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Synergic combination of natural bioactive compounds for preventing and treating human diseases

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Synergic combination of natural bioactive compounds for preventing and treating human diseases

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Editorial: Synergic combination of natural bioactive compounds for preventing and treating human diseases

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Editorial on the Research Topic
Synergic combination of natural bioactive compounds for preventing and treating human diseases

In recent years, the focus on natural molecules and their potential to manage chronic diseases has grown exponentially (1–3), highlighting the complex interactions these substances have with biological pathways and their potential synergistic effects when used in combination (4). This editorial delves into the specifics of several key studies that have demonstrated the potential of natural compound combinations as alternative therapies for the treatment of different types of human diseases (Figure 1). Specifically, a recent study included in this Research Topic explored the combined effects of 3′-sialyl lactose and osteopontin, two human milk oligosaccharides, on influenza virus infection in an *in vitro* model of human laryngeal carcinoma cells (HEP-2). The study highlighted the reduction of pro-inflammatory cytokines, such as TNF-α and interleukin-6 (IL-6), suggesting that the synergy between these molecules may enhance the immune system's response to viral infection (Guo et al.).

In the area of metabolic diseases, a study recently published in the current collection focused on the role of diosgenin, extracted from fenugreek, in a diabetic rat model. The ability of diosgenin to modulate the diabetic state was investigated by evaluating its effects on the expression of the main molecular modulators implicated in glycemic control. Diosgenin appears to modulate the activity of GLUT4, facilitating increased glucose uptake into cells and affecting the insulin signaling cascade by activating IRS and PI3K, leading to the phosphorylation and activation of Akt, which in turn inhibits GSK-3β, resulting in enhanced glycogen synthesis (Tak et al.). Continuing in the context of diabetes, another study of this Research Topic investigated the effects of a multi-component formulation derived from ancient traditional medicine on the management of senile diabetic conditions. The authors find that the tested formulation, composed of a combination of 13 different plant-based extracts, can ameliorate diabetes by reducing islet cell apoptosis and resisting oxidative stress by regulating the insulin-mediated PI3K/AKT/GSK-3β pathway (Zhang Y. et al.). Continuing the discussion on diabetes, a recent review focused on the valuable synergic effects of the different bioactive molecules of *Phyllanthus emblica fruit* extract, highlighting that these compounds could

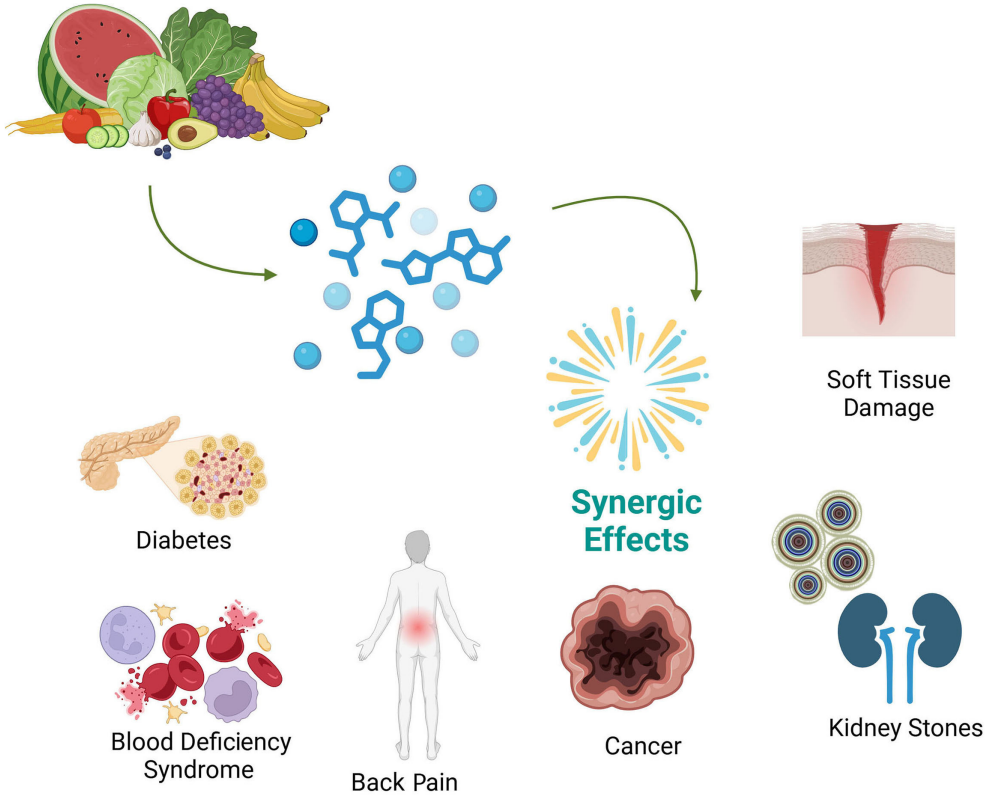

FIGURE 1
Synergic effects of natural bioactive compounds in the management of various human diseases.

potentially enhance diabetic treatment outcomes by leveraging their antioxidant and anti-inflammatory properties (Prananda et al.).

Moving to a completely different pathological setting, a comprehensive review investigated the role of vitamins A, C, D, and E in cancer prevention and therapy, delving into the multifaceted mechanisms by which these vitamins contribute to oncological health. By synthesizing data from a wide range of preclinical and clinical studies, the review highlighted how these vitamins act synergistically to combat cancer progression through several biological pathways. The authors described that, while vitamins C and E can provide a valuable defense against oxidative stress, vitamin A plays a crucial role in the epigenetic regulation of oncogenes and tumor suppressor gene expression, influencing cancer development and progression. Furthermore, vitamin D has been shown to modulate the inflammatory response by regulating cytokine production and inhibiting pathways that lead to inflammation. Based on such considerations, the authors suggest that the synergic combination of vitamins could be a useful multifunctional tool to prevent the progression of cancer (Guo et al.).

In line with this trend, other authors have investigated the effects of a nutraceutical formulation containing a blend of three standardized polyphenolic extracts- rosemary (*Rosmarinus officinalis* L.), ashwagandha (*Withania somnifera* L.), and sesame (*Sesamum indicum* L.) seeds- on the relief of back pain. The formulation was tested in a single-center, randomized double-blind study with three parallel arms. The study resulted in valuable benefits in improving health-related quality of life, mood, and sleep quality in the treated patients (Pérez-Piñero et al.). Other authors have also investigated the effects of a multicomponent formulation consisting of a mixture of various natural extracts, i.e., *Angelica sinensis* (Oliv.), *Carthamus tinctorius* L., *Saposhnikovia divaricata,* Schischk, g; *Arisaema erubescens*, and *Angelica dahurica* on the soft tissue injury. After only 10 days of oral treatment, a valuable pain reduction was observed in the treated group (Zhu H. et al.).

Another example of a synergistic combination of natural bioactive compounds is the combination of melatonin and palmitoylethanolamide in a nutraceutical formulation used for the prevention and treatment of immune disease. The results highlight that such combinations can reduce the release of immune-inflammatory modulators in the human mast cell line (HMC-1.2) at their bioaccessible concentration. Additionally, the nutraceutical formulation tested can reduce COX-2 mRNA transcription levels in stimulated HMC-1.2 and inhibit COX-2 enzymatic activity directly (Maisto et al.).

In contrast, other authors have examined the correlation between the Composite Dietary Antioxidant Index (CDAI) and the prevalence and recurrence of kidney stones. In the study, the

CDAI was derived by standardizing dietary antioxidant intake from 24 h food recalls. The study assessed the prevalence and recurrence of kidney stones based on questionnaire responses. The association between the CDAI and both the prevalence and recurrence of kidney stones was investigated using multivariable logistic regression. The student results suggest that individuals in the top tertile had a 23% lower prevalence of kidney stones (OR = 0.77, 95% CI: 0.66, 0.90, $p = 0.0011$) and a 39% lower recurrence rate (OR = 0.61, 95% CI: 0.47, 0.80, $p = 0.0003$) than those in the bottom tertile. Finally, other studies have investigated the healthy effects of Catalpol (CA), derived from Rehmannia Radix, on blood deficiency syndrome (BDS), using 16S rRNA gene sequencing and metabolomic analysis of serum and spleen (Zhang W. et al.).

Another pivotal study investigated the impact of catalpol, a natural compound derived from *Rehmannia Radix*, on blood deficiency syndrome induced by chemotherapy drugs. This research demonstrated the efficacy of catalpol in mitigating the detrimental effects of these drugs on blood parameters in rats (Zhang W. et al.).

Finally, the multitarget activity of Ophiopogon D, a key natural organic compound found in *Ophiopogon japonicus*, to differentially modulate cardiovascular protection, immune modulation, anti-cancer, anti-atherosclerosis, anti-inflammatory properties, and efficacy against NAFLD (Non-Alcoholic Fatty Liver Disease), was investigated in a systematic review (Chen et al.).

In conclusion, the observed synergy between various natural bioactive compounds offers a promising frontier for the integrative management of health and disease. By harnessing the combined effects of these compounds, it is possible to enhance therapeutic outcomes and provide a basis for the development of novel treatment strategies that are both effective and sustainable. The future of medical treatment may very well depend on our ability to seamlessly integrate these natural elements with traditional medical practices, leading to holistic approaches that are not only preventive but also curative.

Author contributions

MM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AB: Writing – original draft, Writing – review & editing. GT: Writing – original draft, Writing – review & editing.

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.

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References

1. Diederich M. Natural products target the hallmarks of chronic diseases. *Biochem Pharmacol.* (2020) 173:2019–21. doi: 10.1016/j.bcp.2020.113828

2. Amato A. Natural compounds and healthy foods: useful tools against onset and progression of chronic diseases. *Nutrients.* (2023) 15:10–2. doi: 10.3390/nu15132898

3. Owona BA, Abia WA, Moundipa PF. Natural compounds flavonoids as modulators of inflammasomes in chronic diseases. *Int Immunopharmacol.* (2020) 84:106498. doi: 10.1016/j.intimp.2020.106498

4. Hajimehdipoor H, Shahrestani R, Shekarchi M. Investigating the synergistic antioxidant effects of some flavonoid and phenolic compounds. *Res J Pharmacogn.* (2014) 1:35–40.

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Phyllanthus emblica: a comprehensive review of its phytochemical composition and pharmacological properties

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Phyllanthus emblica Linn, a prominent member of the euphorbiaceae family, exhibits extensive distribution across a multitude of tropical and subtropical nations. Referred to as "Balakka" in Indonesia, this plant assumes various names across regions, such as "kimalaka," "balakka," "metengo," "malaka," and "kemloko" in North Sumatra, Ternate, Sundanese, and Java respectively. *Phyllanthus emblica* thrives in tropical locales like Indonesia, Malaysia, and Thailand, while also making its presence felt in subtropical regions like India, China, Uzbekistan, and Sri Lanka. The fruits of Balakka are enriched with bioactive constituents recognized for their wide-ranging benefits, including antioxidant, anti-aging, anti-cholesterol, anti-diabetic, immunomodulatory, antipyretic, analgesic, anti-inflammatory, chemoprotective, hepatoprotective, cardioprotective, antimutagenic, and antimicrobial properties. Comprising a spectrum of phenolic compounds (such as tannins, phenolic acids, and flavonoids), alkaloids, phytosterols, terpenoids, organic acids, amino acids, and vitamins, the bioactive components of Malacca fruit offer a diverse array of health-promoting attributes. In light of these insights, this review aims to comprehensively examine the pharmacological activities associated with *P. emblica* and delve into the intricate composition of its phytochemical constituents.

KEYWORDS

Phyllanthus emblica, phytochemical composition, pharmacological properties, natural product, bioactive substances

1 Introduction

Phyllanthus emblica Linn, a member of the euphorbiaceae family, is extensively distributed throughout the majority of tropical and subtropical nations. Phyllanthus is a very large genus containing approximately 550–750 species and 10 to 11 subgenera. It is endemic to equatorial southeast Asia and is found in the mixed forest of tropical and subtropical regions at elevations between 150 and 1,400 m. In Indonesia *P. emblica* is called balakka, kimalaka, kemlaka, kemloko, or malaka (Summanen, 1999; Mal and Meena, 2022). Natural products have existed since the dawn of humanity, the

significance of traditional systems of medicine and particular traditional medical practices is now acknowledged worldwide. To evaluate selective pharmaceuticals of herbal origin, it is now necessary to adopt an intelligent and pragmatic approach. All parts of *Phyllanthus Emblica*, including its fruits, flowers, seeds, leaves, and bark, have been extensively utilized in numerous traditional remedies. Pharmacological studies reveals that *P. emblica* have antioxidant (Chaphalkar et al., 2017; Sheoran et al., 2019), anticancer (Ngamkitidechakul et al., 2010; Mahata et al., 2013; Gaire and Subedi, 2014; Zhao et al., 2015; Chekdaengphanao et al., 2022; Naik and David, 2023), Immunomodulatory (Jantan et al., 2019), cytoprotective (Zhang et al., 2016), anti-viral (Lv et al., 2014; Lv et al., 2015), anti-jaundice, anti-dyslipidemic (Quranayati et al., 2023), anti-aging (Wu et al., 2022), anti-apoptotic (Chekdaengphanao et al., 2022), anti-inflammatory (Wang et al., 2019), hepatoprotective (Pramyothin et al., 2006), nephroprotective (Huang et al., 2023), and anti-diabetic (Naik and David, 2023). *Phyllanthus emblica* has various constituents have been used in the formulation of numerous herbal and patent medicines (Dinesh et al., 2017). The majority of fixed oils, essential oils, and phosphatides are found in fruit seeds. Fruit, leaves, and bark are rich in tannins; bark also contains leucodelphinidin, while roots are abundant in lupeol and ellagic acid (Hussain et al., 2021). The yellowish-brown seeds of *P. emblica* contain linoleic acid, stearic acid, palmitic acid, linolenic acid, myristic acid, and oleic acid while D-myoinositol, D-fructose, and D-glucose are predominantly found in the ethanol fractions of *P. emblica* (Saini et al., 2022). *Phyllanthus emblica* contains chemopreventive compounds like lupol and glochidone, which belong to the lupine-type triterpenoids (Ramasamy et al., 2012). Additionally, it harbors antioxidant properties from compounds like mallotusinin, isomallotusinin, isostrictinin, and mallonin, along with phyllembilin, cinnamic acid, and chebulagic acid (Ahmad et al., 2021). The vitamin C content in *P. emblica* far surpasses that of common citrus fruits like lemons, oranges, and tangerines (Bajgai et al., 2006). Besides vitamin C, this fruit also contains other essential vitamins including carotene, niacin, riboflavin, and thiamine (Ghosal, 1996). For every 100 g of *P. emblica* fruit, there is an impressive vitamin C quantity ranging from 600 to 1,300 mg. Among the amino acids present, the prominent ones are glutamic acid (29.6%), proline (14.6%), aspartate (8.1%), alanine (5.4%), and lysine (5.3%) (Saini et al., 2022). *Phyllanthus emblica* fruit is rich in vitamin C (70%–72%) and includes a variety of components such as tannins, phembembaic acid (6.3%), gallic acid (5%), lipids (6%), emblicol, flavonoids, and mymic acid. The leaves of *P. emblica* contain gallic acid, chebulic acid, ellagic acid, kaempferol, kaempferol-3-o-glucoside, gallo tannin, and rutin, phosphoric acid, essential oils, linoleic acid, oleic acid, stearic acid, palmitic acid, and mystic acid. The bark of the plant contains proanthocyanidins, tannins, and leucodelphinidin. Moving on to the roots, they contain ellagic acid and lupeol (Saini et al., 2022). This thorough review focuses on the phytochemical makeup and the effects of *P. emblica*. Through an extensive exploration, this study aims to shed light on the numerous health advantages of *P. emblica*, thereby stimulating more research and progress in utilizing this herbal remedy to enhance human health.

FIGURE 1
Phyllanthus emblica.

2 Botanical description and taxonomy

Balakka is a type of plant that lives in a forest on the savanna. The forest has medium-sized trees with lots of branches, and they're about 10–20 m tall. The fruit of the balakka plant is round and has ridges. It is divided into six parts, and each part has a stone in it. The stones are about 1.8–2.5 cm across. The fruit is small and round with a tough covering. Inside, there are six seeds. The fruit looks nice–it is round and yellow. It tastes sour and astringent, which means it makes your mouth pucker a little. The balakka plant grows slowly and can climb things. It is mostly white in color. It is found in certain parts of Sumatra, which is an island in Indonesia. It likes to grow in places that are not very wet, like yards and along roads. It can also grow in places where people plant things, like farms. The balakka plant likes specific types of soil that have a pH between 6.5 and 7. There are different kinds of soil where balakka plants grow. Some soils have a gray or yellowish top layer and a red or yellow lower layer. These soils do not have a lot of nutrients, and they're a bit acidic. The balakka plant also grows in places where the soil has lots of clay. The land where balakka grows comes from rocks and other materials in the ground. It is usually not very high above sea level, maybe around 50–350 m. The balakka plant likes places where it rains between 2,500 and 3,500 mm every year (Gantait et al., 2021). The *P. emblica* Figure 1 can be seen below.

