, S., et al. (2015). Quercetin as an antiviral agent inhibits influenza A virus (IAV) entry. *Viruses* 8 (1), 6. doi:10.3390/v8010006

Yan, H., Ma, L., Wang, H., Wu, S., Huang, H., Gu, Z., et al. (2019). Luteolin decreases the yield of influenza A virus *in vitro* by interfering with the coat protein I complex expression. *J. Nat. Med.* 73 (3), 487–496. doi:10.1007/s11418-019-01287-7

Yang, L.-L., Xiao, N., Li, X.-W., Fan, Y., Alolga, R. N., Sun, X.-Y., et al. (2016). Pharmacokinetic comparison between quercetin and quercetin 3-O-β-Glucuronide in rats by UHPLC-MS/MS. *Sci. Rep.* 6 (1), 35460. doi:10.1038/srep35460

Zakaryan, H., Arabyan, E., Oo, A., and Zandi, K. (2017). Flavonoids: Promising natural compounds against viral infections. *Arch. Virol.* 162 (9), 2539–2551. doi:10.1007/s00705-017-3417-y

Zandi, K., Teoh, B.-T., Sam, S.-S., Wong, P.-F., Mustafa, M. R., and AbuBakar, S. (2011). Antiviral activity of four types of bioflavonoid against dengue virus type-2. *Virol. J.* 8 (1), 560. doi:10.1186/1743-422X-8-560

Zandi, K., Teoh, B.-T., Sam, S.-S., Wong, P.-F., Mustafa, M., and Abu Bakar, S. (2011). Vitro antiviral activity of fisetin, rutin and naringenin against dengue virus type-2. *J. Med. Plants Res.* 5, 5534–5539. doi:10.5897/JMPR11.1046

Zandi, K., Teoh, B.-T., Sam, S.-S., Wong, P.-F., Mustafa, M. R., and AbuBakar, S. (2012). Novel antiviral activity of baicalein against dengue virus. *BMC Complement. Altern. Med.* 12, 214. doi:10.1186/1472-6882-12-214

Zaragozá, C., Villaescusa, L., Monserrat, J., Zaragozá, F., and Álvarez-Mon, M. (2020). Potential therapeutic anti-inflammatory and immunomodulatory effects of dihydroflavones, flavones, and flavonols. *Molecules* 25 (4), E1017. doi:10.3390/molecules25041017

Zhou, Y., Gammeltoft, K. A., Ryberg, L. A., Pham, L. V., Fahnøe, U., Binderup, A., et al. (2022). Nirmatrelvir resistant SARS-CoV-2 variants with high fitness *in vitro*. *bioRxiv June* 7, 494921. doi:10.1101/2022.06.06.494921

Zou, M., Liu, H., Li, J., Yao, X., Chen, Y., Ke, C., et al. (2020). Structure-activity relationship of flavonoid bifunctional inhibitors against zika virus infection. *Biochem. Pharmacol.* 177, 113962. doi:10.1016/j.bcp.2020.113962

frontiers | Frontiers in Pharmacology

TYPE Original Research
PUBLISHED 10 November 2022
DOI 10.3389/fphar.2022.921511

OPEN ACCESS

EDITED BY
Alessandra Russo,
University of Catania, Italy

REVIEWED BY
Samuel Kyerematen,
AbbVie, Germany
Xue-Pin Liao,
Sichuan University, China
Sanjay Tiwari,
National Institute of Pharmaceutical Education and Research, India

*CORRESPONDENCE
Tania F. Bahamondez-Canas,
tania.bahamondez@uv.cl

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Ethnopharmacology, a section of the journal Frontiers in Pharmacology

RECEIVED 19 April 2022
ACCEPTED 24 October 2022
PUBLISHED 10 November 2022

CITATION
Araya N, Leiva-Soto MA, Bruna MV, Castro-Munoz A, Behrend-Keim B, Moraga-Espinoza D and Bahamondez-Canas TF (2022), Formulation of water-soluble *Buddleja globosa* Hope extracts and characterization of their antimicrobial properties against *Pseudomonas aeruginosa*. *Front. Pharmacol.* 13:921511. doi: 10.3389/fphar.2022.921511

Formulation of water-soluble *Buddleja globosa* Hope extracts and characterization of their antimicrobial properties against *Pseudomonas aeruginosa*

Nicolas Araya[1], Martín A. Leiva-Soto[1], Maria V. Bruna[1], Almendra Castro-Munoz[1†], Beatriz Behrend-Keim[1†], Daniel Moraga-Espinoza[1,2] and Tania F. Bahamondez-Canas[1,2]*

[1]Escuela de Química y Farmacia, Facultad de Farmacia, Universidad de Valparaíso, Valparaíso, Chile, [2]Centro de Investigacion Farmacopea Chilena, Universidad de Valparaíso, Valparaíso, Chile

Buddleja globosa Hope (BG) extracts are traditionally used to treat skin and gastric ulcers due to their healing properties. Non-aqueous solvents such as ethanol and DMSO are usually used to extract naturally occurring compounds. However, the cytotoxicity of these solvents and the low water solubility of the extracted compounds can hinder their biomedical applications. To overcome the limited solubility of the BG extracts, we aimed to enhance the solubility by processing a standardized hydroalcoholic extract (BG-126) through spray drying (SD), with and without two solubility enhancers. Spray-dried BG (BG-SD) extracts and spray-dried BG extracts plus polyvinylpyrrolidone (BG-SD PVP) and Soluplus® (BG-SD SP) were developed starting from BG-126 (containing 53% ethanol). These four formulations were characterized by total phenolic content, water solubility at 25°C and 37°C, and antimicrobial properties against *Pseudomonas aeruginosa*. All the SD formulations presented a solubility that allowed them to reach maximum concentrations of 1,024 µg/ml catechin for BG-SD and 2,048 µg/ml catechin for BG-SD PVP and BG-SD SP for antimicrobial testing. BG-SD showed the highest antimicrobial potency with a minimum inhibitory concentration (MIC) of 512 µg/ml catechin, followed by BG-126 with a MIC of 1,024 µg/ml catechin and SP. BG-126 was also shown to inhibit biofilm formation, as well as the excipients PVP and SP. The spray-dried BG (BG-SD) extract represents a promising natural active component with enhanced antimicrobial properties against *P. aeruginosa* for further research and the development of novel phytopharmaceuticals.

KEYWORDS

Buddleja globosa*, natural extracts, phytopharmaceuticals, antimicrobials, *Pseudomonas aeruginosa

1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen commonly isolated from complex wounds (Kirketerp-Moller et al., 2008). Despite the efforts in wound care, chronic wounds remain a clinical challenge due to their susceptibility to complications such as infection or reoccurrence that negatively impact the life quality of these patients. While there is no clear cause of the chronicity of wounds, several identified factors that contribute to this state range from metabolic, nutritional, and local factors (Han and Ceilley, 2017).

Evidence shows that microbial biofilms colonize more than 60% of chronic wounds, and *P. aeruginosa* is one of the most predominant microorganisms (James et al., 2008). Also, colonization by this pathogen is related to larger ulcer sizes and more healing complications (Danielsen et al., 1998; Kirketerp-Moller et al., 2008; Malone et al., 2017). Biofilms are microbial communities that grow adhered to surfaces, protecting themselves from the environment with a self-secreted extracellular polymeric substance (EPS). This EPS, also known as the biofilm matrix, shields the community from external factors such as shear stress, antimicrobial agents, and immune factors (Flemming and Wingender, 2010). Therefore, when these biofilms develop on the surface of wounds, they contribute to a chronic state of inflammation that delays wound healing (Bjarnsholt, 2013). Although biofilms are commonly found in nature, biofilm colonization usually occurs in the tissues of patients affected by underlying diseases such as diabetes or venous ulcers (Wolcott and Ehrlich, 2008; Bjarnsholt, 2013).

As antimicrobial resistance to antibiotics increases, the World Health Organization has made several calls to promote the research and development of new antimicrobial agents, particularly focusing on a detailed list of pathogens (Taconelli and Magrini, 2017). *P. aeruginosa* is included in this list as a pathogen of high priority. In this scenario, natural extracts can still be envisaged as a source of novel antimicrobial compounds.