3 Botanical description and taxonomy

The bioactive components of natural ingredients are defined as secondary metabolites with human and animal pharmacological effects. Bioactive components of *Phyllanthus emblica* fruit include a group of phenolic compounds (tannins, phenolic acids, and flavonoids), alkaloids, phytosterols, terpenoids, organic acids, amino acids, and vitamins (Yang and Liu, 2014). Emblicanin A, B, punigluconin, pedunculagin, geranin, isochorylagin, corylagin, chebulanic acid, chebulagate acid, isostrictinin, gallic acid, mucous acid lactone gallate, digalloylglucose, methyl gallate, ethyl error, monogalloyl glucose, putanjivin A, galloil-HHDP-glucose, and

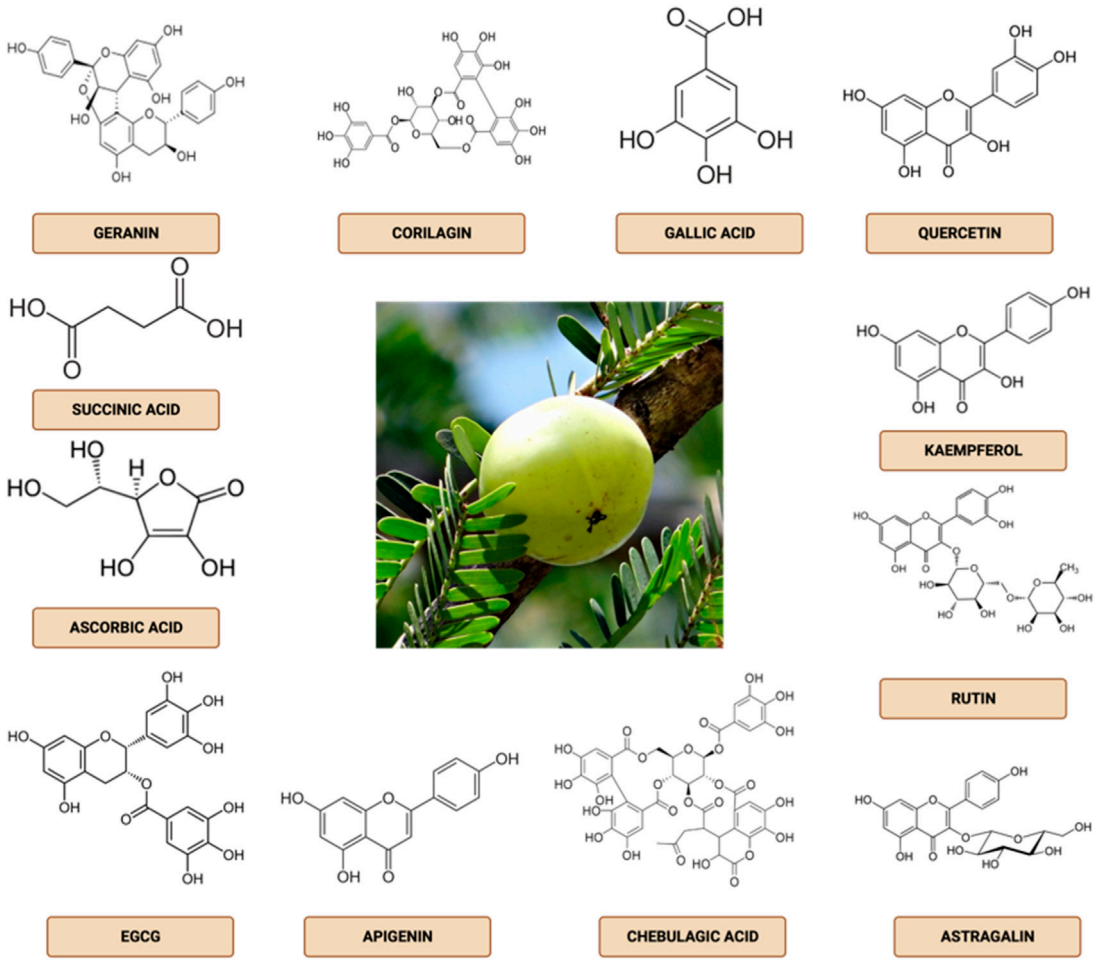

FIGURE 2
Main phytochemical components in *Phyllanthus emblica*.

elaeocarpusin are hydrolyzed tannins found in *P. emblica* fruit (Usharani et al., 2013). Other bioactive substances include gallic acid, ellagic acid, chlorogenic acid, malic acid, chebulic acid, and cinnamic acid. Additionally, *P. emblica* fruit contains flavonoid compounds like quercetin, kaempferol, and routine (Jagdale et al., 2021). This plant contains phytochemicals such as fixed oils, phosphatides, essential oils, tannins, minerals, vitamins, aminoacids, fatty acids, and glycosides, among others (Chanda et al., 2020). *Phyllanthus emblica* has been found to contain linolenic, linoleic, oleic, stearic, palmitic, and myristic acids. Sugar residues consist of D-glucose, D-fructose, D-myo-inositol, D-galacturonic acid, D-arabinosyI, D-rhamnosyl, D-xylosyI, D-glucosyI, D-mannosyl, and D-galactosyI (Ahmad et al., 2021). The results of our previous study indicate the presence of alkaloids (specifically phyllantidine and phyllantine) and tannins (including chebulagic acid, chebulinic acid, punigluconin, emblicanin A, ellagic acid, emblicanin B, 1-O-galloyl-b-D-glucose, ellagic acid, ellagotannin, 3-ethylgallic acid, corilagin, pedunculagin, trigallayl glucose, 3,6-di-O-galloyl-D-glucose, and 1,6-di-O-galloyl-b-D-glucose) as determined by chromatographic and infra-red spectral analysis. Additionally, the presence of flavonoids, including kaempferol-3-O-a-L-(600-ethyl)-rhamnopyranoside, quercetin, acylated apigenin glucoside, and kaempferol-3-O-a-L-(600-methyl)-rhamnopyranoside, was also documented (Halim et al., 2022). The chemical structure of phytochemical composition in *P. emblica* can be seen in Figure 2 and Table 1 below.

4 Traditional medicine use

Balakka is a component that is frequently used in traditional medicine. This plant has been used in India to treat cancer, diabetes, liver, cardiac problems, and anemia. The fruit of the Balakka tree contains chromium, zinc, and copper. Chromium exhibits substantial antidiabetic activity in a variety of experimental diabetes models. Additionally, chromium compounds can enhance the fat metabolism of diabetic rats. The fruit of balakka is used as a treatment for tuberculosis and as an anti-aging agent. The fruit of balakka contains tannin, which has antibacterial properties, and vitamin C, which has antioxidant properties. In addition, it has been demonstrated and investigated that balakka is one of the anti-cancer plants. Balakka plant flavonoids and phenols have antioxidant properties because they can capture free radicals (Vauzour et al., 2008). One

TABLE 1 Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
1	etylacetate	GC-MS	- Citronellyl Propionate	fruit	Acharya (2016)
			- 1-Methyl-4 Isopropyl-Cyclohexyl 2-Hydroperfluorobutanoate		
			- Citronellyl Acetate		
			–3,7,11,15-Tetra Methyl-2 Hexadecen-1-Ol		
			- Bicyclo (2.2.1) - Heptane,1,3,3-Trimethyl-		
			- 7-Octadecyne,2-Methyl		
			- Bicyclo(2.2.1) Heptane, 1,7,7-Trimethyl-		
			- Bicyclo(3.1.1) Heptane,2,6,6-Trimethyl-		
			- Bicyclo(2.2.1) Heptane,2,2,3-Trimethyl-Endo-		
			- Cyclohexane, 1-Methyl-4-(1-Methylethenyl)- Cis-		
2	petroleum ether	GC-MS	- Hentriacontane	Leaf	Elangovan and Irulappan (2015)
			- Dotriacontane		
			- Tetracontane		
			- Tritetracontane		
			- Pentacosane		
			- Octadecanoic Acid		
			- Methyl Ester		
			- Vitamin E		
3	n-hexane, ethyl acetate, and methanol solvents	GC-MS	- 9-Octadecene	Stem bark	Quranayati et al. (2023)
			- Methyl Palmitate		
			- 1-Octadecene		
			- 1-Tetracosanol		
			- Docosanoic Acid		
			- Cyclopropane, 1-(1-Hydroxy-1-Heptyl)-2-Methylene-3-Pentyl		
			- 1-Pentadecene		
			- 1-Hexadecene		
			- 9-Eicosene, (E)-		
			- Neophytadiene		
4	methyl salicylate	GC-MS	- 2-Methyl Butyl Acetate	fruit	Amir, et al. (2014)
			- Isopropyl,2-Methyl Butyrate		
			–2,4-Hexadienol		
			- Benzaldehyde		
			- Menthane		
			- Decane		
			- Butyl Cyclohexane		
			- Butyl Cyclohexene		

(Continued on following page)

TABLE 1 (*Continued*) Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
			- Acetophenone		
			- Undecane		
5	N-Hexane, Ethyl Acetate, and Ethanol	GC-MS	- 2-Furanmethanol	Leaf	Asmilia et al. (2020)
			- 1h-Cyclopropaanaphthalene		
			- Trans-Caryophyllene		
			- Cyclohexane		
			- Caryophyllene		
			- Sativen		
			- Delta-Guaiene		
			- Tetradecanoic Acid		
			- Octadecanal		
			- 3-Eicosyne		
6	Methanol	GC-MS	- Octyl-B-D-Glucopyranoside	fruit	Dinesh et al. (2016)
7	Ethanol	GC-MS	- Galacturonic Acid	fruit	Li et al. (2019)
			- Glucuronic Acid		
			- The Monosaccharide Standard		
			- Hydroxylamine Hydrochloride N-Propylamine		
			- 3-Phenylphenol		
			- Trifluoroacetic Acid		
8	Ethanol and methanol	GC-MS	−1,2,3-Benzenetriol	fruit	Nair et al. (2020)
			- Hexadecanoic Acid		
			- 2-Tert-Butyl-4-Iso Propyl-5 Methyl Phenol		
			- 9-Octadecenoic Acid		
			- Octadecanoic Acid		
9	Methanol	GC-MS	−1,5-Hexanediol	leaf	Abdel-Hady et al. (2022)
			- D-Mannose		
			−1,3-Dimethylindole		
			−24,25-Dihydroxy Vitamin D		
			- Pyrrolizin-1,7-Dione-6- Carboxylic Acid, Methyl (Ester)		
			- Phenol,2,4-Di-Tert-Butyl-		
			- Menthol,1'-[Butyln-3-One-1- Yl)-, (1r, 2s, 5r)		
			−10-Heptadecen-8-Ynoic Acid, Methyl Ester. (E)		
			- Vitamin A Palmitate		
			- Cis-11-Eicosenoic Acid		
10	Methanol	GC-MS	−2,4-Dimethylfuran	fruit	Akter et al. (2022)
			- 4-(2-Hydroxyethyl)-3-Methyl-2-Pyrazolin-5-One		
			- Trans-2,3-Epoxyoctane		

(Continued on following page)

TABLE 1 (*Continued*) Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
			- 2-Furancarboxylic Acid, 2-Ethylhexyl Ester		
			- Heptanoic Acid, 3-Hydroxy-, Methyl Ester		
			- 2-(3-Methylguanidino)Ethanol D-Glycero-D-Ido-Heptose		
			- Paromomycin		
			- Octadecanoic Acid		
			–9,9-Dimethoxybicyclo[3.3.1]Nona-2,4-Dione		
			- N-Propyl Nonyl Ether		
11	Ethanol, acetone, n-hexane	GC-MS	- Succinic Acid Dimethyl Ester	fruit	Harahap et al. (2019)
			- Dimethyl Pentanedioate		
			- Dimethyl Adipate		
			- Methyl Adipate		
			–1,3-Dioxolane-4-Methanol		
12	Ethanol	HPLC	- Quinic Acid	fruit, bark, leaf	Kumar et al. (2017a)
			- Caffeic Acid		
			- Gallic Acid		
			- Vanillic Acid		
			- Gentisic Acid-O-Hexoside -		
			- (+)-Catechin		
			- Brevifolincarboxylic Acid		
			- Epicatechin		
			- Ellagic Acid-O-Dihexoside		
			- Ellagic Acid-O-Hexoside		
13	Ethanol	HPLC	- Mucic Acid- 1,4-Lactone- 3-0-Gallate	fruit	Li et al. (2019)
			- Hamamelitannin		
			- Isocorilagin		
			- Ethyl Gallate		
			- Methyl Gallate		
			- Ellagic Acid		
			- Quercetin-3-O-Rhamnoside		
			- Undefined		
14	methanol	HPLC	- B-Glucogallin	fruit	Kumar et al. (2015)
			- Trigalloylglucose		
			- Geraniin		
			- Gallic Acida		
			- Castalin		
			- Trigalloylglucose (Isomer)		
			- Corilagin		

(Continued on following page)

TABLE 1 (*Continued*) Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
			- Protocatechuic Acida		
			- Methylgallate		
			- P-Coumaric Acid		
15	methanol	HPLC	- Digallic Acid	fruit	Balusamy et al. (2019)
16	Ethanol	HPLC	- Gallic Acid	fruit	Li et al. (2020)
			- Fisetin		
17	Ethanol	LC/MS	- 5-Hydroxyisophthalic Acid	fruit	Wu et al. (2022a)
			- Amlaic Acid		
			–3,5-Dihydroxybenzoic Acid		
			–10-Gingerdione		
			- 6-Methylgingediacetate		
			- Ethyl Gallate		
			- (-)-1,10-Epoxy-Guaia-11-Ene		
			- 7alpha-Hydroxycholesterol		
			- Irisoquin F		
			–2′-Hydroxycinnamaldehyde		
18	Ethanol	HPLC	- Gallic	fruit	Kumnerdkhonkaen et al. (2018)
			- Phydroxybenzoic		
			- Vanillic		
			- Syringic		
			- P-Coumaric		
			- Ferulic		
			- Sinapinic Acids		
19	Methanol	HPLC	- L-Ornithine	fruit	Luo et al. (2022)
			- Taurine		
			- Glucose 1-Phosphate		
			- Deoxycytidine		
			- Methylmalonic Acid		
			- 3-Hydroxy-3- Methylbutanoic Acid		
			- 5-Hydroxytryptamine		
			- N-Lactoyl-Phenylalanine		
			- 2-(3,4-Dimethoxyphenyl) Ethanamine		
			- Indoleacrylic Acid		
20	Methanol	LC/MS	- L-Ornithine	leaf	Kiran et al. (2021)
			- Taurine		
			- Glucose 1-Phosphate		
			- Deoxycytidine		

(Continued on following page)

TABLE 1 (*Continued*) Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
			- Methylmalonic Acid		
			- 3-Hydroxy-3- Methylbutanoic Acid		
			- 5-Hydroxytryptamine		
			- N-Lactoyl-Phenylalanine		
			- 2-(3,4-Dimethoxyphenyl) Ethanamine		
			- Indoleacrylic Acid		
21	Ethanol	HPLC	- Gallic Acid	fruit	Gong et al. (2020)
			- Methyl Gallate		
22	Ethanol and Methanol	LC/MS	- Arbamoyl Phosphate	fruit	Kumar et al. (2017b)
			- L-Methionine; Methionine; L-2-Amino-4methylthiobutyric Acid		
			- Pyridoxamine Phosphate		
			- Sulfate Derivative Of Norepinephrine		
			- Nicotinate D-Ribonucleoside		
			- 4-Hydroxy-All-Trans-Retinyl Acetate		
			–5(S),6(S)-Epoxy-15(S)-Hydroxy-7e,9e,112,13e-Eicosatetraenoic Acid		
			- Prostaglandin B1		
			- N6-D-Biotinyl-L-Lysine; Biocytin; Epsilon-N-Biotinyl- L-Lysine		
			–12-Oxo-20-Dihydroxy-Leukotriene B4		
23	Ethanol	GC-MS	- Fatty Acids And Fatty Acyl Esters	fruit	Khaled et al. (2018)
			- Fatty Acyl Esters		
			- Phthalides		
			- Terpenes		
			- Sterol		
			- Palmitic Acid And Palmitic Acid Methyl Ester		
			- Linoleic Acid		
			- Stearic Acid		
			- Elaidic Acid		
24	Ethanol	HPLC	- Hypophyllanthin	fruit, leaf	Kumar et al. (2017c)
			- Phyllanthin		
			- Gallic Acid		
			- Ellagic Acid		
			- Quinic Acid		
			- Chebulinic Acid		

(Continued on following page)

TABLE 1 (*Continued*) Phytochemical components of *Phyllanthus emblica*.

No.	Extraction method	Analytic Technique	Bioactiv compund identified	Part of the plant	References
25	Methanol	HPLC	- Mucic Acid	fruit	Yang et al. (2012)
			- Mucic Acid Lactone		
			- Malic Acid		
			- Mucic Acid Gallate		
			- Chebulic Acid		
			- Mucic Acid Digallate		
			- Mucic Acid Lactone Gallate		
			- Galloylglucose		
			- Mucic Acid Methyl Ester Gallate		
			- Gallic Acid		
26	Ethanol	HPLC	- Quercetine	fruit	Halim et al. (2022)
			- Betaine		
			- Trigonelline		
			- Stearamide		
			- Ellagic Acid		
			- Myricitrin		
			- Myricetin		
			- Leucine		
			- Kaempferol		
			- A-Linoleic Acid		
27	Methanol	HPLC, LC/MS	- Ellagic Acid	fruit	Muthusamy et al. (2017)
			- Phyllanthin		
			- Hypophyllanthin		
			- Niranthin		

of the benefits of antioxidants is their ability to prevent degenerative diseases such as diabetes mellitus caused by oxidative stress caused by the deterioration of organ cells or body systems. Besides having medicinal properties, the natural benefits of balakka include its fruit for cars, candy, jelly jam, leather contains colorants that can run out as a blue dye in various fabrics only, tanneries, furniture and agricultural implements, and firewood. So balakka can be used for a wide variety of applications healthcare or herbal medicine, food and beverage, cosmetic, industry, dyeing, tanning, etc. According to the Wealth of India, *Phyllanthus emblica* seeds have been documented as a potential remedy for asthma, bronchitis. The juice that is released during the harvesting of fruit is also utilized as an ocular rinse and for the management of ocular inflammation. According to Chopra *P. emblica* has been recognized for its notable wound healing capabilities, making it a potential treatment option for snakebites and scorpion stings. The fixed oil included in FPE has been utilized in traditional formulations as a hair tonic to enhance hair growth and pigmentation (Chopra, 1958; Bruno, 1984).

5 Pharmacological activity of balakka (*Phyllanthus emblica*)

5.1 Antioxidant activity

Oxidative stress is associated with an increased generation of free radicals or a reduction in antioxidant content. The observed phenomenon indicates a disturbance in the equilibrium between pro-oxidant and antioxidant molecules. Pro-oxidants and free radicals are characterized by the presence of several unpaired electrons, rendering them unstable and highly reactive towards other substances. *Phyllanthus emblica* has antixoidant capacity,

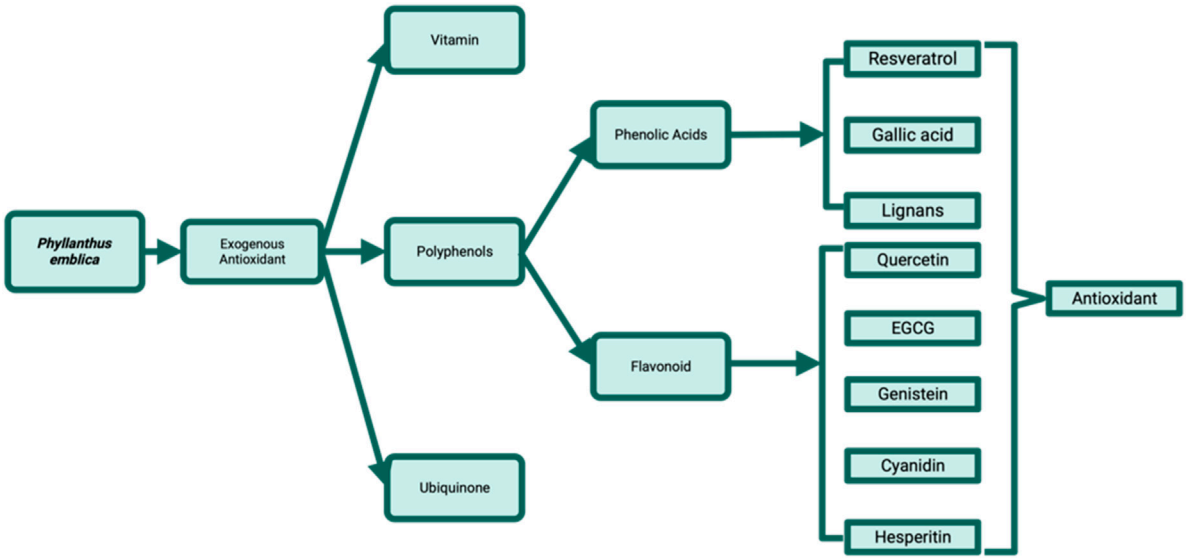

FIGURE 3
Antioxidant of Phyllanthus emblica.

according to previosuly study showed that methanol extract of *P. emblica* exhibited appreciable *in vitro* antioxidant activity for scavenging the DPPH radical (IC_{50} = 39.73 ± 2.12 μg/mL), nitric dioxide (IC_{50} = 39.14 ± 2.31 μg/mL) and moderate antioxidant activity for lipid peroxidation (IC_{50} = 84,10 ± 3.04 μg/mL). *Phyllanthus emblica* also demonstrated recovery ability in CCl_4 treated rats by elevating the level of catalase, superoxide dismutase, glutathione peroxidase and glutathione in the rats' pulmonary. The pharmaceutical activities of *P. emblica* might be attributed to by active phyto-constituents such as gallic acid, caffeic acid, kaempferol and rutin (Tahir et al., 2016). Another study also revelaed that the IC_{50} of the *P. emblica* branches, leaves and barks aqueous extract were respectively (6.92 ± 0.22) μg/mL), (7.72 ± 0.25) μg/mL), and (6.54 ± 0.27) μg/mL). These results showed slightly lower than the IC_{50} of the ascorbic acid (8.06 ± 0.01) μg/mL) (Iamsaard et al., 2014). The antioxidant capacity of *P. emblica* were addressed by phytochemical components such as reseveratrol, gallic acid, lignans, quercetin, EGCG, genistein, cyanidin, and hesperitin (Figure 3).

5.2 Antiinflammatory activity

Inflammation serves as a crucial innate immune response of the organism to external stimuli, such as injury or infection caused by pathogens (Jubaidi et al., 2021). Inflammation is an essential immunological response that enables the body to endure and recover from injury. Inflammation is recognized as a beneficial pathological process due to its significant involvement in restorative, healing, and aggressive mechanisms, particularly in resisting stress generated by pathogens and harmful situations (Rakha et al., 2022). The phenomenon of inflammation is intricate and encompasses a multitude of cellular responses, mostly categorized as acute and chronic inflammation. Acute inflammation serves as a protective mechanism for the body, facilitating the healing of injuries and defending against microbial invasion. In contrast, chronic inflammation specifically targets essential cells, molecules, and organs, thereby contributing to the onset and progression of diverse chronic pathologies such as cardiovascular disease, skeletal muscle disorders, inflammatory bowel disease, diabetes, cancer, and neurological diseases (Ferraz et al., 2020). Consequently, chronic inflammation also exacerbates the aging process The fruit extracts of *Phyllanthus emblica* showed a significantly hight dose-dependent inhibition of Nitric Oxide (NO) and COX-2. NO is a vital immune-signaling pathways molecule. NO overproduction could cause numerous pathological disorders and abnormalities, such as serious inflammation, cardiovascular related injury, and oxidative stress. *Phyllanthus emblica* extracts exhibited dose-dependent NO inhibition in LPS-stimulated RAW264.7. At 50 and 100 μg/mL concentration, the 95% ethanolic extract exhibited significantly higher NO inhibition (49.1%) compared to hot water and commercial extract. The extracts of *P. emblica* exhibited significantly higher COX-2 inhibition compared to hot water and commercial extract. The highest COX-2 inhibition was shown at 10 μg/mL concentration (46.4%). COX-2 inhibition may control inflammation in inflammatory diseases and abnormalities. *Phyllanthus emblica* exhibited its anti-inflammatory activities by inhibiting NO production to avoid excess NO production in macrophage cells and COX-2 enzyme (Li et al., 2022). The mechanism by which Phyllanthus emblica exerts its anti-inflammatory effects involves the inhibition of key enzymes involved in the inflammatory process, namely, COX-1, COX-2, and 5-LOX. These enzymes play pivotal roles in the synthesis of pro-inflammatory mediators, and their suppression by *P. emblica* contributes to the reduction of inflammation. This multifaceted mechanism underscores the potential therapeutic value of Phyllanthus emblica in alleviating inflammatory conditions (Figure 4).

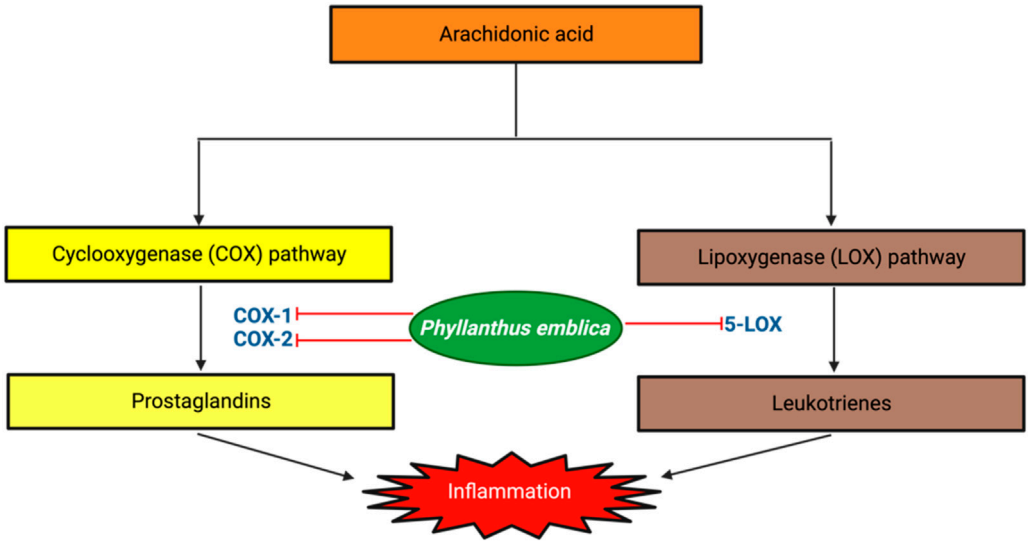

FIGURE 4
Antiinflammatory of *Phyllanthus emblica*.

5.3 Immunomodulatory activity

An immunomodulator is a constituent that possesses the ability to modulate the immune system, encompassing both innate and adaptive immunological responses (Oo et al., 2021). At now, there is a growing interest in investigating the potential of bioactive chemicals obtained from medicinal plants as agents for modulating the immune system in scientific research. Inflammation can be described as a physiological process that occurs within the human body as a result of immunological responses to combat pathogens (Zaragozá et al., 2020). The human body consists of mechanisms that serve to protect against external pathogens, such as bacteria and viruses, as well as to facilitate tissue repair and initiate the healing process following an injury. Nevertheless, an overabundance of inflammation exacerbates functional impairment, causes tissue damage, and leads to pain and discomfort (Bayat et al., 2021; Masad et al., 2021; Han et al., 2022). *Phyllanthus emblica* enhanced the effectiveness of the immunomodulatory system by raising blood levels of CD4, CD8, CD16, CD19, IgM, and IgG as well as albumin and globulin levels in the serum. In comparison to all experimental groups, the *P. emblica* group at a dose of 250 mg/kg b.wt. exhibited the most appreciable outcomes to increase immunity (Bakr and Naga, 2020). The aqueous extract of *P. emblica* fruit possessed a dose-dependent immunomodulatory activity to albino rats with a dose of 100 and 200 mg/kg for 19 days. The fruit extracts significantly increased the hemagglutination antibody titer, leukocytes count, the percentage of lymphocytes distribution, and delayed hypersensitivity in mice (Nirala et al., 2020).

5.4 Antidiabetic activity

Diabetes mellitus, a prevalent endocrine metabolic illness, has resulted in substantial morbidity and death as a consequence of both microvascular consequences (retinopathy, neuropathy, and nephropathy) and macrovascular complications (heart attack, stroke, and peripheral vascular disease) (Hussain et al., 2021). The human body is equipped with both enzymatic and non-enzymatic antioxidative systems that serve to mitigate the production of reactive oxygen species, which have been implicated in the development of several degenerative disorders, such as diabetes. The prevalence of the disease is experiencing a rapid escalation on a global scale, impacting various regions across the globe (Shamsudi et al., 2022). Individuals with diabetes experience elevated levels of blood glucose due to a lack of insulin. Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus, is the predominant manifestation of the condition, including approximately 90%–95% of cases characterized by insufficient insulin production or utilization (Bitew et al., 2021). According to the World Health Organization, it is projected that the diabetic population would experience a substantial growth, potentially reaching 300 million or more individuals by the year 2025 (Ansari et al., 2022). Presently, the therapeutic options for diabetes encompass insulin as well as a range of oral antidiabetic medications, including sulfonylureas, biguanides, and glinides (Kalaitzoglou et al., 2019). The presence of numerous significant unfavorable effects necessitates the exploration of more efficacious and less hazardous hypoglycemic drugs, rendering it a crucial field of inquiry (Islam et al., 2023). According to Bashir et al. (2018), the ethanolic extract of *P. emblica* is a potent remedy to reduce glucose level. In diabetic rats, the glucose level significantly decreased (166 ± 0.7 mg/dL) after being treated with 80 mg/kg *P. emblica* compared to untreated diabetic rats (380 ± 0.7 mg/dL). The ethanolic extract of *P. emblica* contains tannin, an effective agent to prevent adipogenesis and increase glucose uptake by increasing insulin sensitivity towards peripheral tissues (Gul et al., 2022). *Phyllanthus emblica* has several mechanism causes hypoglycemia which are inhibition of digestive

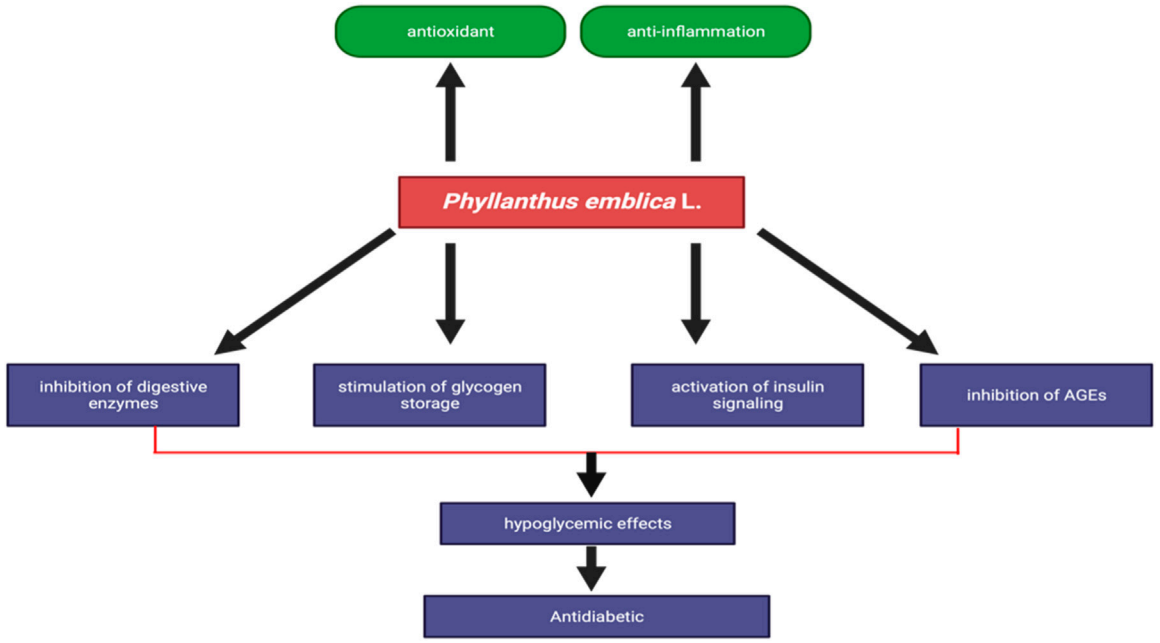

FIGURE 5
Antiinflammatory of *Phyllanthus emblica*.

enzymes, stimulation of glycogen storage, acativation of insulin signaling, and inhibition of AGEs (Figure 5.)

5.5 Hepatoprotective activity

One of the most prevalent chronic liver diseases, NAFLD (Non-alcoholic fatty liver disease) is closely associated with metabolic syndrome and refers to the accumulation of hepatic steatosis that is not brought on by excessive alcohol consumption. *Phyllanthus emblica*, a rich source of gallic acid and many known medically phytochemicals and acids. *Phyllanthus emblica* fruit exhibits *in vitro* inhibitory activity on hepatic steatosis and liver fibrosis (Paik et al., 2020). The gallic acid content is also *in vivo* proven to improve high fat diet (HFD)-induced dyslipidemia, hepatosteatosis, and oxidative stress. Huang et al. initiated a research project aiming to evaluate the hepatoprotective effect of the aqueous extract of *P. emblica* L. fruit (WEPE) on NAFLD in an animal model. The findings revealed that WEPE significantly reduce body weight, peritoneal fat and epididymal fat in rats treated with HFD, as well as increase antioxidant enzyme activities and improve steatosis by increasing *adiponectin* in adipocytes and *PPAR-α* in the liver and decreasing *SREBP-1c* in the liver. This could be the reason for WEPE's ability to reduce hepatic fat deposition. These findings exhibited that WEPE could be beneficial for treating HFD-induced steatosis (Tung et al., 2018).

5.6 Neuroprotective activity

Flavonoids have garnered attention for their ability to alter neuronal activity and prevent age-related neurodegeneration (Dajas et al., 2003). Flavonoid-rich plant or food extracts may preserve fragile neurons, enhance neuronal function, or stimulate neuronal regeneration to improve cognition in people and animals (Hwang et al., 2012). Their neuroprotective properties have been demonstrated in oxidative stress and Aβ-induced neuronal death models. Ginkgo biloba extracts high in flavonoids have been shown to benefit and modulate the brain, especially in Alzheimer's disease and age-related dementia (Calderaro et al., 2022). Individual flavonoids, such as the citrus flavanone tangeretin, have been shown to maintain nigro-striatal integrity and functionality after 6-hydroxydopamine lesioning, suggesting that it may protect against Parkinson's disease pathology (Vauzour et al., 2008). In a recent study conducted by Rajalakshmi et al. (2019), The results of this study on Phyllanthus emblica (Indian gooseberry) offer valuable insights into its potential neuroprotective and antioxidant properties, shedding light on its traditional medicinal uses. This research focused on assessing the radical scavenging capabilities of P. emblica through various assays and its impact on neuroprotection using human neural cell lines (PC12) subjected to glutamate-induced cellular inhibition.

One of the key findings of this investigation was the remarkable antioxidant activity exhibited by P. emblica extract. The DPPH and hydroxyl radical scavenging assays revealed IC50 values of 73.21 μg/mL and 0.426 mg/mL, respectively. This indicates its capacity to effectively neutralize free radicals, which are known to contribute to oxidative stress and various health issues. Moreover, the study observed significant lipid peroxidation activity (IC50: 73.21 μg/mL), further highlighting the potential of P. emblica in preventing oxidative damage to cellular membranes, a crucial factor in maintaining cellular integrity. The neuroprotective

effects of P. emblica were also explored, and the results demonstrated its ability to safeguard PC12 cells against glutamate-induced cytotoxicity. This was confirmed through cell viability assays, which showed that P. emblica extract had a protective effect on neural cells. Additionally, monitoring LDH activity, GSH levels, and ROS levels provided further evidence of its neuroprotective properties. According to Li et al. (2011), the extracts of *P. emblica* were found to provide protection to PC12 cells against cell death triggered by H2O2. All samples derived from *P. emblica* exhibited a protective effect on H2O2-induced PC12 cell death, which was both dose-dependent and consistent across all extracts. The results indicate that the hot water and ethanol extracts exhibited superior PC12 cell protection percentages compared to the commercial extracts. According to Li et al. (2022), the neuroprotective benefits of hydroalcoholic extracts derived from *P. emblica* were shown in rat models with kainic acid-induced seizures. These effects may be attributed to the antioxidant and anti-inflammatory properties of the extracts.