Buddleja globosa Hope (BG) is a native shrub from Chile, Perú, and Argentina, traditionally used to treat skin and gastric ulcers for its analgesic, anti-inflammatory, and antioxidant properties (Backhouse et al., 2008; Bustamante et al., 2015). Also, the antimicrobial properties of several components of BG have been reported against *P. aeruginosa*, *S. aureus*, and *E. coli,* among other microorganisms. Some examples of these compounds are catechin (Zhang et al., 2021), apigenin and its derivatives (Kim et al., 2020), carvacrol (Bhardwaj et al., 2019), verbascoside (Avila et al., 1999), and quercetin (Jaisinghani, 2017a). However, the antimicrobial activity of *B. globosa* extracts against *P. aeruginosa* proliferation and biofilm formation has not been evaluated.

On the other hand, natural extracts are usually obtained using non-aqueous solvents like ethanol, methanol, or DMSO. However, the low solubility of the extract components can hinder their biomedical application in the treatment of chronic or infected wounds as these solvents may exert cytotoxic effects against fibroblasts and keratinocytes (Singh, 2017; Calderon-Montano et al., 2018; Kar et al., 2021). Therefore, increasing the water solubility of natural extracts may maximize their efficacy and minimize or discard the cytotoxicity induced by non-aqueous solvents.

Different strategies have been studied to address these problems, like using nanocarriers and other solubilizing agents (Vanti, 2021). Soluplus® is a graft copolymer of polyvinyl caprolactam, polyvinyl acetate, and polyethylene glycol, capable of solubilizing hydrophobic compounds by forming polymeric nanomicelles (Pignatello et al., 2022). Polyvinylpyrrolidone (PVP) is a versatile excipient useful for encapsulating hydrophilic and hydrophobic compounds in solid dispersions (Kurakula and Rao, 2020). The amphoteric nature of Soluplus® has proven to enhance the solubility of poorly soluble natural extracts. Rajakumari et al. (2020) used Soluplus® for grape seed extract processing by freeze drying and then tested the antioxidant activity of the formulation. This excipient has also been tested for isolated natural compounds like thymoquinone (TQ), a potent antineoplastic component extracted from *Nigella sativa* essential oil. TQ has low aqueous solubility which limits its further applications. Bergonzi et al. (2020) formulated TQ using an association of polymeric solubilizers, where Soluplus® increased 10 times its solubility. This improvement is also related to an increase in its bioactivity against cancer cell migration.

Therefore, this research aimed to explore the antimicrobial properties of BG extracts against *P. aeruginosa* and to develop BG formulations from a hydroalcoholic extract with improved water solubility to enhance its antimicrobial potency without the need for ethanol as a solvent. To enhance the solubility of the BG extract, we developed three dry powder formulations of BG through spray drying (SD), starting from a standardized hydroalcoholic extract (BG-126) with and without two pharmaceutical excipients (PVP and Soluplus®). Then, we evaluated their antimicrobial properties against *P. aeruginosa*, an opportunistic pathogen commonly isolated from chronic wounds.

2 Materials and methods

2.1 Materials

The standardized ethanolic extract of *Buddleja globosa* Hope (BG-126) was kindly donated by Laboratorios Ximena Polanco (Santiago, Chile). Soluplus® and Kollidon® L30 (PVP) were gifted by BASF Chile. Crystal violet, methanol, acetic acid, MOPS buffer, fluorescein diacetate (FDA), tobramycin sulfate, glycerol, catechin hydrate, LB broth and agar, and phosphate-buffered saline (PBS) were provided by Sigma-

TABLE 1 BG formulations studied and prepared by spray drying.

Formulation	Description	Composition	SD parameters	Mass recovered (g)
BG-SD	Spray-dried *B. globosa* ethanolic extract	50 ml extract	IT: 90°C/OT 60°C A 29,75 m^3/h AF 474 L/h F 6 ml/min	1.40
BG-SD SP	Spray-dried *B. globosa* ethanolic extract + Soluplus®	50 ml extract +9.19 g of Soluplus®	IT 90°C and 120°C/OT 60°C A 29,75 m^3/h AF 474 L/h F 6 and 3 ml/min	7.03
BG-SD PVP	Spray-dried *B. globosa* ethanolic extract + PVP	50 ml extract +9.14 g PVP	IT 90°C/OT 60°C A 29,75 m^3/h AF 474 L/h F 6 ml/min	9.42

SD, spray drying; IT, inlet temperature; OT, outer temperature; A, aspirator; AF, atomization gas flow; F, flow of the feeding solution.

Aldrich (St. Louis, MO, United States). Ethanol absolute was obtained from Millipore (Merck KGaA, Darmstadt, Germany), and acetone was obtained from J. T. Baker (Radnor, PA, United States). Verbascoside was provided by MedChemExpress LLC (Monmouth Junction, NJ, United States).

2.2 Spray drying

The hydroalcoholic BG-126 extract [53% (v/v) ethanol] was processed by SD, as received from the provider to obtain BG-SD. Two solutions were prepared with BG-126 and excipients. In total, three formulations were developed, as shown in Table 1. These solutions were spray-dried in a Büchi B-290 Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland). After the drying process, the obtained formulations were stored in amber bottles inside a desiccator until use.

2.3 Characterization of the *Buddleja globosa* Hope formulations

2.3.1 Chemical analysis

All the formulations (BG-126, BG-SD, BG-SD SP, and BG-SD PVP) were characterized according to their total phenolic content expressed as catechins using the Folin–Ciocalteu (FC) method, commonly used to analyze natural products (Chen et al., 2015). For determining the calibration curve, 10.9 mg of catechin hydrate was added to 50-ml volumetric flasks, and the volume was completed with distilled water. Then, five standard solutions were prepared from this stock solution at 10, 20, 30, 40, and 50 ppm in 10-ml volumetric flasks. Then, 0.5 ml of each standard solution was combined and vortexed with 2.5 ml of 10% FC (v/v). After 8 min of vortex mixing, 2.0 ml of 7.5% Na_2CO_3 (w/v) was added, and the mixture was allowed to sit for 1 h protected from light before recording the absorbance at 765 nm. After plotting the absorbance *versus* catechin concentration, a value of R^2 = 0.9927 was obtained. Finally, the following samples of the extracts were analyzed: 4.2 mg of BG-SD, 20.6 mg of BG-SD PVP, and 20.1 mg of BG-SD SP were prepared in 25-ml volumetric flasks, while 266 µl of BG-126 was diluted in a 100-ml volumetric flask. These solutions were analyzed by the FC method, and the catechin content was calculated by linear regression.

BG-126 and BG-SD were additionally characterized due to their verbascoside content using a Shimadzu HPLC system [SIL-20AC HT autosampler, LC-20AD pump, CTO-20AC column oven, DGU-2045 degasser, and SPD-M20A photodiode array detector (PDA)]. Separation was achieved on an Inertsil® ODS-3 column (C18, 4.6 × 250 mm, 5 µm; GL Sciences Inc., Tokyo, Japan) at a flow rate of 1.0 ml/min using a binary gradient with water containing 5% phosphoric acid (mobile phase A) and acetonitrile (mobile phase B). The column oven was set to 30°C, and the PDA detector was operated at 330 nm. Peaks were recorded and integrated using LabSolutions DB (Shimadzu Corporation, Kyoto, Japan). A calibration curve of a verbascoside standard (99.83% purity) was prepared as follows: 8.4 mg of verbascoside was diluted in a 50-ml volumetric flask using 50% ethanol (v/v). A total of five 10 ml standard solutions were prepared from 8.4 to 167.7 mg/ml. These solutions were

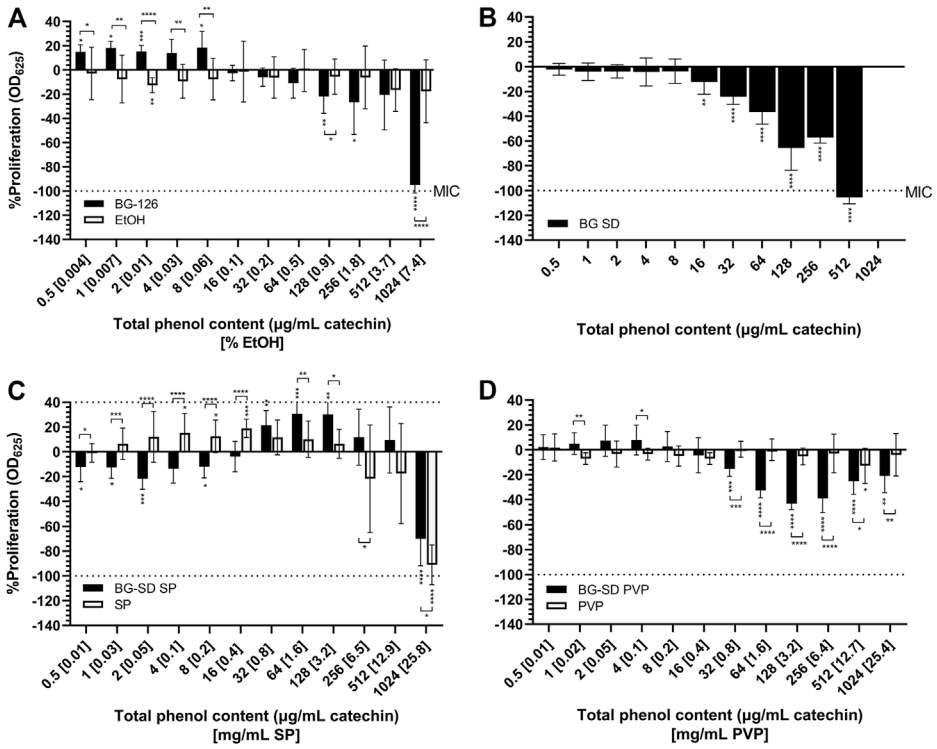


FIGURE 1
Effect of *Buddleja globosa* Hope (BG) extracts on the proliferation of *Pseudomonas aeruginosa*. *P. aeruginosa* was treated for 24 h with a hydroalcoholic BG extract (BG-126) **(A)**, spray-dried BG extract (BG-SD) **(B)**, spray-dried BG extract with Soluplus® (BG-SD SP) **(C)**, and spray-dried BG extract with PVP (BG-SD PVP). **(D)** Asterisks on bars indicate a statistically significant difference with respect to the untreated control, and asterisks on brackets indicate differences between the BG formulations and the excipients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$, and ****$p < 0.01$.

injected (20 µl) into the instrument. The calibration curve obtained had a value of $R^2 = 0.9999$. Then, 0.1 ml of BG-126 was diluted to 5 ml, and 4.5 mg of BG-SD was diluted to 25 ml in volumetric flasks. BG-SD PVP and BG-SD SP were not analyzed by this method due to their high polymeric content.

2.3.2 Solubility and flowability

To determine the solubility of the SD formulations, 200 mg of BG-SD, BG-SD PVP, and BG-SD SP were weighed in 200-ml beakers. Then, known volumes of distilled water were added at 15 min intervals while mixing on a magnetic stirrer at 25°C until complete dissolution was observed, followed by the FC assay described previously. This test was also performed at 37°C considering further conditions used in bacterial culture.

The angle of repose was assessed using a glass funnel loaded with 1 g of the SD formulations at 6 cm from the surface. The angles of repose were captured using a monochromatic camera (Flea3®, FLIR Integrated Imaging Solutions, Inc.) and calculated by ImageJ software. The flowability of the dry powder formulations was described from "excellent" to "very, very poor," as described by chapter 1,174 of the USP.

2.4 Antimicrobial properties against *Pseudomonas aeruginosa*

2.4.1 Bacterial culture

Pseudomonas aeruginosa ATCC 27853 (Microbiologics, Inc., St. Cloud, MN, United States) was maintained in 50% glycerol stocks at −80°C. Fresh agar cultures were prepared in LB agar for each experiment starting from frozen stocks, and then, the plates were incubated overnight in a static incubator at 37°C (Heratherm IGS60, Thermo Fisher Scientific, Columbus, OH, United States). Then, bacterial suspensions were prepared in LB medium and adjusted to 3×10^7 CFU/ml by spectrophotometry (at 600 nm, Synergy H1M, BioTek Instruments, Santa Clara, CA, United States). For the broth microdilution method, this suspension was diluted in 1: 100 dilutions and added to tissue culture-treated 96-well plates (Corning Inc., Corning, NY, United States). For biofilm formation, 100 µl of the adjusted suspension (3×10^7 CFU/ml) was added to black, flat-bottomed, tissue culture-treated 96-well plates (Corning Inc., Corning, NY, United States). After 1.5 h of incubation at 37°C and 75 rpm, we discarded the supernatant and

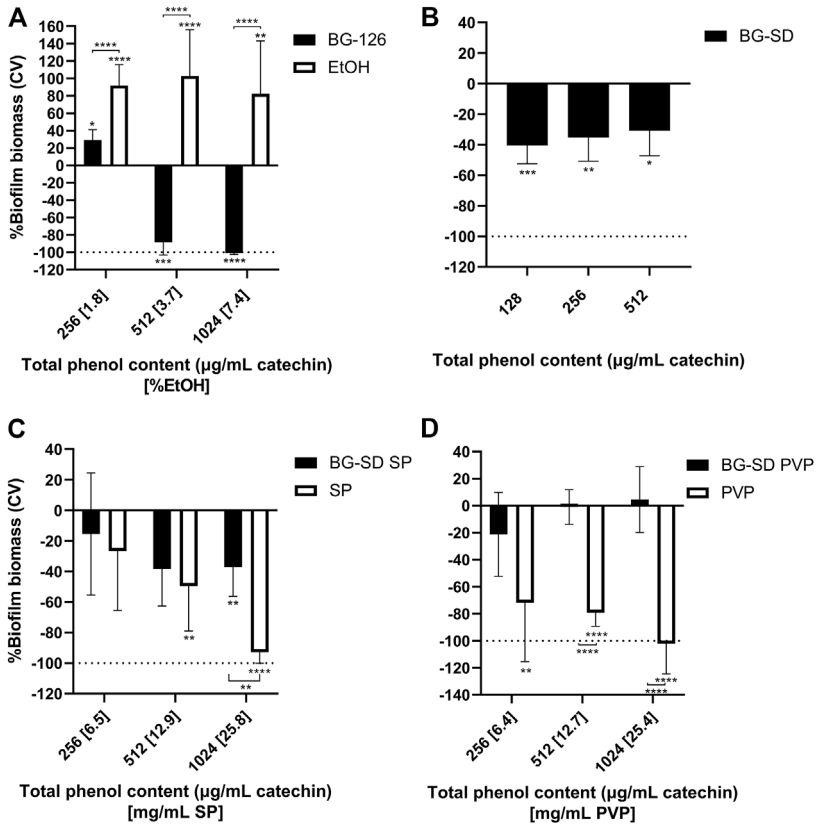


FIGURE 2
Effect of *Buddleja globosa* Hope (BG) extracts on *Pseudomonas aeruginosa* biofilm formation. *P. aeruginosa* was treated for 24 h with a hydroalcoholic BG extract (BG-126) **(A)**, spray-dried BG extract (BG-SD) **(B)**, spray-dried BG extract with Soluplus® (BG-SD SP) **(C)**, and spray-dried BG extract with PVP (BG-SD PVP) **(D)** were stained with crystal violet (CV) for the determination of the biomass of biofilms based on the amount of CV retained by the stained wells. The results are expressed as changes in %biomass with respect to the control group. Asterisks on bars indicate a statistically significant difference with respect to the untreated control, and asterisks on brackets indicate differences between the BG formulations and the excipients. $*p < 0.05$, $**p < 0.01$, $***p < 0.01$, and $****p < 0.01$.

rinsed the wells twice with 100 µl of sterile PBS. Finally, 200 µl of LB broth was added before a second incubation at 37°C and 75 rpm for 24 h.