5.7 Cardioprotective potential

Globally, there is a significant escalation in the prevalence of chronic diseases, including cardiovascular diseases, cancer, diabetes, and obesity. In the year 2001, chronic diseases accounted for nearly 59% of the total recorded deaths worldwide, amounting to 56.5 million fatalities. Furthermore, these disorders were responsible for 46% of the overall burden of disease on a global scale. Cardiovascular diseases (CVD) encompass a range of conditions that affect the heart and blood arteries, such as hypertension (elevated blood pressure) and coronary heart disease (myocardial infarction) (Mayakrishnan et al., 2013). According to Ekta in Pria et al, oral administration of *P. emblica* fruit extract at a dose of 50 and 100 mg/kg BW twice a day for 2 weeks significantly reversed the effects of IRI (ischaemia reperfusion injury), a disease occurs due to oxidative stress. This cardioprotective effect occurs due to the emblicanin A and B contents of *P. emblica* fruit extract (Rajak et al., 2004).

Globally, there has been a concerning surge in the prevalence of chronic diseases, encompassing conditions such as cardiovascular diseases, cancer, diabetes, and obesity. In 2001, chronic diseases accounted for a staggering 59% of total recorded deaths worldwide, resulting in 56.5 million fatalities. Additionally, these ailments imposed a substantial burden, constituting 46% of the overall global disease burden. Among chronic diseases, cardiovascular diseases (CVD) comprise a spectrum of conditions affecting the heart and blood vessels, including hypertension (elevated blood pressure) and coronary heart disease (myocardial infarction) (Mayakrishnan et al., 2013). In a noteworthy study by Ekta in Pria et al., it was demonstrated that the oral administration of *P. emblica* fruit extract at doses of 50 and 100 mg/kg body weight, twice a day for 2 weeks, led to a significant reversal of the effects of ischemia-reperfusion injury (IRI), a condition arising from oxidative stress. This observed cardioprotective effect is attributed to the presence of emblicanin A and B within *P. emblica* fruit extract (Rajak et al., 2004).

5.8 Anticancer activity

Cancer remains a prominent global health challenge, compelling researchers to explore novel substances and treatments aimed at diminishing cancer cell survival, impeding angiogenesis, thwarting proliferation, and restraining metastasis. In the past decade, specific phytochemical compounds and flavonoids have emerged as promising candidates for cancer therapy. In a study conducted by Mahata et al. (2013), the mechanism of action of Phyllanthus emblica fruit extract was meticulously examined. The investigation focused on its impact on activator protein-1 (AP-1) activity and its relevance to cervical cancer cells driven by human papillomavirus (HPV). The findings unveiled a compelling pattern: the Phyllanthus emblica fruit extract exhibited dose- and time-dependent inhibition of DNA binding in both constitutively active AP-1 HPV16-positive (SiHa) and HPV18-positive (HeLa) cervical cancer cells. This AP-1 inhibition, accompanied by the suppression of viral transcription, resulted in the inhibition of cervical cancer cell growth. Moreover, the growth-inhibitory effect of Phyllanthus emblica was primarily attributed to the induction of apoptotic cell death. These findings collectively suggest that Phyllanthus emblica demonstrates its anticancer efficacy by concurrently inhibiting AP-1 and targeting the transcription of viral oncogenes responsible for the development of cervical cancer, thereby indicating its potential utility in the treatment of HPV-induced cervical cancer cells (Mahata et al., 2013; Quranayati et al., 2022). In a separate investigation by Samatiwat et al. (2021), the anti-proliferative activity of the ethanolic extract of Phyllanthus emblica bark was assessed in the context of cholangiocarcinoma. The results highlighted the extract's cytotoxic potential on the KKU-452 CCA cell line, with an IC50 value of 52.2 μg/mL, accompanied by a significant induction of apoptosis. Furthermore, the ethanolic extract of Phyllanthus emblica bark substantially inhibited cell migration at concentrations of 25 and 50 μg/mL, with reductions of 425% and 32.9%, respectively, compared to untreated cells. These anticancer effects were attributed to the phenolic acid and flavonoid content present in the bark extract of Phyllanthus emblica (Samatiwat et al., 2021). The anticancer potential of Phyllanthus emblica is graphically illustrated in Figure 6.

5.9 Antihyperlipidemic and atherogenic activity

In recent years, hyperlipidemia and oxidative stress have emerged as significant health concerns, acknowledged as primary risk factors in the development and progression of atherosclerosis, as well as cardiovascular and cerebrovascular diseases (Li et al., 2011). Consequently, there has been a growing interest in exploring the potential of new natural antioxidants, predominantly of plant origin (Sun et al., 2021). Flavonoids and phenolic compounds derived from plants have gained recognition for their multifaceted pharmacological properties, including antioxidant and anti-hyperlipidemic effects (Gong et al., 2020). The powdered extract of *Phyllanthus emblica* has demonstrated remarkable anti-hyperlipidemic, hypolipidemic, and anti-atherogenic effects, substantiated by statistically significant differences when compared to control groups (Santoshkumar et al., 2013). These

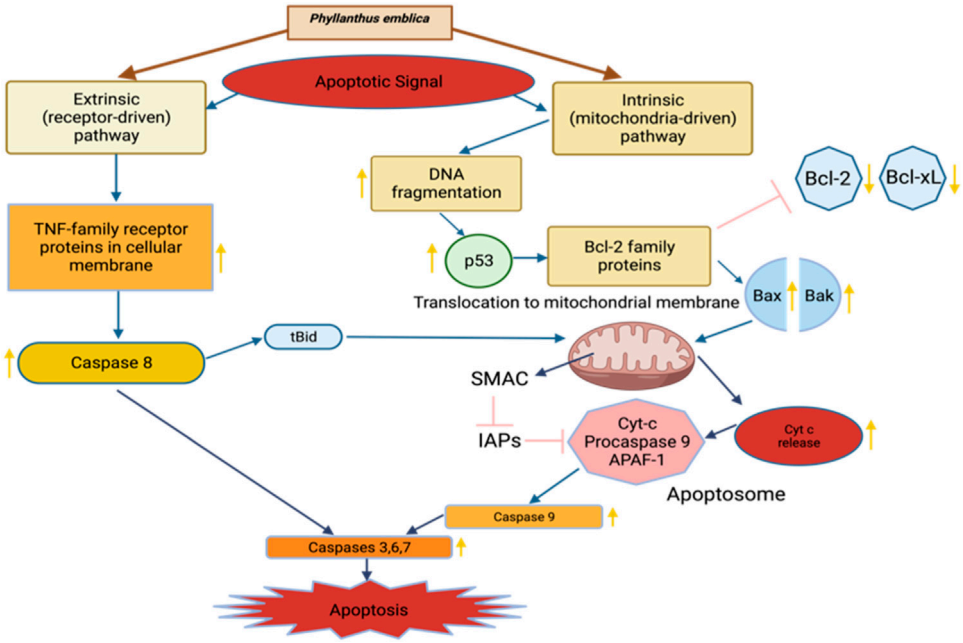

FIGURE 6
Anticancer of *Phyllanthus emblica*.

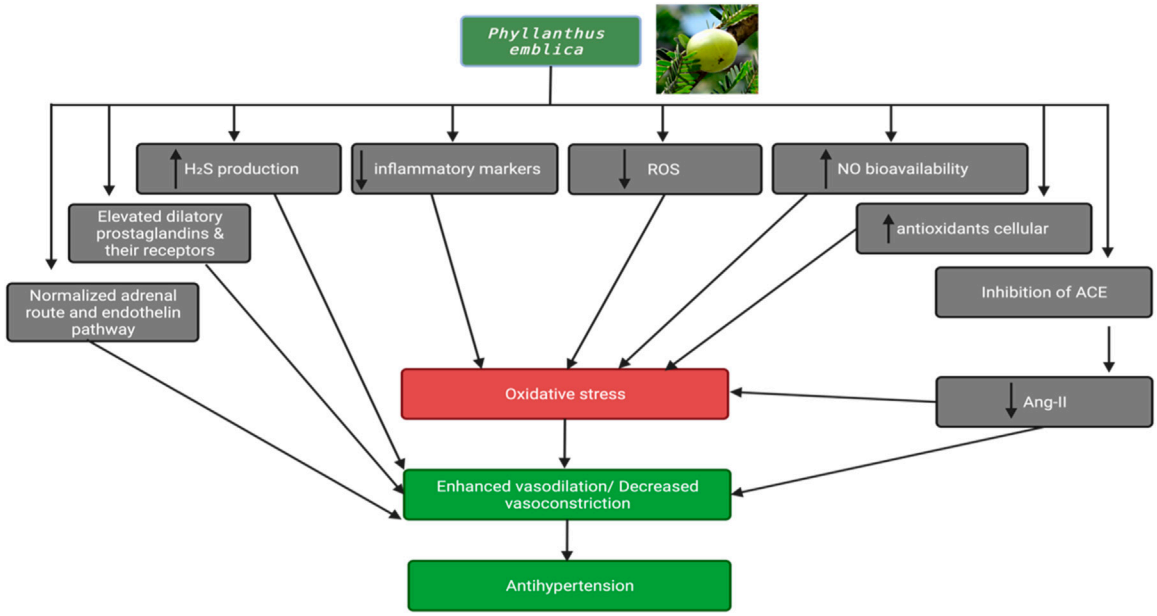

FIGURE 7
Antihypertension activity of *Phyllanthus emblica*.

effects can be conceivably attributed to the flavonoid content found in Phyllanthus emblica, which exhibits a hypolipidemic effect in atherogenic albino rats. The flavonoids in Phyllanthus emblica function as hypolipidemic agents by inhibiting the activity of HMG-CoA reductase while concurrently increasing the activity of plasma lecithin cholesterol acyl transferase (LCAT) (Sobhani et al., 2017).

5.10 Antihypertensive activity

In a rigorously conducted study by Ghaffari et al. (2020), a randomized, triple-blind, and placebo-controlled research approach was employed to investigate the antihypertensive effects of *P. emblica*. The study included 92 patients who were randomly

assigned to two groups. Each group received either *P. emblica* at a dosage of 500 mg/three times daily after meals or a placebo, in addition to their standard antihypertensive treatments. The findings of this study were quite compelling. After 8 weeks of treatment, the systolic blood pressure exhibited a significant reduction of 15.6% ± 8.23% in the *P. emblica* group, as compared to a 6.3% ± 7.49% decrease in the placebo group. Likewise, diastolic blood pressure showed a notable decrease of 12.3% ± 7.87% in the *P. emblica* group, in contrast to a 3.88% ± 7.98% decrease in the placebo group. Importantly, throughout the study duration, no toxic side effects were observed in any of the patients, underscoring the safety profile of *P. emblica* in this context (Ghaffari et al., 2020). For a visual representation of the mechanism, please refer to Figure 7.

5.11 Antimicrobial activity

Jahan and Akter (2015) conducted research to evaluate the antimicrobial activity of *Phyllanthus emblica*. This research was conducted utilizing disc diffusion method. The methanolic extract of *P. emblica* was assayed at 500 µg/disc concentration with standard kanamycin disc against gram positive, gram negative and multi drug resistant strains. The results demonstrated that the ethanolic extract of PE exhibited significant antimicrobial effect againts some microorganisms especially, *Bacillus subtilis* (25 mm), *Staphylococcus aureus* (20 mm) and *Shigella dysenteriae* (17 mm). Both aqueous and ethanolic extract of *P. emblica* was proven effective against *Pseudomonas aeruginosa* in disc diffusion method (Farhana et al., 2022). The methanolic extract of *P. emblica* at a dose rate of 50 mg/mL and 25 mg/mL, respectively, had a complete bactericidal effect on AMR (antimicrobial-resistant) *S. Typhi* and S. Enteritidis (Nair et al., 2020).

5.12 Anti-diarrheal activity

According to Afrin et al. (2016), *P. emblica* extract exhibited a significant anti-diarrheal effect. These authors conducted research to evaluate the anti-diarrheal activity towards diarrheal (castor oil induced) mice at a dose of 2 mL/mouse. The extracts of *P. emblica* at a dose of 500 mg/kg BW were given 1 h before the mice were induced with castor oil orally. The results showed that *P. emblica* significantly inhibited the defecation mean number compared to control group administrated with standard anti-diarrheal drug. The methanolic extract of *P. emblica* at a dose of 25% exhibited 42.86% inhibition while at a dose of 500 mg/kg BW, it showed 64.29% inhibition. The anti-diarrheal standard drug (loperamide) at a dose of 5 mg/kg exhibited 71.43% inhibition. As *P. emblica* succesfully inhibited the castor oil induced diarrhea, it might have exhibited its antidiarrheal effect by inhibiting the biosynthesis of prostaglandin through antisecretory mechanism (Afrin et al., 2016).

5.13 Anti-aging activity

In a study conducted by Wu et al. (2022), it was observed that the polyphenols found in the fruit of *P. emblica* demonstrated a notable protective effect against the aging process in the *Caenorhabditis elegans* model. The anti-aging properties were evidenced through the augmentation of thermal resistance, as well as the significant reduction in the activity levels of acetylcholinesterase (AChE) by 34.71% and butyrylcholinesterase (BuChE) by 45.38%. These findings were accompanied by a significant increase in the activity of antioxidant enzymes, specifically superoxide dismutase (SOD) by 30.74% and catalase (CAT) by 8.42%. Additionally, there was a notable decrease in the amount of malondialdehyde (MDA) by 36.25%. The presence of rich flavonols and phenolic acids, including quercetin, myricetin, ellagic, gallic, and chlorogenic acids, together with their glycosides, may potentially be associated with the interrelation of these qualities (Wu et al., 2022).

5.14 Laxative

Mehmood et al. (2013) reported that the crude extract derived from *P. emblica* exhibited prokinetic and laxative properties in mice. The extract facilitated the movement of charcoal meal through the small intestine and resulted in an increase in the production of wet feces. These effects were comparable to the effects observed with carbachol, a commonly used cholinergic agonist known for its ability to accelerate intestinal contents. The extract's gut stimulatory activities were observed to be slightly influenced by atropine, a blocker of muscarinic receptors. The mice exhibited wet feces at a rate of 35.7% and 44.1% when administered with a crude extract of *P. emblica* at doses of 100 and 300 mg/kg, respectively. The experimental group receiving the positive control, CCh (1 mg/kg), exhibited a moist feces percentage of 48.2%, whereas the group treated with saline solution displayed a wet feces percentage of just 10.5%. The laxative effect was determined by calculating the percentage increase in moist feces compared to the total fecal production, as described by Mehmood et al. (2013). The pharmacological activity of *P. emblica* can be seen in Table 2.

6 Safety and toxicity

Plants especially medicinal plants have been used for treatments in humans. Since the prehistoric era, raw parts or extract of *Phyllanthus emblica* have been used to treat various diseases and *in vitro* and *in vivo* studies have proven its effectiveness to inhibit various pathogenesis. Chronic toxicity studies by inducting *P. emblica* oral doses of 300, 600 and 1,200 mg/kg for 270 days resulted in no evident changes in treated animals pathologically. Previous studies reported the absence of toxicity of *P. emblica* fruit extract at doses of 200, 400, 300, and 500 mg/kg (Golechha et al., 2014; Middha et al., 2015; Anto et al., 2022). Furthermore, Uddin et al. (2016) reported that the ethanolic extract of Phyllanthus emblica is safe with a dose up to 2000 mg/kg b.w. in rats. The hematological examination, behavioral observation, and biochemical test of *P. emblica* demonstrated the absence of toxic effect. Thiennimitr et al. (2018) reported that consuming *Lactobacillus* sp. mediated fermented *P. emblica* fruit juice up to 9 mL/kg/day for 60 days to both male and female rats is safe and no rat exhibited any remarkable changes in the body weights, internal organs, hematology, and biochemical parameters.

TABLE 2 Pharmacological effects and mechanisms of action of extract from *Phyllanthus emblica*.

No.	Dose	Pharmacology activity	Mechanism	References
1	50 and 100 μg/mL	Antioxidant	- enhancing reducing power and total antioxidant capacity, scavenging hydroxil radical and superoxide anion	Uddin et al. (2016); Li et al. (2020)
			- Reduced peroxidation level	
			- Induced defense system (GSH, SOD, CAT, GPx, GSH reductase, and GSH S-transferase)	
2	50 and 100 μg/mL	Antiinflammatory	- Inhibited Nitric Oxide (NO) and COX-2 (dose-dependent)	Li et al. (2022)
3	100; 200; 500 mg/kg BW	Immunomodulatory	- Induced the activity of GSH, CAT, and SOD in the thymus of mice	Ghosh et al. (2013); Saini et al. (2022)
			- increased the hemagglutination antibody titer, leukocytes count, the percentage of lymphocytes distribution, and delayed hypersensitivity in mice	
4	500 mg/kg BW	Hepatoprotective	- Gallic acid content improves high fat diet induced dyslipidemia, hepatosteatosis, and oxidative stress	Huang et al. (2017)
			- increasing *adiponectin* in adipocytes and *PPAR-α* in the liver and decreasing *SREBP-1c* in the liver	
5	Dose dependent	Neuroprotective	- Conferred PC12 cells protection against H_2O_2-induced cell death	Li et al. (2022)
6	50 and 100 mg/kg BW	Cardioprotective	- The emblicanin A and B contents reversed the effects of IRI (ischaemia reperfusion injury)	Priya and Islam (2019)
7	25 and 50 μg/mL	Anticancer	- Phenolic acid and flavonoid content inhibited cell migration	Samatiwat et al. (2021)
8	2 mL/kg/day	Antihyperlipidemic	- Activated PPARα and carnitine palmitoyl transferase (involved in lipid oxidation)	Variya et al. (2016)
			- Reduced the serum triglycerides	
9	500 mg/TDS	Antihypertensive	- Reduced systolic and diastolic blood pressure	Ghaffari et al. (2020)
10	50 and 25 mg/mL	Antimicrobial	- Methanolic extract showed potent antimicrobial activity to antimicrobial-resistant (AMR) *Salmonella Typhi* and *Salmonella Enteritidis*; *Bacillus subtilis*, *Staphylococcus aureus* and *Shigella dysenteriae*; *Pseudomonas aeruginosa*	Nair et al. (2020); Jahan and Akter (2015); Farhana et al. (2022)
11	250 and 500 mg/kg BW	Anti-diarrheal	- Inhibited the biosynthesis of prostaglandin through antisecretory mechanism	Afrin et al. (2016)
12	1.2 mg/mL	Anti-aging	- Reduced the activity of AchE, BuChE; enhanced antioxidant enzymes activity of SOD and CAT; decrease MDA level	Wu et al. (2022b)
13	100 and 300 mg/kg BW	Laxative	- The crude extract increased the production of wet feces at a mechanism similar to the effect of standard drug carbachol	Mehmood et al. (2013)

7 Future perspective and challaenges

Plants have been used as remedial agents throughout history. In the last two centuries, scientists have found a way to utilize secondary metabolites contents in medicinal plants for drug manufacturing. *Phyllanthus emblica*, is a widely used herb in the Indian medicinal system. Numerous researches have proven therapeutic properties using different extracts and herbal preparations of *P. emblica*. A variety of well-established beneficial health effects and pharmacological activities was ascribed to *P. emblica*, namely, antioxidant, anti-cancer, hepatoprotective, neuroprotective, immunomodulatory, anti-inflammatory, anti-diabetic, and anti-hyperlipidemic effects. *Phyllanthus emblica* is traditionally used to address numerous disorders along with food ingredients. In spite of the fact that various modern research techniques have been established to validate the medicinal uses of *P. emblica* traditionally, some aspects including its contents and its applications need to be further investigated scientifically. For example, only a few studies reported *P. emblica* antimalarial, antiviral, anti-venom, and insecticidal properties. Some of its properties were also reported with other parts of *P. emblica*. Accordingly, it is imperative that the agents, molecules or parts mediating its therapeutic activities be identified. Additionally, more extensive research, such large-scale evidence-based trials, must be done to examine the medical benefits of *P. emblica*.

8 Conclusion

In conclusion, our comprehensive review of *P. emblica*, commonly known as Indian gooseberry or Amla, has shed light on its remarkable phytochemical composition and extensive pharmacological properties. This indigenous fruit, deeply rooted in traditional medicine, has proven to be a valuable source of

bioactive compounds that hold immense potential for various therapeutic applications. The phytochemical analysis of Phyllanthus emblica revealed a rich array of secondary metabolites, including flavonoids, tannins, polyphenols, and ascorbic acid. These compounds collectively contribute to the antioxidant, anti-inflammatory, and antimicrobial activities of the plant, making it a promising candidate for the development of natural remedies and pharmaceutical agents. Regarding its pharmacological properties, Phyllanthus emblica has exhibited a wide spectrum of effects, ranging from its hepatoprotective and immunomodulatory actions to its antidiabetic, anticancer, and cardioprotective potentials. The extensive research conducted in this area has highlighted the versatility of this botanical treasure, providing avenues for future investigations and applications in the realm of medicine and healthcare. However, it is essential to acknowledge the limitations of our review. Firstly, while we have compiled a comprehensive overview of the available literature, the field of *P. emblica* research is continuously evolving, and new discoveries may have emerged since the completion of this review. Additionally, the majority of studies have been conducted *in vitro* or in animal models, necessitating further clinical trials to establish the efficacy and safety of Phyllanthus emblica-based interventions in humans. Moreover, variations in the phytochemical composition of *P. emblica* due to geographical and environmental factors pose a challenge in standardizing its therapeutic applications. *Phyllanthus emblica* holds great promise as a source of bioactive compounds with diverse pharmacological properties. While this review serves as a valuable resource for understanding its potential benefits, further research and clinical investigations are warranted to fully harness the therapeutic potential of this remarkable botanical species. Despite the limitations, the compelling evidence presented in this review underscores the significance of Phyllanthus emblica in the realm of natural medicine and inspires continued exploration in the pursuit of improved healthcare solutions.

Author contributions

AP: Funding acquisition, Methodology, Writing–original draft. AD: Software, Supervision, Writing–original draft. UH: Conceptualization, Visualization, Writing–original draft. YS: Formal analysis, Writing–review and editing. EP: Validation, Writing–review and editing. NK: Investigation, Writing–original draft. PH: Formal analysis, Writing–review and editing. RS: Conceptualization, Data-curation, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. PS: Formal analysis, Writing–original draft. FN: Data-curation, Formal analysis, Supervision, Visualization, Writing–review and editing.

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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.

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References

Abdel-Hady, H., Abdallah morsi, E., and Ahmed El-wakil, E. (2022). *In-vitro* antimicrobial potentialities of phylunthus emblica leaf extract against some human pathogens. *Egypt. J. Chem.* 65 (7), 705. doi:10.21608/EJCHEM.2021.109577.4998

Acharya, C. D. (2016). Ethnicity and Scientific validation of West Bengal Amla (*Phyllanthus emblica* L.) with special reference to GC-MS screening. *Int. J. Exp. Res. Rev.* 3 (1), 54. doi:10.52756/ijerr.2016.v03.006

Afrin, F., Banik, S., and Hossain, M. S. (2016). Pharmacological activities of methanol extract of Phyllanthus acidus pulp. *J. Med. Plants Res.* 10 (43), 790–795. doi:10.5897/JMPR2015.5806

Ahmad, B., Hafeez, N., Rauf, A., Bashir, S., Linfang, H., Rehman, M. U., et al. (2021). *Phyllanthus emblica*: a comprehensive review of its therapeutic benefits. *South Afr. J. Bot.* 138, 278–310. doi:10.1016/j.sajb.2020.12.028

Akter, R., Khan, S. S., Kabir, M. T., and Halder, S. (2022). GC-MS-employed phytochemical characterization, synergistic antioxidant, and cytotoxic potential of Triphala methanol extract at non-equivalent ratios of its constituents. *Saudi J. Biol. Sci.* 29 (1), 103287. doi:10.1016/j.sjbs.2022.103287

Amir, D. (2014). Composition of the essential oil of the fruits of *Phyllanthus emblica* cultivated in Egypt. *J. Pharm. Chem. Biol. Sci.* 2 (3), 205.

Ansari, P., Akther, S., Hannan, J. M. A., Seidel, V., Nujat, N. J., and Abdel-Wahab, Y. H. (2022). Pharmacologically active phytomolecules isolated from traditional antidiabetic plants and their therapeutic role for the management of diabetes mellitus. *Molecules* 27 (13), 4278. doi:10.3390/molecules27134278

Anto, E. J., Syahputra, R. A., Silitonga, H. A., Situmorang, P. C., and Nugaraha, S. E. (2022). Oral acute toxicity study extract ethanol of balakka fruit (*Phyllanthus emblica*). *Pharmacia* 69 (1), 187–194. doi:10.3897/pharmacia.69.e81280

Asmilia, N., Fahrimal, Y., Abrar, M., and Rinidar, R. (2020). Chemical compounds of Malacca leaf(*Phyllanthus emblica*) after triple extraction with N-hexane, ethyl acetate, and ethanol. *e Sci. World J.* 1 (1), 2739056. doi:10.1155/2020/2739056

Bajgai, T. R., Hashinaga, F., Isobe, S., Raghavan, G. V., and Ngadi, M. O. (2006). Application of high electric field (HEF) on the shelf-life extension of emblic fruit (*Phyllanthus emblica* L.). *J. Food Eng.* 74 (3), 308–313. doi:10.1016/j.jfoodeng.2005.03.023

Bakr, E. H., and Naga, M. (2020). Immunomodulatory efficacy of *Phyllanthus emblica* and Costus speciosus aqueous extracts for immunosuppressive rats. *Egypt. J. Nutr.* 35 (2), 101–123. doi:10.21608/ENJ.2020.144766

Balusamy, S. R., Veerappan, K., and Ranjanm, A. (2019). *Phyllanthus emblica* fruit extract attenuates lipid metabolism in 3T3-L1 adipocytes via activating apoptosis mediated cell death. *J. Pre-proof* 1 (1), 6. doi:10.1016/j.phymed.2019.153129

Bashir, A. S. I. F. A., Mushtaq, A. A. M. I. R., and Mehboob, T. O. O. B. A. (2018). Evaluation of antioxidant and antidiabetic activity of Phyllanthus emblica (fruit). *Biol. Pak.* 64 (1), 85–91.

Bayat, P., Farshchi, M., Yousefian, M., Mahmoudi, M., and Yazdian-Robati, R. (2021). Flavonoids, the compounds with anti-inflammatory and immunomodulatory properties, as promising tools in multiple sclerosis (MS) therapy: a systematic review of preclinical evidence. *Int. Immunopharmacol.* 95, 107562. doi:10.1016/j.intimp.2021.107562

Bitew, M., Desalegn, T., Demissie, T. B., Belayneh, A., Endale, M., and Eswaramoorthy, R. (2021). Pharmacokinetics and drug-likeness of antidiabetic flavonoids: molecular docking and DFT study. *Plos one* 16 (12), e0260853. doi:10.1371/journal.pone.0260853

Bruno, M. (1984). Raw materials, profits, and the productivity slowdown. *Q. J. Econ.* 99 (1), 1–29. doi:10.2307/1885718

Calderaro, A., Patanè, G. T., Tellone, E., Barreca, D., Ficarra, S., Misiti, F., et al. (2022). The neuroprotective potentiality of flavonoids on alzheimer's disease. *Int. J. Mol. Sci.* 23 (23), 14835. doi:10.3390/ijms232314835

Chanda, S., Biswas, S. M., and Sarkar, P. K. (2020). Phytochemicals and antiviral properties of five dominant medicinal plant species in Bankura district, West Bengal: an overview. *J. Pharmacogn. Phytochemistry* 9 (6), 1420–1427. doi:10.22271/phyto.2020.v9.i6u.13146

Chaphalkar, R., Apte, K. G., Talekar, Y., Ojha, S. K., and Nandave, M. (2017). Antioxidants of *Phyllanthus emblica* L. bark extract provide hepatoprotection against ethanol-induced hepatic damage: a comparison with silymarin. *Oxidative Med. Cell. Longev.* 2017, 3876040. doi:10.1155/2017/3876040

Chekdaengphanao, P., Jaiseri, D., Sriraj, P., Aukkanimart, R., Prathumtet, J., Udonsan, P., et al. (2022). Anticancer activity of Terminalia chebula, Terminalia bellirica, and *Phyllanthus emblica* extracts on cholangiocarcinoma cell proliferation and induction of apoptosis. *J. Herb. Med.* 35, 100582. doi:10.1016/j.hermed.2022.100582

Chopra, R. N. (1958). *Chopra's indigenous drugs of India, 284*. Academic Publishers, 1994, 503–505.

Dajas, F., Rivera, F., Blasina, F., Arredondo, F., Echeverry, C., Lafon, L., et al. (2003). Cell culture protection and *in vivo* neuroprotective capacity of flavonoids. *Neurotox. Res.* 5, 425–432. doi:10.1007/BF03033172

Dinesh, M., Roopan, S. M., and Selvaraj, C. I. (2016). Photocatalytic degradation of nitrophenol using biologically active *Phyllanthus emblica* seed extract. *J. Photochem. Photobiol.* 1 (1), 273–278. doi:10.1016/j.jphotobiol.2016.05.033

Dinesh, M., Roopan, S. M., Selvaraj, C. I., and Arunachalam, P. (2017). *Phyllanthus emblica* seed extract mediated synthesis of PdNPs against antibacterial, heamolytic and cytotoxic studies. *J. Photochem. Photobiol. B Biol.* 167, 64–71. doi:10.1016/j.jphotobiol.2016.12.012

Elangovan, M., and Irulappan, E. (2015). Determination of bioactive compounds from the petroleum ether leaf extract of moringa oleifera and *Phyllnthus emblica* using GC-MS analysis. *World J. Pharm. Res.* 4 (3), 1290.

Farhana, F., Mosaddek, A. S. M., Joynal, B. J., Sharmin, H., and Mosaddek, N. (2022). Antibacterial effect of Amlaki (Phyllanthus emblica) extract against *Pseudomonas aeruginosa*. *J. Clin. images Med. case Rep.* 3, 6. doi:10.52768/2766-7820/1886

Ferraz, C. R., Carvalho, T. T., Manchope, M. F., Artero, N. A., Rasquel-Oliveira, F. S., Fattori, V., et al. (2020). Therapeutic potential of flavonoids in pain and inflammation: mechanisms of action, pre-clinical and clinical data, and pharmaceutical development. *Molecules* 25 (3), 762. doi:10.3390/molecules25030762

Gaire, B. P., and Subedi, L. (2014). Phytochemistry, pharmacology and medicinal properties of *Phyllanthus emblica* Linn. *Chin. J. Integr. Med.* 2, 014. doi:10.1007/s11655-014-1984-2

Gantait, S., Mahanta, M., Bera, S., and Verma, S. K. (2021). Advances in biotechnology of Emblica officinalis Gaertn. syn. *Phyllanthus emblica* L.: a nutraceuticals-rich fruit tree with multifaceted ethnomedicinal uses. *3 Biotech.* 11, 62–25. doi:10.1007/s13205-020-02615-5

Ghaffari, S., Navabzadeh, M., Ziaee, M., Ghobadi, A., Ghods, R., and Hashem-Dabaghian, F. (2020). A randomized, triple-blind, placebo-controlled, add-on clinical trial to evaluate the efficacy of Emblica officinalis in uncontrolled hypertension. *Evidence-Based Complementary Altern. Med.* 2020, 8592869. doi:10.1155/2020/8592869

Ghosal, S. (1996). Active constituents of Emblica officinalis: Part I. The chemistry and antioxidative effects of two new hydrolysable tannins. *Emblicanin A B. Indian J. Chem.* 35, 941–948. doi:10.1002/chin.199647279

Ghosh, A. R. K. A., Laloo, D. A. M. I. K. I., and Singh, N. K. (2013). Comparative estimation and chemical standardization of new and old sample of Chyawanprash. *Int. J. Pharm. Pharm. Sci.* 5, 801–804.

Golechha, M., Sarangal, V., Ojha, S., Bhatia, J., and Arya, D. S. (2014). Anti-inflammatory effect of Emblica officinalis in rodent models of acute and chronic inflammation: involvement of possible mechanisms. *Int. J. Inflamm.* 2014, 178408. doi:10.1155/2014/178408

Gong, X., Li, X., Xia, Y., Xu, J., Li, Q., Zhang, C., et al. (2020). Effects of phytochemicals from plant-based functional foods on hyperlipidemia and their underpinning mechanisms. *Trends food Sci. Technol.* 103, 304–320. doi:10.1016/j.tifs.2020.07.026

Gul, M., Liu, Z. W., Rabail, R., Faheem, F., Walayat, N., Nawaz, A., et al. (2022). Functional and nutraceutical significance of Amla (*Phyllanthus emblica* L.): a review. *Antioxidants* 11 (5), 816. doi:10.3390/antiox11050816

Halim, B., Syahputra, R. A., Adenin, I., Lubis, H., Mendrofa, F., Lie, S., et al. (2022). Determination of phytochemical constituent, antioxidant activity, total phenol and total flavonoid of extract ethanol *Phyllanthus emblica* fruit. *Pharmacogn. J.* 14 (1), 63–67. doi:10.5530/pj.2022.14.9

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

Harahap, F. S., Atifah, Y., and Ginting, N. (2019). Comparison of the chemical compounds of Malacca bark and Malacca fruit (*Phyllanthus emblica*) with gas chromatography-mass spectrometer (GC-MS). *J. Phys. Conf. Ser.* 1 (1), 4. doi:10.1088/1742-6596/1477/7/072011

Huang, C. Z., Tung, Y. T., Hsia, S. M., Wu, C. H., and Yen, G. C. (2017). The hepatoprotective effect of Phyllanthus emblica L. fruit on high fat diet-induced non-alcoholic fatty liver disease (NAFLD) in SD rats. *Food & Funct.* 8 (2), 842–850. doi:10.1039/C6FO01585A

Huang, Y. N., Chen, S. Y., Lin, J. A., Chiang, I. C., and Yen, G. C. (2023). *Phyllanthus emblica* L. extract alleviates leptin resistance and lipid accumulation by inhibiting methylglyoxal production. *Food Biosci.* 53, 102619. doi:10.1016/j.fbio.2023.102619

Hussain, N., Kakoti, B. B., Rudrapal, M., Sarwa, K. K., Celik, I., Attah, E. I., et al. (2021a). Bioactive antidiabetic flavonoids from the stem bark of *Cordia dichotoma* Forst.: identification, docking and ADMET studies. *Molbank* 2021 (2), M1234. doi:10.3390/m1234

Hussain, S. Z., Naseer, B., Qadri, T., Fatima, T., and Bhat, T. A. (2021b). "Anola (emblica officinalis): morphology, taxonomy, composition and health benefits," in *Fruits grown in highland regions of the himalayas: nutritional and health benefits* (Cham: Springer International Publishing), 193–206.

Hwang, S. L., Shih, P. H., and Yen, G. C. (2012). Neuroprotective effects of citrus flavonoids. *J. Agric. Food Chem.* 60 (4), 877–885. doi:10.1021/jf204452y

Iamsaard, S., Arun, S., Burawat, J., Sukhorum, W., Wattanathorn, J., Nualkaew, S., et al. (2014). Phenolic contents and antioxidant capacities of Thai-Makham Pom (*Phyllanthus emblica* L.) aqueous extracts. *J. Zhejiang Univ. Sci. B* 15 (4), 405–408. doi:10.1631/jzus.B1300284

Islam, M. R., Akash, S., Jony, M. H., Alam, M. N., Nowrin, F. T., Rahman, M. M., et al. (2023). Identifying novel therapeutic inhibitors to target FMS-like tyrosine kinase-3 (FLT3) against acute myeloid leukemia: a molecular docking, molecular dynamics, and DFT study. *Mol. Cell. Biochem.* 7, 12–16. doi:10.1080/07391102.2023.2192798

Jagdale, Y. D., Mahale, S. V., Zohra, B., Nayik, G. A., Dar, A. H., Khan, K. A., et al. (2021). Nutritional profile and potential health benefits of super foods: a review. *Sustainability* 13 (16), 9240. doi:10.3390/su13169240

Jahan, N., and Akter, S. (2015). Assessment of the antimicrobial activity of the ethanolic extract of Phyllanthus emblica in combination with different classes of antibiotics against single and multi-drug resistant strains. *J. Pharmacogn. Phytochemistry* 4 (4), 142–155.