2.4.2 Determination of the minimum inhibitory concentration of the formulations in *Pseudomonas aeruginosa*

The formulations were tested using the broth microdilution method (EUCAST, 2003). Briefly, the formulations were prepared in 96-well plates and diluted in LB broth in serial two-fold dilutions, reaching a final volume of 100 µl of each concentration at 2x. Then, 100 µl of the bacterial suspension previously described was added to the multiwell plate with the treatment solutions to obtain the final concentrations ranging from 0.5 to 1,024 µg/ml catechin. The plates were incubated at 37°C and 180 rpm for 24 h before recording the absorbance at 625 nm as a measurement of bacterial proliferation. Tobramycin sulfate (1 µg/ml) was used as a positive control. Sterile formulation blanks were also included. Results were reported as % proliferation with respect to the untreated bacterial suspensions (negative control).

2.4.3 Determination of the effect of the formulations on *Pseudomonas aeruginosa* biofilm formation

The three highest concentrations of the formulations (around the determined MICs) were tested against *P. aeruginosa,* as described previously. After 24 h of incubation, the supernatants were discarded and the plates were thoroughly rinsed with distilled water and air-dried over a paper towel. Then, 200 µl of methanol was added for biofilm fixation, followed by an incubation of 30 min at room temperature. Then, methanol was discarded, and the plate was allowed to air-dry overnight. A measure of 200 µl of 0.1% (w/v) crystal violet solution was added to each well of the plate for 15 min at room temperature. After rinsing with distilled water and air-drying, the retained crystal violet was dissolved with 30% (v/v) acetic acid for 30 min before a

TABLE 2 Characterization of BG formulations.

Formulation	Type of formulation	Catechin		Verbascoside		SD yield (expressed as catechin)	Solubility (expressed as μg/ml catechin)	
		% (w/w)	(μg/ml)	% (w/w)	(μg/ml)	%	25°C	37°C
BG-126	Solution	—	7,378	—	3,696	—	—	—
BG-SD	Dry powder	16.9	—	11.0	—	64.0	132.7	1,024
BG-SD SP	Dry powder	3.4	—	—	—	66.4	176.4	2,048
BG-SD PVP	Dry powder	3.5	—	—	—	87.0	175.0	4,116

BG, *Buddleja globosa* Hope extract; SD, spray-dried; PVP, polyvinylpyrrolidone; SP, Soluplus®

step of linear shaking for 10 s and recording the absorbance at 570 nm. The results were reported as %biofilm biomass using untreated *P. aeruginosa* as a negative control.

2.5 Statistical analysis

For bacterial proliferation and biofilm biomass determination, each condition was tested in quadruplicate in at least two independent experiments. Results were expressed as the mean ± standard deviation, and significant differences (α = 0.05) were analyzed by Tukey–Kramer honestly significant difference (HSD) test by JMP 10.0.0 software (SAS Institute Inc.).

3 Results

3.1 Total phenolic content and solubility of the *Buddleja globosa* Hope formulations

BG-126 and the three SD formulations were analyzed for total phenolic content using catechin as the standard. For BG-126, we determined a total phenolic content of 7,378 ± 45 μg/ml catechin that was close to that reported in the certificate of analysis of BG-126 (7,551 μg/ml catechin). On the other hand, the BG formulations developed by SD showed total phenolic contents from about 3.4% to 16.9% (Table 2). The lowest catechin content in BG-SD PVP and BG-SD SP compared to BG-SD was expected since the excipients added more volume to the powder formulations. We also determined the yield of the spray drying process using the catechin content of BG-126 as a reference. All formulations were prepared by spray drying 50 ml of BG-126 with or without the addition of excipients that contain 369 mg of catechin. The yield of the process was high, ranging from 64 to 87%.

All the SD formulations showed poor flowability. The powder flow fluctuates from passable (BG-SD SP and BG-SD PVP) to poor (BG-SD) with angles of repose of 42, 44, and 50, respectively. The SD formulations as a total mass were very slightly or slightly soluble (‘10 mg/ml) at 25°C and soluble at 37°C. When comparing these solubilities expressed as μg/ml catechin, BG-126 retains the highest solubility as a hydroalcoholic extract, followed by the aqueous extracts BG-SD PVP, BG-SD SP, and BG-SD with the lowest solubility (Table 2). The solubility at room temperature improved slightly with the addition of excipients from 132 to 175 μg/ml catechin. PVP improved the solubility at 37°C, reaching a maximum of 4,116 μg/ml catechin (about 56% of the content of BG-126).

3.2 Determination of the minimum inhibitory concentration of the *Buddleja globosa* Hope formulations in *Pseudomonas aeruginosa*

The antimicrobial properties of the BG formulations were tested *in vitro*. Figure 1 shows the effect of the formulations on bacterial proliferation. Only BG-126 (−95.0% at 1,024 μg/ml catechin and 7.4% ethanol) and BG-SD (−105.6% at 512 μg/ml catechin) (Figures 1A,B) caused complete inhibition of bacterial proliferation equivalent to that of 1 μg/ml of tobramycin sulfate (positive control; not plotted). When comparing these MICs, BG-SD showed the highest potency. When expressed as verbascoside content, the MIC of BG-126 is equivalent to 513 μg/ml, while that of BG-SD is 333 μg/ml.

BG-126 also showed an increase in bacterial proliferation at low concentrations (≤8 μg/ml catechin and 0.06% EtOH). The MIC for BG-SD SP and BG-SD PVP could not be determined at the tested concentrations (Figures 1C,D). BG-SD SP reached a maximum inhibition of −70.1% (at 1,024 μg/ml catechin and 25.8 mg/ml SP), and BG-SD PVP reached −40% (near 128 μg/ml catechin and 3.2 mg/ml).

For the tested excipients, ethanol (Figure 1A) and PVP (Figure 1D) did not impact bacterial proliferation. On the other hand, SP did show the opposite effect, causing an

increase in bacterial proliferation at a lower concentration (1–128 μg/ml catechin) and inhibition at 1,024 μg/ml (−91%).

3.3 Determination of the effect of the *Buddleja globosa* Hope formulations on *Pseudomonas aeruginosa* biofilm formation

After 24 h of incubation of *P. aeruginosa* with the different BG extracts and the studied excipients, the microplates were stained with crystal violet for biofilm biomass determination. BG-126 inhibited biofilm formation completely at the determined MIC (equivalent to a total phenolic content of 1,024 μg/ml catechin and 7.4% ethanol) (Figure 2A). At the MIC of BG-SD, it reached about 40% inhibition of biofilm formation (Figure 2B). Similar inhibition was achieved with BG-SD SP (Figure 2C). BG-SD PVP did not affect *P. aeruginosa* biofilm formation (Figure 2D).

On the other hand, the tested excipients showed significant effects on biofilm formation (Figure 2, white bars). SP and PVP caused significant reductions in *P. aeruginosa* biofilm formation (Figures 2C,D), while ethanol caused a highly significant increase in biofilm formation (Figure 2A).

4 Discussion

4.1 Spray-dried *B. globosa* extract showed the highest potency to inhibit *P. aeruginosa* proliferation, while the hydroalcoholic extract BG-126 caused complete inhibition of biofilm formation

Using the broth microdilution method, we were able to determine the MIC for BG-126 and BG-SD against *P. aeruginosa*. These MICs were reported in terms of the total phenolic content expressed as the concentration of catechin. BG-SD caused bacterial inhibition at half the concentration needed for BG-126 (Figure 1). To the best of our knowledge, this is the first report on the inhibitory effect of a BG extract against *P. aeruginosa*. Mølgaard et al. tested the antimicrobial activity of BG and other methanolic extracts against seven microorganisms (including four strains of *P. aeruginosa*), but the authors did not observe relevant results with BG in this or other species (Molgaard et al., 2011). We also tested biofilm formation by BG-treated *P. aeruginosa* by crystal violet staining. BG-126 showed the highest potency to prevent biofilm formation (Figure 2A). The prevention of biofilm formation is an important attribute of a formulation aimed to treat chronic wounds. A high percentage of these wounds are colonized by biofilm-growing microorganisms such as *P. aeruginosa* (James et al., 2008).