Jantan, I., Haque, M. A., Ilangkovan, M., and Arshad, L. (2019). An insight into the modulatory effects and mechanisms of action of phyllanthus species and their bioactive metabolites on the immune system. *Front. Pharmacol.* 10, 878. doi:10.3389/fphar.2019.00878

Jubaidi, F. F., Zainalabidin, S., Taib, I. S., Hamid, Z. A., and Budin, S. B. (2021). The potential role of flavonoids in ameliorating diabetic cardiomyopathy via alleviation of cardiac oxidative stress, inflammation and apoptosis. *Int. J. Mol. Sci.* 22 (10), 5094. doi:10.3390/ijms22105094

Kalaitzoglou, E., Fowlkes, J. L., Popescu, I., and Thrailkill, K. M. (2019). Diabetes pharmacotherapy and effects on the musculoskeletal system. *Diabetes/metabolism Res. Rev.* 35 (2), e3100. doi:10.1002/dmrr.3100

Khaled, S. E., Hashem, F. A., and Shabana, M. H. (2018). A biochemometric approach for the assessment of *Phyllanthus emblica* female fertility effects as determined via UPLC-ESI-qTOFMS and GC-MS. *food Funct.* 1 (1), 9. doi:10.1039/c9fo00767a

Kiran, K. R., Swathy, P. S., Paul, B., Shama Prasada, K., Radhakrishna Rao, M., Joshi, M. B., et al. (2021). Untargeted metabolomics and DNA barcoding for discrimination of Phyllanthus species. *J. Ethnopharmacol.* 1 (1), 113928. doi:10.1016/j.jep.2021.113928

Kumar, S., Chandra, P., Bajpai, V., Singh, A., Srivastava, M., Mishra, D., et al. (2015). Rapid qualitative and quantitative analysis of bioactive compounds from Phyllanthus amarus using LC/MS/MS technique, s. *Industrial Crops Prod.* 69 (1), 143–152. doi:10.1016/j.indcrop.2015.02.012

Kumar, S., Singh, A., and Bajpai, V. (2017c). Development of a UHPLC–MS/McS method for the quantitation of bioactive compounds in Phyllanthus species and its herbal formulations. *J. Sep. Sci.* 1 (1), 5. doi:10.1002/jssc.201601361

Kumar, S., Singh, A., and Kumar, C. (2017b). Identification and characterization of phenolics and terpenoids from ethanolic extracts of Phyllanthus species by HPLC-ESI-QTOF-MS/MS. *J. Pharm. Analysis* 7 (1), 214–222. doi:10.1016/j.jpha.2017.01.005

Kumar, V., Aneesh, K. A., Kshemada, K., Ajith, K. G. S., Binil, R. S. S., Deora, N., et al. (2017a). Amalaki rasayana, a traditional Indian drug enhances cardiac mitochondrial and contractile functions and improves cardiac function in rats with hypertrophy. *Scientific* 1 (1), 8588. doi:10.1038/s41598-017-09225-x

Kumnerdkhonkaen, P., Saenglee, S., Asgar, M. A., Senawong, G., Khongsukwiwat, K., and Senawong, T. (2018). Antiproliferative activities and phenolic acid content of water and ethanolic extracts of the powdered formula of Houttuynia cordata Thunb. fermented broth and *Phyllanthus emblica* Linn. fruit. *BMC Complementary Altern. Med.* 18 (130), 130. doi:10.1186/s12906-018-2185-x

Li, C. H. E. N., Xin-Bo, M. A., Liang, Y. H., Shi-Cheng, P. E. I., Yi-Ping, F. E. N. G., and Min, W. E. I. (2011). Effects of persimmon leaf total flavonoid on enzyme of lipoprotein

metabolism and antioxidation in hyperlipidemia rats. *Chin. J. Nat. Med.* 9 (1), 74–77. doi:10.1016/s1875-5364(11)60024-1

Li, P. H., Wang, C. W., Lu, W. C., Song, T. Y., and Wang, C. C. (2022). Antioxidant, anti-inflammatory activities, and neuroprotective behaviors of *Phyllanthus emblica* L. Fruit extracts. *Agriculture* 12 (5), 588. doi:10.3390/agriculture12050588

Li, W., Zhu, H. W., Chen, Y. J., Xiao, H., Ge, Y. Z., Hu, H. E., et al. (2020). Bioactivity-guided isolation of anti-inflammatory components from *Phyllanthus emblica*. *Food Sci. Nutr.* 8 (6), 2670–2679. doi:10.1002/fsn3.1553

Li, Y., Guo, B., Wang, W., Li, L., Cao, L., Yang, C., et al. (2019). Characterization of phenolic compounds from *Phyllanthus emblica* fruits using HPLC-ESI-TOF-MS as affected by an optimized microwave-assisted extraction. *Int. J. Food Prop.* 22 (1), 330–342. doi:10.1080/10942912.2019.1583249

Luo, X., Zhang, B., Pan, Y., Gu, J., Tan, R., and Gong, P. (2022). *Phyllanthus emblica* aqueous extract retards hepatic steatosis and fibrosis in NAFLD mice in association with the reshaping of intestinal microecology. *Front. Pharmacol.* 1 (1), 893561. doi:10.3389/fphar.2022.893561

Lv, J. J., Wang, Y. F., Zhang, J. M., Yu, S., Wang, D., Zhu, H. T., et al. (2014). Anti-hepatitis B virus activities and absolute configurations of sesquiterpenoid glycosides from *Phyllanthus emblica*. *Org. Biomol. Chem.* 12 (43), 8764–8774. doi:10.1039/c4ob01196a

Lv, J. J., Yu, S., Xin, Y., Cheng, R. R., Zhu, H. T., Wang, D., et al. (2015). Anti-viral and cytotoxic norbisabolane sesquiterpenoid glycosides from *Phyllanthus emblica* and their absolute configurations. *Phytochemistry* 117, 123–134. doi:10.1016/j.phytochem.2015.06.001

Mahata, S., Pandey, A., Shukla, S., Tyagi, A., Husain, S. A., Das, B. C., et al. (2013). Anticancer activity of *Phyllanthus emblica* Linn (Indian gooseberry): inhibition of transcription factor AP-1 and HPV gene expression in cervical cancer cells. *Nutr. cancer* 65 (1), 88–97. doi:10.1080/01635581.2013.785008

Mal, A., and Meena, D. S. (2022). *Phyllanthus emblica*: a herbal remedy for healthy life. *ECS Trans.* 107 (1), 3199–3206. doi:10.1149/10701.3199ecst

Masad, R. J., Haneefa, S. M., Mohamed, Y. A., Al-Sbiei, A., Bashir, G., Fernandez-Cabezudo, M. J., et al. (2021). The immunomodulatory effects of honey and associated flavonoids in cancer. *Nutrients* 13 (4), 1269. doi:10.3390/nu13041269

Mayakrishnan, V., Kannappan, P., Abdullah, N., and Ahmed, A. B. A. (2013). Cardioprotective activity of polysaccharides derived from marine algae: an overview. *Trends Food Sci. Technol.* 30 (2), 98–104. doi:10.1016/j.tifs.2013.01.007

Mehmood, M. H., Rehman, A., Rehman, N. U., and Gilani, A. H. (2013). Studies on prokinetic, laxative and spasmodic activities of Phyllanthus emblica in experimental animals. *Phytotherapy Res.* 27 (7), 1054–1060. doi:10.1002/ptr.4821

Middha, S. K., Goyal, A. K., Lokesh, P., Yardi, V., Mojamdar, L., Keni, D. S., et al. (2015). Toxicological evaluation of Emblica officinalis fruit extract and its anti-inflammatory and free radical scavenging properties. *Pharmacogn. Mag.* 11 (Suppl. 3), S427. doi:10.4103/0973-1296.168982

Muthusamy, A., Sanjay, E. R., and Prasad, N. (2017). Quantitative analysis of Phyllanthus species for bioactive molecules using high-pressure liquid chromatography and liquid chromatography–mass spectrometry. *Quantitative Analysis Phyllanthus* 1 (1), 2. doi:10.1007/s40011-017-0839-y

Naik, J., and David, M. (2023). Phytofabrication of silver and zinc oxide nanoparticles using the fruit extract of *Phyllanthus emblica* and its potential anti-diabetic and anti-cancer activity. *Part. Sci. Technol.* 41 (6), 761–773. doi:10.1080/02726351.2022.2141668

Nair, A., Balasaravanan, T., Jadhav, S., Mohan, V., and Kumar, C. (2020). Harnessing the antibacterial activity of Quercus infectoria and *Phyllanthus emblica* against antibiotic-resistant Salmonella Typhi and Salmonella Enteritidis of poultry origin. *Veterinary World* 13 (1), 1388–1396. doi:10.14202/vetworld.2020.1388-1396

Ngamkitidechakul, C., Jaijoy, K., Hansakul, P., Soonthornchareonnon, N., and Sireeratawong, S. (2010). Antitumour effects of *Phyllanthus emblica* L.: induction of cancer cell apoptosis and inhibition of *in vivo* tumour promotion and *in vitro* invasion of human cancer cells. *Phytotherapy Res.* 24 (9), 1405–1413. doi:10.1002/ptr.3127

Nirala, R. K., Raj, P., Anjana, K., and Mandal, K. G. (2020). A review on immunomodulatory activity of amla and Aloe vera. *J. Pharmacogn. Phytochemistry* 9 (5), 2014–2016.

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

Paik, J. M., Golabi, P., Younossi, Y., Mishra, A., and Younossi, Z. M. (2020). Changes in the global burden of chronic liver diseases from 2012 to 2017: the growing impact of NAFLD. *Hepatology* 72 (5), 1605–1616. doi:10.1002/hep.31173

Pramyothin, P., Samosorn, P., Poungshompoo, S., and Chaichantipyuth, C. (2006). The protective effects of *Phyllanthus emblica* Linn. extract on ethanol induced rat hepatic injury. *J. Ethnopharmacol.* 107 (3), 361–364. doi:10.1016/j.jep.2006.03.035

Priya, F. F., and Islam, M. S. (2019). Phyllanthus emblica Linn.(Amla)—a natural gift to humans: an overview. *J. Dis. Med. Plants* 5, 1–9. doi:10.11648/j.jdmp.20190501.11

Quranayati, Q., Iqhrammullah, M., Saidi, N., Nurliana, N., Idroes, R., and Nasution, R. (2022). Cytotoxicity and phytochemical profiles of *Phyllanthus emblica* stem barks with *in silico* drug-likeliness: focusing on antidiabetic potentials. *J. Adv. Pharm. Technol. Res.* 13 (4), 281–285. doi:10.4103/japtr.japtr_319_22

Quranayati, Q., Saidi, N., Nurliana, N., Idroes, R., Gusti, N., and Nasution, R. (2023). Effect of *Phyllanthus emblica* L. stem bark extract on diabetic nephropathy and hyperlipidemia in rats. *J. Pharm. Pharmacogn. Res.* 11 (2), 308–314. doi:10.56499/jppres23.1595_11.2.308

Rajak, S., Banerjee, S. K., Sood, S., Dinda, A. K., Gupta, Y. K., Gupta, S. K., et al. (2004). Emblica officinalis causes myocardial adaptation and protects against oxidative stress in ischemic-reperfusion injury in ratsficinalis causes myocardial adaptation and protects against oxidative stress in ischemic-reperfusion injury in rats. *Phytotherapy Res. Int. J. Devoted Pharmacol. Toxicol. Eval. Nat. Prod. Deriv.* 18 (1), 54–60. doi:10.1002/ptr.1367

Rajalakshmi, S., Vijayakumar, S., and Praseetha, P. K. (2019). Neuroprotective behaviour of *Phyllanthus emblica* (L) on human neural cell lineage (PC12) against glutamate-induced cytotoxicity. *Gene Rep.* 17, 100545. doi:10.1016/j.genrep.2019.100545

Rakha, A., Umar, N., Rabail, R., Butt, M. S., Kieliszek, M., Hassoun, A., et al. (2022). Anti-inflammatory and anti-allergic potential of dietary flavonoids: a review. *Biomed. Pharmacother.* 156, 113945. doi:10.1016/j.biopha.2022.113945

Ramasamy, S., Abdul Wahab, N., Zainal Abidin, N., Manickam, S., and Zakaria, Z. (2012). Growth inhibition of human gynecologic and colon cancer cells by Phyllanthus watsonii through apoptosis induction. *PLoS One* 7 (4), e34793. doi:10.1371/journal.pone.0034793

Saini, R., Sharma, N., Oladeji, O. S., Sourirajan, A., Dev, K., Zengin, G., et al. (2022). Traditional uses, bioactive composition, pharmacology, and toxicology of *Phyllanthus emblica* fruits: a comprehensive review. *J. Ethnopharmacol.* 282, 114570. doi:10.1016/j.jep.2021.114570

Samatiwat, P., Tabtimmai, L., Suphakun, P., Jiwacharoenchai, N., Toviwek, B., Kukongviriyapan, V., et al. (2021). The effect of the EGFR-targeting compound 3-[(4-Phenylpyrimidin-2-yl) amino] benzene-1-sulfonamide (13f) against cholangiocarcinoma cell lines. *Asian Pac. J. Cancer Prev. APJCP* 22 (2), 381. doi:10.31557/APJCP.2021.22.2.381

Santoshkumar, J., Devarmani, M. S., Sajjanar, M., Pranavakumar, M. S., and Dass, P. J. M. I. (2013). A study of anti-inflammatory activity of fruit of Emblica officinalis (Amla) in Albino rats. *Medica Innov.* 2 (1), 17–26.

Shamsudin, N. F., Ahmed, Q. U., Mahmood, S., Shah, S. A. A., Sarian, M. N., Khattak, M. M. A. K., et al. (2022). Flavonoids as antidiabetic and anti-inflammatory agents: a review on structural activity relationship-based studies and meta-analysis. *Int. J. Mol. Sci.* 23 (20), 12605. doi:10.3390/ijms232012605

Sheoran, S., Nidhi, P., Kumar, V., Singh, G., Lal, U. R., Sourirajan, A., et al. (2019). Altitudinal variation in gallic acid content in fruits of *Phyllanthus emblica* L. and its correlation with antioxidant and antimicrobial activity. *Vegetos* 32, 387–396. doi:10.1007/s42535-019-00048-x

Sobhani, Z., Reza Nami, S., Ahmad Emami, S., Sahebkar, A., and Javadi, B. (2017). Medicinal plants targeting cardiovascular diseases in view of Avicenna. *Curr. Pharm. Des.* 23 (17), 2428–2443. doi:10.2174/1381612823666170215104101

Summanen, J. O. (1999). A chemical and ethnopharmacological study on Phyllanthus emblica L.(Euphorbiaceae). *Zhongguo Zhong Yao Za Zhi* 22, 515–8.

Sun, J., Wang, Z., Chen, L., and Sun, G. (2021). Hypolipidemic effects and preliminary mechanism of chrysanthemum flavonoids, its main components luteolin and luteoloside in hyperlipidemia rats. *Antioxidants* 10 (8), 13CC09. doi:10.3390/antiox10081309

Tahir, I., Khan, M. R., Shah, N. A., and Aftab, M. (2016). Evaluation of phytochemicals, antioxidant activity and amelioration of pulmonary fibrosis with *Phyllanthus emblica* leaves. *BMC complementary Altern. Med.* 16, 406–41C2. doi:10.1186/s12906-016-1387-3

Thiennimitr, P., Yasom, S., Tunapong, W., Chunchai, T., Wanchai, K., Pongchaidecha, A., et al. (2018). Lactobacillus paracasei HII01, xylooligosaccharides, and synbiotics reduce gut disturbance in obese rats. *Nutrition* 54, 40–47. doi:10.1016/j.nut.2018.03.005

Tung, Y. T., Huang, C. Z., Lin, J. H., and Yen, G. C. (2018). Effect of *Phyllanthus emblica* L. fruit on methionine and choline-deficiency diet-induced nonalcoholic steatohepatitis. *J. food drug analysis* 26 (4), 1245–1252. doi:10.1016/j.jfda.2017.12.005

Uddin, M. S., Mamun, A. A., Hossain, M. S., Akter, F., Iqbal, M. A., and Asaduzzaman, M. (2016). Exploring the effect of Phyllanthus emblica L. on cognitive performance, brain antioxidant markers and acetylcholinesterase activity in rats: promising natural gift for the mitigation of Alzheimer's disease. *Ann. Neurosci.* 23 (4), 218–229. doi:10.1159/000449482

Usharani, P., Fatima, N., and Muralidhar, N. (2013). Effects of *Phyllanthus emblica* extract on endothelial dysfunction and biomarkers of oxidative stress in patients with type 2 diabetes mellitus: a randomized, double-blind, controlled study. *Diabetes, Metabolic Syndrome Obes. Targets Ther.* 6, 275–284. doi:10.2147/DMSO.S46341

Variya, B. C., Bakrania, A. K., and Patel, S. S. (2016). Emblica officinalis (Amla): a review for its phytochemistry, ethnomedicinal uses and medicinal potentials with respect to molecular mechanisms. *Pharmacol. Res.* 111, 180–200. doi:10.1016/j.phrs.2016.06.013

Vauzour, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C., and Spencer, J. P. (2008). The neuroprotective potential of flavonoids: a multiplicity of effects. *Genes. & Nutr.* 3 (3), 115–126. doi:10.1007/s12263-008-0091-4

Wang, H. M. D., Fu, L., Cheng, C. C., Gao, R., Lin, M. Y., Su, H. L., et al. (2019). Inhibition of LPS-induced oxidative damages and potential anti-inflammatory effects of *Phyllanthus emblica* extract via down-regulating NF-κB, COX-2, and iNOS in RAW 264.7 cells. *Antioxidants* 8 (8), 270. doi:10.3390/antiox8080270

Wu, M., Cai, J., Fang, Z., Li, S., Huang, Z., Tang, Z., et al. (2022b). The composition and anti-aging activities of polyphenol extract from *Phyllanthus emblica* L. fruit. *Nutrients* 14 (4), 857. doi:10.3390/nu14040857

Wu, T., Liu, X., and Sun, Z. (2022a). Fruit of Phyllanthus emblica L. suppresses macrophage foam-cell genesis and vascular lipid deposition using *in vivo* and *in vitro* models of early atherosclerosis development. *Food Sci. Technol. Res.* 28 (4), 325. doi:10.3136/fstr.FSTR-D-22-00002

Yang, B., Kortesniemi, M., Liu, P., Karonen, M., and Salminen, J. P. (2012). Analysis of hydrolyzable tannins and other phenolic compounds in emblic leafflower (*Phyllanthus emblica* L.) fruits by high performance liquid Chromatography–Electrospray ionization mass spectrometry. *J. Agric. Food Chem.* 1 (1), 8672–8683. doi:10.1021/jf302925v

Yang, B., and Liu, P. (2014). Composition and biological activities of hydrolyzable tannins of fruits of *Phyllanthus emblica*. *J. Agric. food Chem.* 62 (3), 529–541. doi:10.1021/jf404703k

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), 1017. doi:10.3390/molecules25041017

Zhang, Y., Zhao, L., Guo, X., Li, C., Li, H., Lou, H., et al. (2016). Chemical constituents from *Phyllanthus emblica* and the cytoprotective effects on H 2 O 2-induced PC12 cell injuries. *Archives Pharmacal Res.* 39, 1202–1211. doi:10.1007/s12272-014-0433-2

Zhao, T., Sun, Q., Marques, M., and Witcher, M. (2015). Anticancer properties of Phyllanthus emblica (Indian gooseberry). *Oxid. Med. Cell. Longev.* 2015, 950890. doi:10.1155/2015/950890

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Serum, spleen metabolomics and gut microbiota reveals effect of catalpol on blood deficiency syndrome caused by cyclophosphamide and acetylphenylhydrazine

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Catalpol (CA), extracted from *Rehmannia Radix*, holds extensive promise as a natural medicinal compound. This study employed 16S rRNA gene sequencing and combined serum and spleen metabolomics to profoundly investigate the therapeutic effects of CA on blood deficiency syndrome (BDS) and the underlying mechanisms. Notably, CA exhibited effectiveness against BDS induced by cyclophosphamide (CP) and acetylphenylhydrazine (APH) in rats-CA substantially elevated levels of crucial indicators such as erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor-alpha (TNF-a), and interleukin-6 (IL-6). Additionally, CA could alleviate peripheral blood cytopenia. Furthermore, the analysis of 16S rRNA revealed that CA had the potential to reverse the Firmicutes/Bacteroidetes (F/B) ratio associated with BDS. Through comprehensive serum and spleen metabolomic profiling, we successfully identified 22 significant biomarkers in the serum and 23 in the spleen, respectively. Enrichment analysis underscored Glycerophospholipid metabolism and Sphingolipid metabolism as potential pathways through which CA exerts its therapeutic effects on BDS.

KEYWORDS

metabolomics, gut microbiota, catalpol, cyclophosphamide, rehmannia radix

Abbreviations: CP, cyclophosphamide; APH, Acetylphenylhydrazine; SD, Standard deviation; BDS, Blood deficiency syndrome; CA, Catalpol; KEGG, Kyoto Encyclopedia of Genes and Genomes; VIP, Variable importance in the projection; RBC, Red blood cells; WBC, White blood cells; HGB, Hemoglobin; HCT, Hematocrit; Rt, Retention time; SphK1, Sphingosine in the presence of sphingosine kinase 1; LPC, lysophosphatidylcholine; Mfsd2b, Major facilitator superfamily transporter 2b; S1P, Sphingosine-1-phosphate.

1 Introduction

Blood deficiency is a prevalent syndrome in clinical medicine, attributed to causes such as substantial blood loss, nutritional insufficiencies, inadequate hematogenesis, and iron depletion (1). In a modern medical context, it refers to the decrease in hemoglobin concentration and the blood pancytopenia. Traditional Chinese medicine (TCM) characterizes a pathological condition involving blood dysfunction and organ deterioration (2). Acetylphenylhydrazine (APH) is a potent oxidantthat gradually inflicts oxidative damage to red blood cells (RBC), leading to hemolytic anemia (3). In the field of cancer treatment, chemotherapy stands as the prevailing approach. Cyclophosphamide (CP), a broad-spectrum anticancer agent, exerts formidable cytotoxic effects on hematopoietic stem cells both in the bone marrow and circulating peripheral blood, culminating in anemia (due to hematopoietic inhibition) and compromised immune function (4). Commonly, CP, combined with antibiotic treatment, is used in cancer therapy due to the complications associated with bacterial translocation and infections caused by the mucosal barrier disruption (5). Chemotherapy alone alters the fecal microbiota in patients, including decrease species richness and absolute bacterial load (6). Consequently, an urgent need arises to identify a therapeutic agent capable of alleviating blood deficiency induced by chemotherapeutic interventions. A recent investigation established a hemolytic and aplastic anemia model using APH in combination with CP (7). This model closely mirrors the *in vivo* conditions characteristic of blood deficiency syndrome (BDS) in clinical scenarios.

Rehmanniae Radix (RR) is a TCM distributed in many provinces, such as Henan, Hebei, and Shanxi in China (8). Many clinical and experimental studies have shown that RR is an important TCM for treating bone loss by improving bone mineral density in patients with osteoporosis (9). RR's steamed product, *Rehmanniae Radix* Praeparata (RRP), historically known for its " nourish the bone marrow " properties, has attracted contemporary attention for its bioactive ingredients, such as catalpol (CA; 10). Relevant pharmacokinetic studies have shown that CA can pass the blood-brain barrier, has the potential for oral administration, can be rapidly absorbed, and exhibits higher absolute bioavailability and a relatively long half-life (11). CA has many biological activities, such as antioxidant, anti-inflammatory, anticancer, antiapoptotic, and neuroprotective (12, 13).

In recent years, metabolomics has emerged as a powerful tool for understanding complex biological systems, offering valuable insights into metabolic pathways and their dynamic alterations. Concurrently, research into the gut microbiota has unveiled its pivotal role in human health and disease. An integrated analysis approach has emerged, fusing metabolomics and gut microbiota research to yield more profound and comprehensive insights. Metabolomics systematically studies small molecules, known as metabolites, within a biological system. These molecules are markers of biochemical processes and can provide a snapshot of an organism's physiological state. Furthermore, integrated metabolomics and gut microbiota analysis offer promising avenues for personalized medicine. By elucidating individual-specific metabolic signatures linked to distinct microbiota compositions, clinicians can develop targeted interventions and therapies tailored to an individual's unique physiological makeup.

2 Materials and methods

2.1 Chemicals and reagents

APH was provided by Aladdin; CP was provided by MACKLIN; Interleukin-6 (IL-6), Tumor Necrosis Factor-alpha (TNF-α), Erythropoietin (EPO), and Granulocyte Colony-Stimulating Factor (G-CSF) ELISA kit was provided by CUSABIO; CA was provided by Jingzhu bio-technology; Phospholipase D (PLD), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), and Sphingosine-1-Phosphate (S1P) as provided by Jiangsu Meimian Industrial Co., Ltd.

2.2 Animals and ethic statement

Animal experiments were approved by the Ethics Committee of Heilongjiang University of Chinese Medicine (approval number: 2019121101) in this study. Forty male SD rats (180-220 g) were purchased from Guangdong Medical Experimental Animal Center (SCXK (Guangdong) 2017-0125). Rats breeding environment: temperature 24 ± 2°C, relative humidity 50 ± 2%, light and dark cycle 12-24 hours. After one week of adaptation, 40 rats were randomly divided into five groups: Control group; Model group; CA high dose (CA-H; 9.7 mg/kg) group; CA medium dose (CA-M; 4.85 mg/kg) group; CA low dose (CA-L; 2.425 mg/kg) group (14). The CA was reconfigured when it was used. Rats in the control and model groups were fed distilled water daily for 14 consecutive days. Except for the control group, rats in each group were subcutaneously injected with APH on day 1 (20 mg/kg) and day 4 (10 mg/kg). Two hours after the second APH injection, rats were intraperitoneally injected with CP (20 mg/kg) for four consecutive days. The control group received an equal volume of 0.9% saline following the same procedure (Figure 1).

2.3 Routine blood test

After being anesthetized with 20% uratan, blood was collected from the rats using vacuum blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) to obtain plasma. We analyzed white blood cell (WBC), RBC, hematocrit (HCT), and hemoglobin (HGB) levels using a HEMAVET 950 automated hematology analyzer (Drew Scientific Group, Dallas, TX, USA).

2.4 ELISA kit detection

We collected rat blood using blood collection tubes without filler. The blood was allowed to stand at 4°C for 30 min and then centrifuged at 3500 rpm/min for 10 min. The serum was analyzed

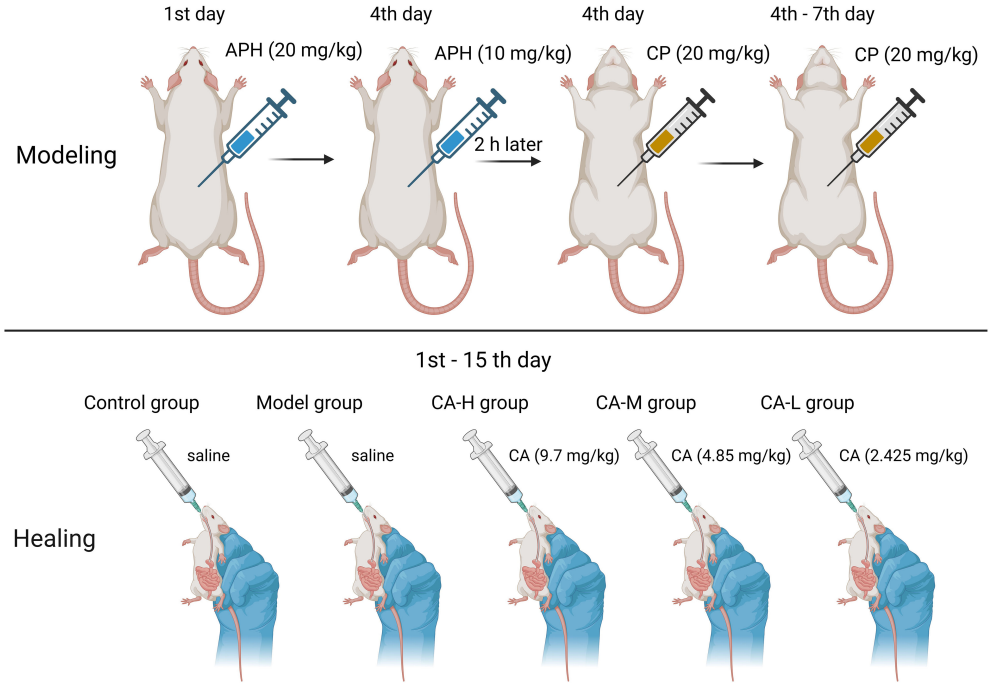

FIGURE 1
Modeling method in BDS rats.

using an ELISA kit according to the manufacturer's instructions, and the absorbance (OA) value was measured at 450 nm using a microplate reader. The levels of S1P, GAPDH, PLD, TNF-α, IL-6, EPO and G-CSF in serum were then calculated.

2.5 HE staining

The extent of spleen injury in rats was assessed using HE staining. Parts of the spleen tissue were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin blocks were cut into 10 μm thick sections with a microtome and stained with hematoxylin. The remaining unfixed spleen tissue was immediately transferred to liquid nitrogen and stored at -80°C for further analysis.

2.6 Metabolomics analysis

2.6.1 Metabolomics sample preparation

Serum metabolomics: We mixed serum (100 μL) with acetonitrile (300 μL) and vortexed the mixture for 30 s. Then, the samples were placed in a high-speed centrifuge and centrifuged at 13500 rpm/min for 20 min at 4°C. Next, we collected 300 μL of the upper layer solution, which was evaporated and dried. Then, a mixture of 50% acetonitrile and water (150 μL) to redissolve the sample. Finally, we took 10 μL of each sample and pooled them together as a quality control sample for metabolomic analysis.

Spleen metabolomics: We weighed 100 mg of spleen tissue and added a mixed solution of 50% acetonitrile and water (300 μL). Placed into a tissue grinder and grind at 3500rpm/min for 3 min, repeat 3 times. Place the ground spleen tissue into a centrifuge and centrifuge at 13500 rpm/min for 15 min at 4°C. Then, the samples were placed in a high-speed centrifuge and centrifuged at 13500 rpm/min for 20 min at 4°C. Next, we collected 300 μL of the upper layer solution, which was evaporated and dried. Then, a mixture of 50% acetonitrile and water (150 μL) to redissolve the sample. Finally, we took 10 μL of each sample and pooled them together as a quality control sample for metabolomic analysis.

2.6.2 Chromatography conditions

The treated samples were subjected to chromatography using a Waters ACQUITY UPLC HSS T3 column with gradient elution at a flow rate of 0.4 mL/min. The mobile phase A consisted of water containing 0.1% formic acid, while mobile phase B was acetonitrile containing 0.1% formic acid. The elution conditions for phase B were as follows: 0-2% (0-1 min), 2-35% (1-4 min), 35-100% (4-13 min), 100-100% (13-15.5 min), and 100-2% (15.5-19 min).

2.6.3 MS conditions

We used an ultra-high performance liquid phase (Dionex Ultimate 3000, USA), samples were analyzed with a tandem electrostatic field orbitrap high-resolution mass spectrometer (Thermo orbitrap Fusion, USA). The spray voltage was set to +3.5 KV and −2.5 KV, respectively. The capillary voltage was set to 325°C, with an intrathecal gas volume flow rate of 50 arb and an auxiliary gas volume flow rate of 10 arb. The resolution was set to 120 000, and the acquisition range was m/z: 100-800. The HRMS2 intensity threshold was set to 2.0×10^4. HRMS2 scanning high-energy collisional dissociation was set to 20%, 35%, 50% with a

resolution of 30 000. Collision-induced dissociation was set to 15%, 30%, 45% with a resolution of 30 000.

2.6.4 Biomarker identification and pathway analysis

Compound Discoverer 3.2 software was used for chromatographic peak identification, alignment, and normalization, and files containing m/z, retention time (Rt), and peak area were obtained. The original files were introduced into SIMCA 14.1 data processing software, and principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to analyze the rats in each group. Serum and spleen metabolites were analyzed. Based on the variable VIP > 1 and $p < 0.05$, the serum and spleen metabolites of rats in each group were compared to screen out potential biomarkers related to BDS. Based on the relative molecular mass and tandem mass spectrometry results, the mass spectrum information is matched with the mzCloud, Masslist Search, mzVault, and ChemSpider database to identify potential markers.

2.6.5 DNA extraction and PCR amplification

Total microbial genomic DNA was extracted from colon contents samples using the PF Mag-Bind Stool DNA Kit (Omega Bio-tek, GA, USA) according to the manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., USA) and stored at −80°C before further use. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified by the ABI GeneAmp® 9700 PCR thermal cycler using the primer pair 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (California, USA State ABI). The PCR reaction mixture included 4 μL 5 × Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL forward and reverse primers (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng template DNA, and ddH_2O in a final volume of 20 μL. The PCR amplification cycle conditions were: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and 27 cycles of single extension. 72°C 10 min, end at 4°C. All samples were amplified in triplicate. PCR products were extracted and purified from 2% agarose gel. Quantification was then performed using a Quantus™ fluorometer (Promega, USA).

2.6.6 Data processing

Raw FASTQ files were demultiplexed using an in-house perl script, then quality filtered via fastp version 0.19.6 and merged via FLASH version 1.2.11, meeting the following criteria: (i) Truncate 300 bp reads at any site with an average quality score < 20 on a 50 bp sliding window, and discard truncated reads shorter than 50 bp, and reads containing ambiguous characters are also discarded; (ii) Assemble based on overlapping sequences only Overlapping sequences longer than 10 bp. The maximum mismatch ratio in the overlapping area is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished based on barcodes and primers, and sequence directions were adjusted so that barcodes matched accurately and primers matched with 2 nucleotide mismatches. The optimized sequences were then clustered into operational taxonomic units (OTUs) using UPARSE 11 with a sequence similarity of 97%. The most abundant sequence for each OTU was selected as the representative sequence.

2.7 Statistical analysis

Based on OTU information, Mothur v1.30.2 was used to calculate rarefaction curves and alpha diversity indexes, including observed OTUs, Chao richness, Shannon index, and Simpson index. The similarity of microbial communities in different samples was determined by principal coordinate analysis (PCoA) based on Bray-curtis dissimilarity using the Vegan v2.4.3 package. The PERMANOVA test was used to assess the percentage of variation explained by treatment and its statistical significance using the Vegan v2.4.3 software package. Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify taxa (phylum to genus) that were significantly enriched by bacteria in different groups (LDA score > 2, $p < 0.05$). Due to multicollinearity issues among clinical parameters, the variance inflation factor (VIF) of each variable was estimated using the vif function in the Vegan v2.4.3 package (https://cran.r-project.org/web/packages/car/car.pdf). Distance-based redundancy analysis (db-RDA) was performed using the Vegan v2.4.3 software package to study the impact of clinical parameters on intestinal bacterial community structure. Forward selection is based on a Monte Carlo permutation test (permutation = 9999). The values of the x- and y-axes and the length of the corresponding arrows represent the importance of each clinical parameter in explaining the distribution of taxa across communities. Linear regression analysis was applied to determine the association between the main clinical parameters determined by db-RDA analysis and the microbial alpha diversity index. The co-occurrence network is constructed to explore the internal community relationships between samples.