It is essential to highlight that the antimicrobial properties of a natural extract can be a result of the activity of specific compounds and synergistic interactions between said compounds. Here, we used catechin to standardize the BG extract concentrations (Letelier et al., 2012; Letelier et al., 2017; Zamorano-Aguilar et al., 2020). This flavonoid has shown antimicrobial properties and synergism with antibiotics against Gram-positive and Gram-negative bacteria (Gomes et al., 2018; Ibitoye and Ajiboye, 2019; Zhang et al., 2021), and the reported MIC of pure catechin against *S. aureus* was around 78–156 μg/ml (Zhang et al., 2021). However, to date, there are no reports on the antimicrobial activity of catechin against *P. aeruginosa*, but there are reports on their effect against biofilm formation and proliferation (Lekbach et al., 2019). On the other hand, other BG components have been tested in *P. aeruginosa* cultures, showing inhibitory activity. Apigenin is probably the compound with the highest antimicrobial evidence of its inhibitory effect against *P. aeruginosa* (Basile et al., 2000; Ozcelik et al., 2011; Liu et al., 2013; Nayaka et al., 2014; Lucarini et al., 2015), followed by quercetin (Basile et al., 2000; Ozcelik et al., 2011; Jaisinghani, 2017b; Wang et al., 2018) and linalool (Silva et al., 2015; Abd El-Baky and Hashem, 2016; Liu et al., 2020). Therefore, the antimicrobial effect exerted by the BG formulations might result from the activity of the combination and synergisms of different compounds such as apigenin, quercetin, and linalool and not by only catechin (Caesar and Cech, 2019). We used catechin as a referential compound to dose comparable concentrations of the extract. Additionally, we determined the verbascoside content for BG-126 and BG-SD. We did not express the concentration as mass/volume since the formulations with excipients (BG-SD PVP and BG-SD SP) would have a comparatively low concentration of the BG extract due to the diluting effect of the solubilizers in the formulation.

Dry powder formulations can be advantageous as powders have higher stability than solutions. Therefore, these spray-dried BG formulations have the potential to present higher stability. Spray drying has been used to encapsulate natural extracts showing great stability when stored in closed containers at room temperature (Cortes-Rojas et al., 2016). Additionally, these dry powder formulations can be used within capsules for oral administration (Letelier et al., 2017), to study their performance for inhalation delivery (Shetty et al., 2020), or used as ingredients for solutions or semisolid antimicrobial formulations for future applications. However, considering the poor flowability exhibited by the formulations, this property should be optimized for dosage forms that require fast filling steps, such as hard capsules or tableting, to prevent dose variability.

On the other hand, one of the disadvantages of spray drying is the low yield of recovery. Most of the powder is recovered from the collection vessel. However, there is a loss of powder that deposits along the system (drying chamber, connectors,

cyclone, and filter). Since the commercial hydroalcoholic extract was obtained directly from the plant material, we could not quantify the yield in mass, but we obtained a high yield expressed as catechin content (64%–87%). Additionally, some loss of the material can be attributed to thermal degradation. Encapsulation of antioxidants has been shown to protect the integrity of compounds such as catechin and epigallocatechin gallate from thermal stress (Peres et al., 2011; Ho et al., 2017; Cruz-Molina et al., 2021). In our study, we used PVP as the encapsulating agent for the BG extract, and this BG-SD PVP formulation showed the highest yield, which could be attributed to the protective effect of PVP (da Fonseca Machado et al., 2018).

4.2 Tested excipients had significant effects on *P. aeruginosa* proliferation and biofilm formation

We used PVP (polyvinylpyrrolidone) and SP (Soluplus®) as solubility enhancers to improve the water solubility of spray-dried BG extracts. PVP is widely used as a pharmaceutical excipient, as a solubilizer to enhance the dissolution of drugs with low water solubility, and as a coating agent (Rowe et al., 2009). Soluplus® is the commercial name of a co-polymeric solubilizer with an amphiphilic structure capable of forming colloidal micelles. Using PVP and SP, we reached higher testing concentrations in terms of the total phenolic content compared to BG-SD but without the ethanolic content of BG-126.

Previously, we found that pharmaceutical excipients, while considering inactive ingredients, may present antimicrobial effects that can synergize with antibiotics against biofilm-growing bacteria (Bahamondez-Canas and Smyth, 2018). Some pharmaceutical excipients, such as citric acid, succinic acid, glutamic, xylitol, and EDTA, present antibiofilm, bactericidal, and biofilm dispersion activities (Arias-Moliz et al., 2008; Badet et al., 2008; Ammons et al., 2011; Sommerfeld Ross and Fiegel, 2012; Khayyat et al., 2021). These excipients could also potentiate the activity of antibiotics (Allison et al., 2011; Sommerfeld Ross et al., 2017; Bahamondez-Canas et al., 2018). These authors proposed that these ingredients could act as nutrients reversing the quiescent state of a biofilm subpopulation that makes bacteria more tolerant to antimicrobials, may induce the dispersion of biofilms, or may prevent bacterial adhesion to surfaces. Therefore, in this study, we also tested the effect of these excipients alone on *P. aeruginosa* proliferation and biofilm formation. We observed that PVP significantly reduced biofilm formation (Figure 2D). PVP has been studied as a coating for prosthesis and tympanostomy tubes, showing the prevention of *S. aureus* and *P. aeruginosa* biofilm formation, respectively (Wolter and Hellstrom, 2004; Antonelli et al., 2011). Therefore, PVP could act as a coating agent on the surface of the multiwell plates used in these studies. However, we found no significant effect on biofilm formation with BG-SD PVP, indicating that the BG extract components might hinder this inhibitory effect of PVP. We also found that SP inhibited *P. aeruginosa* proliferation at 25.8 mg/ml (Figure 1C) and caused significant inhibition of biofilm formation from 12.9 mg/ml (Figure 2C), while BG-SD SP caused a moderate inhibitory effect at these concentrations (512 and 1,024 μg/ml catechin) (Figure 2C). These results indicate that SP can significantly reduce biofilm formation below the inhibitory concentration (25.8 mg/ml). This inhibitory effect of SP on biofilms has been described against *Staphylococcus epidermidis* when used as a polymeric nanocarrier (Takahashi et al., 2015; Albayaty et al., 2019; Takahashi et al., 2021). The amphiphilic nature of SP might contribute to this inhibition of biofilm formation as certain surfactants are known to weaken the biofilm matrix, causing biofilm disruption (Nguyen et al., 2020). Therefore, these excipients are good candidates to continue exploring the biofilm inhibitory effects in formulations.

Finally, we found that ethanol induced biofilm formation (Figure 2A). The treatment for 24 h with ethanol resulted in higher biofilm biomasses than the untreated control. Ethanol is well known as a disinfectant, which could be a desirable property but it has cytotoxic effects on mammalian cells (Farkas et al., 2003; Calderon-Montano et al., 2018; Kar et al., 2021). On the other hand, studies have shown that low ethanol concentrations (1 or 2%) are considered stress factors that induce biofilm formation (Chaieb et al., 2007; Tashiro et al., 2014) by increased cell aggregation (He et al., 2022) and induction of exopolysaccharide production (Chen et al., 2014). Our results using ethanol from 1.8 to 7.4% are correlated with this evidence. We also observed that diluted BG-126, containing 1.8% ethanol, caused an increase in biofilm formation with respect to the untreated control. This result could be attributed to the biofilm induction caused by a low ethanolic content, which might counteract the inhibitory effect of the BG-126 extract observed at higher concentrations. On the other hand, none of the three SD formulations showed induction of biofilm formation, indicating that the residual ethanolic content in the powders is not significant, which is, indeed, another advantage of formulating BG extracts by spray drying. The residual solvent content of spray-dried powders is usually below 5% (Bahamondez-Canas et al., 2018). Da Fonseca Machado compared three techniques for PVP encapsulation of natural ethanolic extracts finding that spray drying resulted in the lowest residual ethanolic content (2%) when compared to freeze drying (5%) and supercritical antisolvent (8%) (da Fonseca Machado et al., 2018).

4.3 Formulation with the excipients PVP and SP improved the water solubility of the spray-dried BG extract at 37°C

The improvement in the water solubility was the first aim of this research to achieve higher concentrations of the actives without the ethanolic content. As expected, we found a low aqueous solubility for BG-SD at 25°C that increased with the addition of excipients and

at 37°C (Table 2). Determining the solubility at 37°C was necessary due to the characteristics of the wound site as the actives intended for wound healing must be able to dissolve in the wound fluid at body temperature. The wound fluid or exudate represents a small volume of liquid below 6 ml (Gohel et al., 2008), with reports on exudate production of 0.2 ml/h for pressure ulcers (Iizaka et al., 2010). Therefore, improving the solubility of the BG components is critical to exploiting their antimicrobial properties for wound care.