All data were expressed as mean ± standard deviation. A t-test was performed using GraphPad Prism 7 software (GraphPad Software, United States). A value of $p < 0.05$ was considered significant, while a value of $p < 0.01$ was considered highly significant. Histograms were generated using GraphPad Prism 7 software.

3 Results

3.1 Results of spleen coefficient

Compared with the control group, the model group exhibited a significant increase in spleen index ($p < 0.01$). Compared with the model group, the spleen index of the CA-H ($p < 0.01$) and CA-M ($p < 0.05$) groups significantly decreased. There was no significant difference in the spleen index of the CA-L group (Figure 2A). These findings suggest that the effect of CA on splenomegaly was dose-dependent.

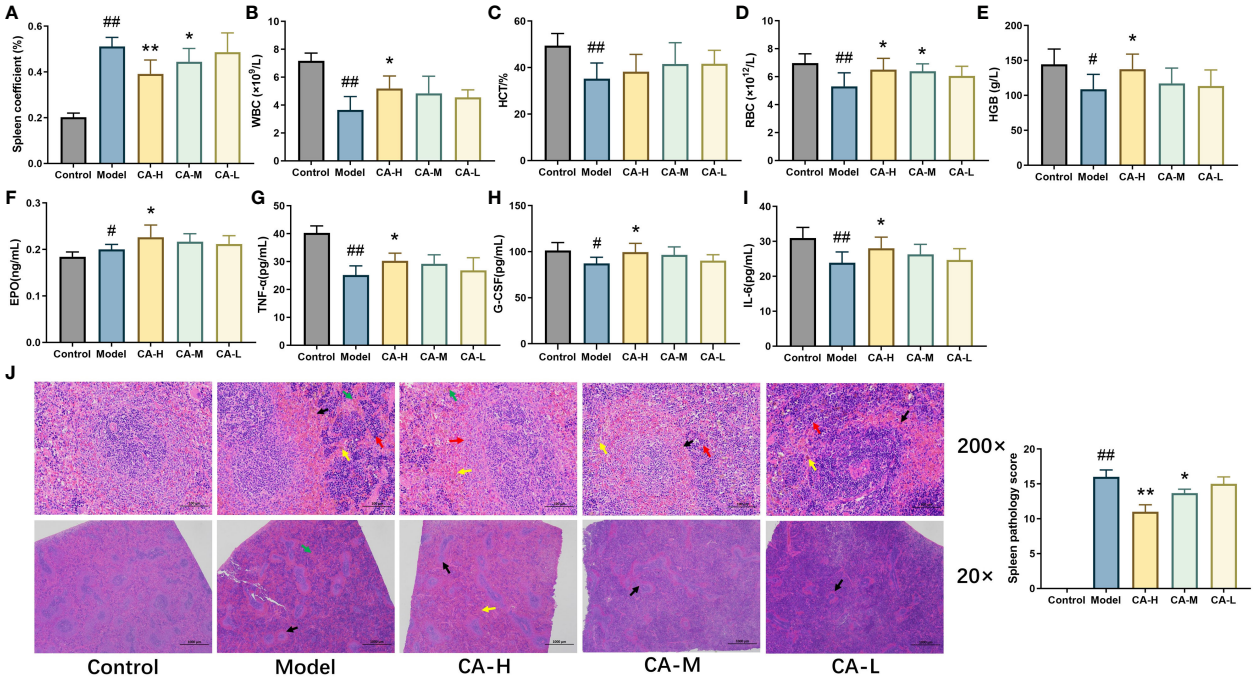

FIGURE 2
The effect of CA on physiological and biochemical indicators in BDS rats. **(A)** Rat spleen coefficient; **(B)** The level of WBC; **(C)** The level of HCT; **(D)** The level of RBC; **(E)** The level of HGB; **(F)** The level of EPO; **(G)** The level of TNF-α; **(H)** The level of G-CSF; **(I)** The level of IL-6; **(J)** HE staining (200 × and 20 ×), CA treatment of CP and APH induced spleen injury. Each value represents the mean ± SD (n = 3). $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$, compared with the control group; $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with the model group.

3.2 Results of routine blood test

After 15 days of administration, we measured the rats' WBC, RBC, HGB, and HCT levels (Figures 2B–E). Compared with the control group, the levels of RBC, WBC, HCT, and HGB were significantly decreased ($p < 0.01$) in the model group. Compared with the model group, the levels of WBC, RBC, and HCT in the CA-H group were significantly increased ($p < 0.05$), and the level of HGB was remarkably increased ($p < 0.01$).

3.3 Results of ELISA kit

We used ELISA kits to detect the levels of EPO, G-CSF, TNF-α, and IL-6 in serum. The data showed that compared with the control group, the levels of G-CSF, TNF-α, and IL-6 were significantly decreased ($p < 0.05$ or $p < 0.01$), and the level of EPO was increased ($p < 0.05$) in the model group. Compared with the model group, the levels of EPO, G-CSF, TNF-α, and IL-6 were significantly increased ($p < 0.05$ or $p < 0.01$) in the CA-H group, while in the CA-M group, the levels of TNF-α were significantly increased ($p < 0.05$) (Figures 2F–I).

3.4 Results of HE staining

Compared to the control group, the model group exhibited widespread white pulp atrophy with smaller cross-sections and loss of the peripheral marginal zone. At the same time, alongside peripheral capillary congestion, an increase in red blood cells (indicated by a black arrow), a moderate rise in extramedullary hematopoietic cells in the red pulp (red arrow), and more hemosiderin deposition (yellow arrow). Additionally, CP and APH expanded blood sinuses and increased red blood cells (green arrow) in the red pulp.

Compared with the model group, white pulp atrophy was mitigated in the CA-H group, and the cross-section was expanded (black arrow). The marginal zone around the white pulp was noticeable, broader, and relatively even in thickness (red arrow) compared to the model group. The red pulp demonstrated a reduction; in blood sinus congestion and lessened expansion, decreasing the number of red blood cells (yellow arrow). Moreover, there was a significant decrease in extramedullary hematopoietic cells, and less hemosiderin deposition was observable (green arrow; Figure 2J).

3.5 Results of metabolomics analysis

We employed an untargeted metabolomics approach to identify rat serum and spleen metabolites to investigate changes in host metabolism. Compounds detected in positive and negative modes used electrospray ionization (ESI+ and ESI−) in rat serum metabolism for multivariate statistical analysis. The QC samples are closely distributed and highly correlated, indicating that the entire detection process is stable. Principal component analysis

(PCA) of ESI+ metabolites is shown in Figure 3A. The PCA plot shows that the serum samples of the control, model, CA-H, CA-M, and CA-L groups can be divided into clusters. Likewise, a clear separation was observed in the PLS-DA plot (Figure 3B). In order to verify the accuracy of PCA or PLS-DA, permutation test analysis (Q^2 = 0.00461, R^2 = -0.407, Figure 3C) was used, PCA and PLS-DA analysis of ESI− metabolites also showed significant differences among the control, model, CA-H, CA-M, and CA-L groups (Figures 3D, E). At VIP≥1.0, $p < 0.05$, 22 differential metabolites were obtained (Table 1; Figure 3G). The permutation test analysis (Q^2 = 0.231, R^2 = - 0.415, Figure 3F) was used. The fold change (FC) value is shown in Figure 3H. In addition, KEGG pathway enrichment analysis showed that in $p < 0.05$, differential metabolites were closely related to 4 metabolic pathways (Figure 3I), including Sphingolipid metabolism, Biosynthesis of unsaturated fatty acids, Arginine biosynthesis, and Primary bile acid biosynthesis.

Compounds detected in positive and negative modes used ESI+ and ESI− in rat spleen metabolism for multivariate statistical analysis. PCA of ESI+ metabolites was shown in Figure 4A. The PCA plot shows that the spleen samples of the control, model, CA-H, CA-M, and CA-L groups can be divided into clusters. Likewise, a clear separation was observed in the PLS-DA scoring scatter plot (Figure 4B). To verify the accuracy of PCA or PLS-DA, permutation test analysis (Q^2 = 0.204, R^2 = - 0.498, Figure 4C) was used. PCA and PLS-DA analysis of ESI− metabolites also showed significant differences among the control, model, CA-H, CA-M, and CA-L groups (Figures 4D, E). At VIP≥1.0, $p < 0.05$, 23 differential metabolites were obtained (Table 2; Figure 4G). The permutation test analysis (Q^2 = 0.0206, R^2 = - 0.277, Figure 4F) was used. The fold change (FC) value is shown in Figure 4H. In addition, KEGG pathway enrichment analysis showed that in $p < 0.05$, differential metabolites were closely related to 5 metabolic pathways (Figure 4I), including Glycerophospholipid metabolism, Arginine and proline metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Glycine, serine and threonine metabolism, and Biosynthesis of unsaturated fatty acids.

3.6 Results of gut microbiota analysis

3.6.1 The effects of CA on the structure of the gut microbiota

To investigate the impact of CA on the gut microbiota of BDS rats, we performed 16s RNA sequencing on the colonic contents of control, model, CA-H, CA-M, and CA-L groups rats. The sequencing reads were sufficient for subsequent analysis, as shown in Figure 5 (n = 3), with 687403 sequencing reads obtained from 15 samples. After removing ineligible sequences, Venn diagrams were used to represent the characteristics of different treatment groups and common taxonomic groups. Based on > 97% similarity between sequences, 567 OTUs in the gut microbiota of all groups (Figure 5C). The species diversity of each sample was assessed using the alpha diversity statistical

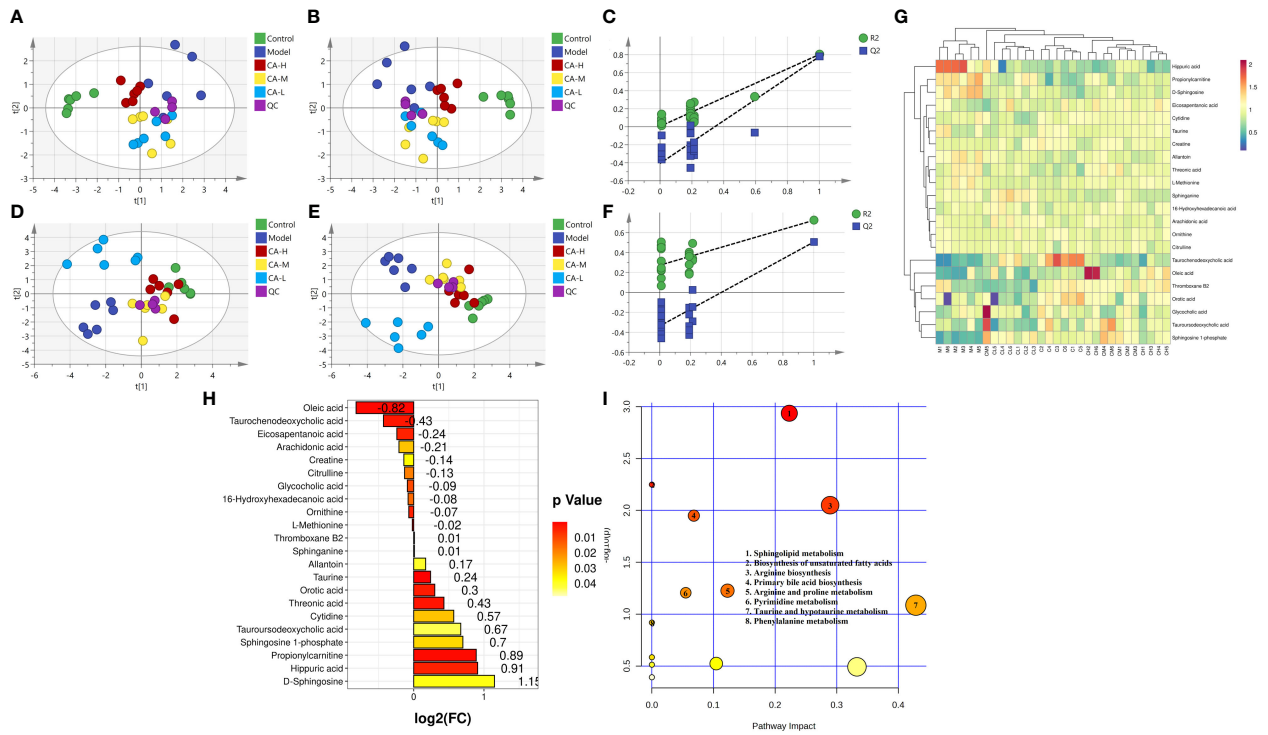

FIGURE 3
Serum metabolomics. **(A, D)** The PCA analysis represents the positive and negative ion modes of the ESI; **(B, E)** The PLS-DA analysis represents the positive and negative ion modes of the ESI (n = 6); **(C, F)** The permutation test analysis represents the positive and negative ion modes; **(G)** The heatmap and cluster analysis for the potential biomarker; **(H)** Folding change chart; **(I)** Bubble chart.

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TABLE 1 Detection of BDS related metabolites in serum metabolomics.

Metabolite	HMDB ID	Adducts	Formula	m/z	RT (min)	a	b	b	b
						Model	CA-H	CA-M	CA-L
Thromboxane B2	3252	M-H	$C_{20}H_{34}O_6$	370.2364	7.15	↑	↑	↑	↑
Threonic acid	943	M-H	$C_4H_8O_5$	136.0367	0.66	↑	↓	↓	↓
Tauroursodeoxycholic acid	874	M-H	$C_{26}H_{45}NO_6S$	499.2977	7.23	↑	↓	↑	↑
Taurochenodeoxycholic acid	951	M-H	$C_{26}H_{45}NO_6S$	499.2974	7.45	↓	↑	↑	↑
Taurine	251	M-H	$C_2H_7NO_3S$	125.0142	0.62	↑	↓	↓	↓
Sphingosine 1-phosphate	277	M+H	$C_{18}H_{38}NO_5P$	379.2511	8.95	↓	↑	↑	↑
Sphinganine	269	M+H	$C_{18}H_{39}NO_2$	301.2995	8.74	↑	↓	↓	↓
Propionylcarnitine	824	M+H	$C_{10}H_{19}NO_4$	217.1325	2.07	↑	↓	↓	↓
Orotic acid	226	M-H	$C_5H_4N_2O_4$	156.0166	0.79	↑	↑	↑	↑
Ornithine	214	M-H	$C_5H_{12}N_2O_2$	132.0894	0.63	↓	↑	↑	↑
Oleic acid	207	M-Na	$C_{18}H_{34}O_2$	282.2564	13.88	↓	↑	↑	↑
L-Methionine	696	M+H	$C_5H_{11}NO_2S$	149.0519	0.82	↓	↑	↓	↓
Hippuric acid	714	M-H	$C_9H_9NO_3$	179.0578	4.44	↓	↓	↓	↓
Glycocholic acid	138	M-H	$C_{26}H_{43}NO_6$	465.3099	7.08	↓	↓	↓	↓
Eicosapentanoic acid	1999	M-H	$C_{20}H_{30}O_2$	302.2247	10.86	↓	↑	↑	↑
D-Sphingosine	252	M+H	$C_{18}H_{37}NO_2$	299.2837	9.11	↑	↓	↓	↓
Cytidine	89	M+Na	$C_9H_{13}N_3O_5$	243.0869	0.79	↑	↓	↓	↓
Creatine	64	M-H	$C_4H_9N_3O_2$	131.0690	0.66	↓	↑	↑	↑
Citrulline	904	M-H	$C_6H_{13}N_3O_3$	175.0954	0.64	↓	↑	↑	↑
Arachidonic acid	1043	M-Na	$C_{20}H_{32}O_2$	304.2409	12.98	↓	↓	↓	↓
Allantoin	462	M-H	$C_4H_6N_4O_3$	158.0435	0.66	↑	↓	↓	↓
16-Hydroxyhexadecanoic acid	6294	M-H	$C_{16}H_{32}O_3$	272.2354	12.46	↓	↑	↑	↑

a, represents comparison with the control group; b, represents comparison with the model group. ↑, represents an increase in content; ↓, represents a decrease in content.

analysis index. The rarefaction curves (Sobs index at the OTU level) tended to be stable (Figure 5A), and the rank-abundance curves tended to be smooth (Figure 5B). The Chao, Simpson, and Shannon indexes were negatively correlated with diversity. The model group's Shannon, Shannon, and Chao indexes were significantly lower than the control group. However, the Shannon, Simpson, and Chao indexes were significantly higher in the CA-H group compared with the model group (Figures 5D–F).

The heatmap plots of the dominant gut microbiota composition at the phylum level (Figure S1A) and the genus level (Figure S1B) reveal variations in the abundance of prominent species through an analysis of the gut microbiota. Compared with the model group, the relative abundance of *Proteobacteria*, *Bacteroidota*, and *Verrucomicrobiota* is significantly increased in the CA-H group, while the relative abundance of *Firmicutes* at the phylum level is notably decreased. When compared to the model group, the CA-H group shows a substantial increase in the relative abundance of *Lactobacillus* and *Bifidobacterium*, accompanied by a significant decrease in the relative abundance of *Corynebacterium* and *Staphylococcus* at the genus level (Figures 5G, H).

Based on unweighted uniFrac distances, we utilized PCoA analysis to assess gut microbiota diversity in BDS rats. The outcomes revealed significant distinctions across the five groups (Figures 6B, F), indicative of gut microbial dysbiosis in BDS rats. Corresponding observations were evident in the NMDS analysis (Figure 6C). The PCA plot featured MetaStats analysis of the sequencing data (Figures 6A, E), aligning with the findings from PCoA and NMDS analyses. PLS-DA analysis (Figure 6D) enhanced these results and illustrated improved group dispersion. We generated a heatmap (Figure 6G) rooted in the unweighted uniFrac distances between samples to provide additional validation. This heatmap highlighted the substantial treatment effect of BDS within the CA-H group, surpassing that of the CA-M group, while the influence in the CA-M group exceeded that in the CA-L group.

We employed the linear discriminant analysis effect size (LEfSe) technique to discern specific bacterial taxa within distinct

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