To determine the solubility of a compound, solubility studies are routinely performed during preformulation studies. This determination is achieved by adding an excess of a compound in a fixed volume (25–50 ml), followed by agitation at room temperature that can last several days. This study is conducted using water and different buffers to fully characterize the solubility of a said active ingredient (Bonfilio et al., 2018). In our research, we evaluated the antimicrobial properties of the BG extract based on the evidence of its wound healing properties (Backhouse et al., 2008; Bustamante et al., 2015). Therefore, with this application in mind and the incubation temperatures needed for antimicrobial testing, we also conducted this solubility test at a physiologically relevant temperature of 37°C and in smaller volumes. The United States Pharmacopeia describes the solubility of a compound, regardless of the solvent used (Savjani et al., 2012). All SD formulations can be classified as very slightly and slightly soluble. When comparing solubilities in terms of the total phenol content expressed as catechins, then the SD formulations can be described as freely to very soluble. Cuevas-Valenzuela et al. determined that the water solubility of catechin increases with temperature and ethanol concentration (Cuevas-Valenzuela et al., 2014), supporting the relation between solubilities we obtained for BG-SD and BG-126.

5 Conclusion

The BG extract obtained by spray drying (BG-SD) was capable of inhibiting *P. aeruginosa* proliferation with a higher potency than the hydroalcoholic extract BG-126. On the other hand, BG-126 prevented biofilm formation and the excipients PVP and Soluplus® alone. We also observed that low concentrations of ethanol alone or in BG-126 induced *P. aeruginosa* biofilm formation. Although the spray-dried BG extracts with excipients showed lower antimicrobial efficacy than BG-126 or BG-SD, these BG formulations can be used to investigate biocompatible topical therapies for wound healing as non-ethanolic alternatives. Spray drying allowed the development of a water-soluble BG extract with promising antimicrobial efficacy for further research and development of novel phytopharmaceuticals to treat *P. aeruginosa* infections.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

NA, ML, and MB developed, characterized, and evaluated the antimicrobial properties of the formulations. AC and BB contributed to the development and characterization of the formulations. DM-E, as the senior author, designed the formulations and revised the manuscript. TFB-C is the principal investigator of the grants that funded this research.

Funding

This work was funded by the National Agency for Research and Development (ANID) grants FONDECYT 11190348 (2019) and PAI 77190010 (2019) given to TFB-C.

Acknowledgments

The authors acknowledge Laboratorios Ximena Polanco and BASF Chile for kindly donating the reagents used in this research. Also, the authors thank Maria Isabel Chavez (from Farmacopea Chilena) for her guidance in conducting the chemical characterization of the extracts. AC and BB acknowledge the program Bioactivity of Natural and Synthetic Products from Universidad de Valparaiso for their scholarship to pursue their master's degree. DM-E acknowledges the ANID for the grant FONDECYT 11190987.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.921511/full#supplementary-material

References

Abd El-Baky, R. M., and Hashem, Z. S. (2016). Eugenol and linalool: Comparison of their antibacterial and antifungal activities. *Afr. J. Microbiol. Res.* 10, 1860–1872. doi:10.5897/ajmr2016.8283

Albayaty, Y. N., Thomas, N., Jambhrunkar, M., Al-Hawwas, M., Kral, A., Thorn, C. R., et al. (2019). Enzyme responsive copolymer micelles enhance the anti-biofilm efficacy of the antiseptic chlorhexidine. *Int. J. Pharm.* 566, 329–341. doi:10.1016/j.ijpharm.2019.05.069

Allison, K. R., Brynildsen, M. P., and Collins, J. J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 473, 216–220. doi:10.1038/nature10069

Ammons, M. C. B., Ward, L. S., and James, G. A. (2011). Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. *Int. Wound J.* 8, 268–273. doi:10.1111/j.1742-481X.2011.00781.x

Antonelli, P. J., Sampson, E. M., and Ojano-Dirain, C. (2011). biofilm formation on silicone tympanostomy tubes with polyvinylpyrrolidone coating. *Arch. Otolaryngol. Head. Neck Surg.* 137, 19–23. doi:10.1001/archoto.2010.205

Arias-Moliz, M. T., Ferrer-Luque, C. M., Espigares-Rodríguez, E., Liébana-Ureña, J., and Espigares-García, M. (2008). Bactericidal activity of phosphoric acid, citric acid, and EDTA solutions against Enterococcus faecalis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 106, e84–e89. doi:10.1016/j.tripleo.2008.04.002

Avila, J. G., De Liverant, J. G., Martínez, A., Martínez, G., Muñoz, J. L., Arciniegas, A., et al. (1999). Mode of action of Buddleja cordata verbascoside against Staphylococcus aureus. *J. Ethnopharmacol.* 66, 75–78. doi:10.1016/s0378-8741(98)00203-7

Backhouse, N., Rosales, L., Apablaza, C., Goïty, L., Erazo, S., Negrete, R., et al. (2008). Analgesic, anti-inflammatory and antioxidant properties of Buddleja globosa, Buddlejaceae. *J. Ethnopharmacol.* 116, 263–269. doi:10.1016/j.jep.2007.11.025

Badet, C., Furiga, A., and Thébaud, N. (2008). Effect of xylitol on an *in vitro* model of oral biofilm. *Oral Health Prev. Dent.* 6, 337–341.

Bahamondez-Canas, T. F., Ferrati, S., Moraga-Espinoza, D. F., and Smyth, H. D. C. (2018). Development, characterization, and *in vitro* testing of Co-delivered antimicrobial dry powder formulation for the treatment of Pseudomonas aeruginosa biofilms. *J. Pharm. Sci.* 107, 2172–2178. doi:10.1016/j.xphs.2018.04.015

Bahamondez-Canas, T., and Smyth, H. D. C. (2018). Influence of excipients on the antimicrobial activity of tobramycin against Pseudomonas aeruginosa biofilms. *Pharm. Res.* 35, 10. doi:10.1007/s11095-017-2301-5

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., et al. (2000). Antibacterial and allelopathic activity of extract from Castanea sativa leaves. *Fitoterapia* 71 (1), S110–S116. doi:10.1016/s0367-326x(00)00185-4

Bergonzi, M. C., Vasarri, M., Marroncini, G., Barletta, E., and Degl'Innocenti, D. (2020). Thymoquinone-loaded soluplus(®)-solutol(®) HS15 mixed micelles: Preparation, *in vitro* characterization, and effect on the SH-SY5Y cell migration. *Molecules* 25, E4707. doi:10.3390/molecules25204707

Bhardwaj, M., Singh, B. R., Sinha, D. K., Or, V. K., Vadhana, P., Singh, S. V., et al. (2019). Evaluation of carvacrol as an antibacterial agent against *Escherichia coli* isolated from different animal species. *J. entomology zoology Stud.* 7, 911–914.

Bjarnsholt, T. (2013). The role of bacterial biofilms in chronic infections. *APMIS. Suppl.* 121, 1–58. doi:10.1111/apm.12099

Bonfilio, R., Souza, M. C. O., Leal, J. S., Viana, O. M. M. S., Doriguetto, A. C., and Araújo, M. B. D. (2018). Solubility and dissolution studies of tibolone polymorphs. *Braz. J. Pharm. Sci.* 53. doi:10.1590/s2175-97902017000400233

Bustamante, S., Álvarez, N., Mendiburen, R., Vergara, F., Zárate, I., Collado, C., et al. (2015). Preclinical support for ethnomedical use of matico (*Buddleja globosa* Hope). *Rev. Fitoter.* 15, 37–51.

Caesar, L. K., and Cech, N. B. (2019). Synergy and antagonism in natural product extracts: When 1+ 1 does not equal 2. *Nat. Prod. Rep.* 36, 869–888. doi:10.1039/c9np00011a

Calderón-Montaño, J. M., Jiménez-Alonso, J. J., Guillén-Mancina, E., Burgos-Morón, E., and López-Lázaro, M. (2018). A 30-s exposure to ethanol 20% is cytotoxic to human keratinocytes: Possible mechanistic link between alcohol-containing mouthwashes and oral cancer. *Clin. Oral Investig.* 22, 2943–2946. doi:10.1007/s00784-018-2602-z

Chaieb, K., Chehab, O., Zmantar, T., Rouabhia, M., Mahdouani, K., and Bakhrouf, A. (2007). *In vitro* effect of pH and ethanol on biofilm formation by clinicalica-positive Staphylococcus epidermidis strains. *Ann. Microbiol.* 57, 431–437. doi:10.1007/bf03175085

Chen, A. I., Dolben, E. F., Okegbe, C., Harty, C. E., Golub, Y., Thao, S., et al. (2014). *Candida albicans* ethanol stimulates *Pseudomonas aeruginosa* WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog.* 10, e1004480. doi:10.1371/journal.ppat.1004480

Chen, L.-Y., Cheng, C.-W., and Liang, J.-Y. (2015). Effect of esterification condensation on the Folin–Ciocalteu method for the quantitative measurement of total phenols. *Food Chem.* 170, 10–15. doi:10.1016/j.foodchem.2014.08.038

Cortés-Rojas, D. F., Souza, C. R. F., and Oliveira, W. P. (2016). Assessment of stability of a spray dried extract from the medicinal plant *Bidens pilosa* L. *J. King Saud Univ. - Eng. Sci.* 28, 141–146. doi:10.1016/j.jksues.2014.04.004

Cruz-Molina, A. V. D. L., Ayala Zavala, J. F., Bernal Mercado, A. T., Cruz Valenzuela, M. R., González-Aguilar, G. A., Lizardi-Mendoza, J., et al. (2021). Maltodextrin encapsulation improves thermal and pH stability of green tea extract catechins. *J. Food Process. Preserv.* 45, e15729. doi:10.1111/jfpp.15729

Cuevas-Valenzuela, J., González-Rojas, Á., Wisniak, J., Apelblat, A., and Pérez-Correa, J. R. (2014). Solubility of (+)-catechin in water and water-ethanol mixtures within the temperature range 277.6–331.2K: Fundamental data to design polyphenol extraction processes. *Fluid Phase Equilibria* 382, 279–285. doi:10.1016/j.fluid.2014.09.013

Da Fonseca Machado, A. P., Alves Rezende, C., Alexandre Rodrigues, R., Fernández Barbero, G., De Tarso Vieira E Rosa, P., and Martínez, J. (2018). Encapsulation of anthocyanin-rich extract from blackberry residues by spray-drying, freeze-drying and supercritical antisolvent. *Powder Technol.* 340, 553–562. doi:10.1016/j.powtec.2018.09.063

Danielsen, L., Balslev, E., Dörng, G., Hoiby, N., Madsen, S. M., Ågren, M., et al. (1998). Ulcer bed infection. *APMIS* 106, 721–726. doi:10.1111/j.1699-0463.1998.tb00218.x

Farkas, Á., Kemény, L., Széll, M., Dobozy, A., and Bata-Csörgő, Z. (2003). Ethanol and acetone stimulate the proliferation of HaCaT keratinocytes: The possible role of alcohol in exacerbating psoriasis. *Arch. Dermatol. Res.* 295, 56–62. doi:10.1007/s00403-003-0399-2

Flemming, H.-C., and Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–633. doi:10.1038/nrmicro2415

Gohel, M. S., Windhaber, R. A. J., Tarlton, J. F., Whyman, M. R., and Poskitt, K. R. (2008). The relationship between cytokine concentrations and wound healing in chronic venous ulceration. *J. Vasc. Surg.* 48, 1272–1277. doi:10.1016/j.jvs.2008.06.042

Gomes, F. M. S., Da Cunha Xavier, J., Dos Santos, J. F. S., De Matos, Y., Tintino, S. R., De Freitas, T. S., et al. (2018). Evaluation of antibacterial and modifying action of catechin antibiotics in resistant strains. *Microb. Pathog.* 115, 175–178. doi:10.1016/j.micpath.2017.12.058

Han, G., and Ceilley, R. (2017). Chronic wound healing: A review of current management and treatments. *Adv. Ther.* 34, 599–610. doi:10.1007/s12325-017-0478-y

He, S., Zhan, Z., Shi, C., Wang, S., and Shi, X. (2022). Ethanol at subinhibitory concentrations enhances biofilm formation in *Salmonella enteritidis*. *Foods* 11, 2237. doi:10.3390/foods11152237

Ho, S., Thoo, Y. Y., Young, D. J., and Siow, L. F. (2017). Inclusion complexation of catechin by β-cyclodextrins: Characterization and storage stability. *LWT* 86, 555–565. doi:10.1016/j.lwt.2017.08.041

Ibitoye, O. B., and Ajiboye, T. O. (2019). (+)-Catechin potentiates the oxidative response of *Acinetobacter baumannii* to quinolone-based antibiotics. *Microb. Pathog.* 127, 239–245. doi:10.1016/j.micpath.2018.12.012

Iizaka, S., Sanada, H., Nakagami, G., Sekine, R., Koyanagi, H., Konya, C., et al. (2010). Estimation of protein loss from wound fluid in older patients with severe pressure ulcers. *Nutrition* 26, 890–895. doi:10.1016/j.nut.2009.09.008

Jaisinghani, R. N. (2017a). Antibacterial properties of quercetin. *Microbiol. Res. (Pavia).* 8, 6877. doi:10.4081/mr.2017.6877

Jaisinghani, R. N. (2017b). Antibacterial properties of quercetin. *Microbiol. Res. (Pavia).* 8, 13–14. doi:10.4081/mr.2017.6877

James, G. A., Swogger, E., Wolcott, R., Pulcini, E. D., Secor, P., Sestrich, J., et al. (2008). Biofilms in chronic wounds. *Wound Repair Regen.* 16, 37–44. doi:10.1111/j.1524-475X.2007.00321.x

Kar, N., Gupta, D., and Bellare, J. (2021). Ethanol affects fibroblast behavior differentially at low and high doses: A comprehensive, dose-response evaluation. *Toxicol. Rep.* 8, 1054–1066. doi:10.1016/j.toxrep.2021.05.007

Khayyat, A. N., Hegazy, W. A. H., Shaldam, M. A., Mosbah, R., Almalki, A. J., Ibrahim, T. S., et al. (2021). Xylitol inhibits growth and blocks virulence in *Serratia marcescens*. *Microorganisms* 9, 1083. doi:10.3390/microorganisms9051083

Kim, S., Woo, E.-R., and Lee, D. G. (2020). Apigenin promotes antibacterial activity via regulation of nitric oxide and superoxide anion production. *J. Basic Microbiol.* 60, 862–872. doi:10.1002/jobm.202000432

Kirketerp-Møller, K., Jensen, P. Ø., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., et al. (2008). Distribution, organization, and ecology of bacteria in chronic wounds. *J. Clin. Microbiol.* 46, 2717–2722. doi:10.1128/JCM.00501-08

Kurakula, M., and Rao, G. (2020). Pharmaceutical assessment of polyvinylpyrrolidone (PVP): As excipient from conventional to controlled delivery systems with a spotlight on COVID-19 inhibition. *J. Drug Deliv. Sci. Technol.* 60, 102046. doi:10.1016/j.jddst.2020.102046

Lekbach, Y., Dong, Y., Li, Z., Xu, D., El Abed, S., Yi, Y., et al. (2019). Catechin hydrate as an eco-friendly biocorrosion inhibitor for 304L stainless steel with dual-action antibacterial properties against *Pseudomonas aeruginosa* biofilm. *Corros. Sci.* 157, 98–108. doi:10.1016/j.corsci.2019.05.021

Letelier, M. E., Jones, R., López, C., Palma, K., Aracena, P., Razmilic, I., et al. (2012). Safety profile and wound healing properties of a standardized Buddleja globosa hope (matico) extract in sprague-dawley rats. *Rev. Farmacol. Chile* 5, 13–19.

Letelier, M., Hidalgo-Castro, F., López-Valladares, M., Ibacache, N., Pérez, C., Brunner, J., et al. (2017). BG126® phytodrug improves urinary tract infection treatment with nitrofurantoin in adult women in a double-blind randomized clinical trial. *J. Herb. Med.* 9, 60–67. doi:10.1016/j.hermed.2017.03.001

Liu, R., Zhang, H., Yuan, M., Zhou, J., Tu, Q., Liu, J. J., et al. (2013). Synthesis and biological evaluation of apigenin derivatives as antibacterial and antiproliferative agents. *Molecules* 18, 11496–11511. doi:10.3390/molecules180911496

Liu, X., Cai, J., Chen, H., Zhong, Q., Hou, Y., Chen, W., et al. (2020). Antibacterial activity and mechanism of linalool against *Pseudomonas aeruginosa*. *Microb. Pathog.* 141, 103980. doi:10.1016/j.micpath.2020.103980

Lucarini, R., Tozatti, M. G., Silva, M. L. A., Gimenez, V. M. M., Pauletti, P. M., Groppo, M., et al. (2015). Antibacterial and anti-inflammatory activities of an extract, fractions, and compounds isolated from *Gochnatia pulchra* aerial parts. *Braz. J. Med. Biol. Res.* 48, 822–830. doi:10.1590/1414-431X20154410

Malone, M., Bjarnsholt, T., Mcbain, A. J., James, G. A., Stoodley, P., Leaper, D., et al. (2017). The prevalence of biofilms in chronic wounds: A systematic review and meta-analysis of published data. *J. Wound Care* 26, 20–25. doi:10.12968/jowc.2017.26.1.20

Mølgaard, P., Holler, J. G., Asar, B., Liberna, I., Rosenbæk, L. B., Jebjerg, C. P., et al. (2011). Antimicrobial evaluation of Huilliche plant medicine used to treat wounds. *J. Ethnopharmacol.* 138, 219–227. doi:10.1016/j.jep.2011.09.006

Nayaka, H. B., Londonkar, R. L., Umesh, M. K., and Tukappa, A. (2014). Antibacterial attributes of apigenin, isolated from *Portulaca oleracea* L. *Int. J. Bacteriol.* 2014, 175851. doi:10.1155/2014/175851

Nguyen, B. V., Nagakubo, T., Toyofuku, M., Nomura, N., and Utada, A. S. (2020). Synergy between sophorolipid biosurfactant and SDS increases the efficiency of *P. aeruginosa* biofilm disruption. *Langmuir* 36, 6411–6420. doi:10.1021/acs.langmuir.0c00643

Ozçelik, B., Kartal, M., and Orhan, I. (2011). Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. *Pharm. Biol.* 49, 396–402. doi:10.3109/13880209.2010.519390

Peres, I., Rocha, S., Gomes, J., Morais, S., Pereira, M. C., and Coelho, M. (2011). Preservation of catechin antioxidant properties loaded in carbohydrate nanoparticles. *Carbohydr. Polym.* 86, 147–153. doi:10.1016/j.carbpol.2011.04.029

Pignatello, R., Corsaro, R., Bonaccorso, A., Zingale, E., Carbone, C., and Musumeci, T. (2022). Soluplus® polymeric nanomicelles improve solubility of BCS-class II drugs. *Drug Deliv. Transl. Res.* 12, 1991–2006. doi:10.1007/s13346-022-01182-x

Rajakumari, R., Volova, T., Oluwafemi, O. S., Rajesh Kumar, S., Thomas, S., and Kalarikkal, N. (2020). Grape seed extract-soluplus dispersion and its antioxidant activity. *Drug Dev. Ind. Pharm.* 46, 1219–1229. doi:10.1080/03639045.2020.1788059

Rowe, R. C., Sheskey, P., and Quinn, M. (2009). *Handbook of pharmaceutical excipients*. London, England: Libros Digitales-Pharmaceutical Press.

Savjani, K. T., Gajjar, A. K., and Savjani, J. K. (2012). Drug solubility: Importance and enhancement techniques. *ISRN Pharm.* 2012, 195727. doi:10.5402/2012/195727

Shetty, N., Cipolla, D., Park, H., and Zhou, Q. T. (2020). Physical stability of dry powder inhaler formulations. *Expert Opin. Drug Deliv.* 17, 77–96. doi:10.1080/17425247.2020.1702643

Silva, V., Sousa, J., Guerra, F., Pessôa, H., Freitas, A., Alves, L., et al. (2015). Antibacterial activity of Ocimum basilicum essential oil and linalool on bacterial isolates of clinical importance. *Int. J. Pharmacogn. Phytochemical Res.* 7, 1066–1071.

Singh, M. (2017). Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. *J. Biotech Res.* 8, 78.

Sommerfeld Ross, S., and Fiegel, J. (2012). Nutrient dispersion enhances conventional antibiotic activity against *Pseudomonas aeruginosa* biofilms. *Int. J. Antimicrob. Agents* 40, 177–181. doi:10.1016/j.ijantimicag.2012.04.015

Sommerfeld Ross, S., Gharse, S., Sanchez, L., and Fiegel, J. (2017). Dry powder aerosols to co-deliver antibiotics and nutrient dispersion compounds for enhanced bacterial biofilm eradication. *Int. J. Pharm.* 531, 14–23. doi:10.1016/j.ijpharm.2017.08.060

Taconelli, E., and Magrini, N. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Available at: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (Accessed February 27, 2017).

Takahashi, C., Saito, S., Suda, A., Ogawa, N., Kawashima, Y., and Yamamoto, H. (2015). Antibacterial activities of polymeric poly (dl-lactide-co-glycolide) nanoparticles and Soluplus® micelles against Staphylococcus epidermidis biofilm and their characterization. *RSC Adv.* 5, 71709–71717. doi:10.1039/c5ra13885j

Takahashi, C., Yamada, T., Yagi, S., Murai, T., and Muto, S. (2021). Preparation of silver-decorated Soluplus® nanoparticles and antibacterial activity towards S. epidermidis biofilms as characterized by STEM-CL spectroscopy. *Mat. Sci. Eng. C Mat. Biol. Appl.* 121, 111718. doi:10.1016/j.msec.2020.111718

Tashiro, Y., Inagaki, A., Ono, K., Inaba, T., Yawata, Y., Uchiyama, H., et al. (2014). Low concentrations of ethanol stimulate biofilm and pellicle formation in *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* 78, 178–181. doi:10.1080/09168451.2014.877828

Vanti, G. (2021). Recent strategies in nanodelivery systems for natural products: A review. *Environ. Chem. Lett.* 19, 4311–4326. doi:10.1007/s10311-021-01276-x

Wang, S., Yao, J., Zhou, B., Yang, J., Chaudry, M. T., Wang, M., et al. (2018). Bacteriostatic effect of quercetin as an antibiotic alternative *in vivo* and its antibacterial mechanism *in vitro*. *J. Food Prot.* 81, 68–78. doi:10.4315/0362-028X.JFP-17-214

Wolcott, R. D., and Ehrlich, G. D. (2008). Biofilms and chronic infections. *Jama* 299, 2682–2684. doi:10.1001/jama.299.22.2682

Wolter, C. E., and Hellstrom, W. J. G. (2004). The hydrophilic-coated inflatable penile prosthesis: 1-Year experience. *J. Sex. Med.* 1, 221–224. doi:10.1111/j.1743-6109.2004.04032.x

Zamorano-Aguilar, P., Morales, M., Rivillas, Y., Lopez, J., and Rojano, B. A. (2020). Antioxidant activity and cytotoxic effect of chilean *Buddleja globosa* (Matico) and *Ribes magellanicum* (Zarzaparrilla) flower extracts. *asphc.* 19, 59–70. doi:10.24326/asphc.2020.6.5

Zhang, G., Tan, Y., Yu, T., Wang, S., Liu, L., and Li, C. (2021). Synergistic antibacterial effects of reuterin and catechin against *Streptococcus mutans*. *LWT* 139, 110527. doi:10.1016/j.lwt.2020.110527

Frontiers in Pharmacology

Explores the interactions between chemicals and living beings

The most cited journal in its field, which advances access to pharmacological discoveries to prevent and treat human disease.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact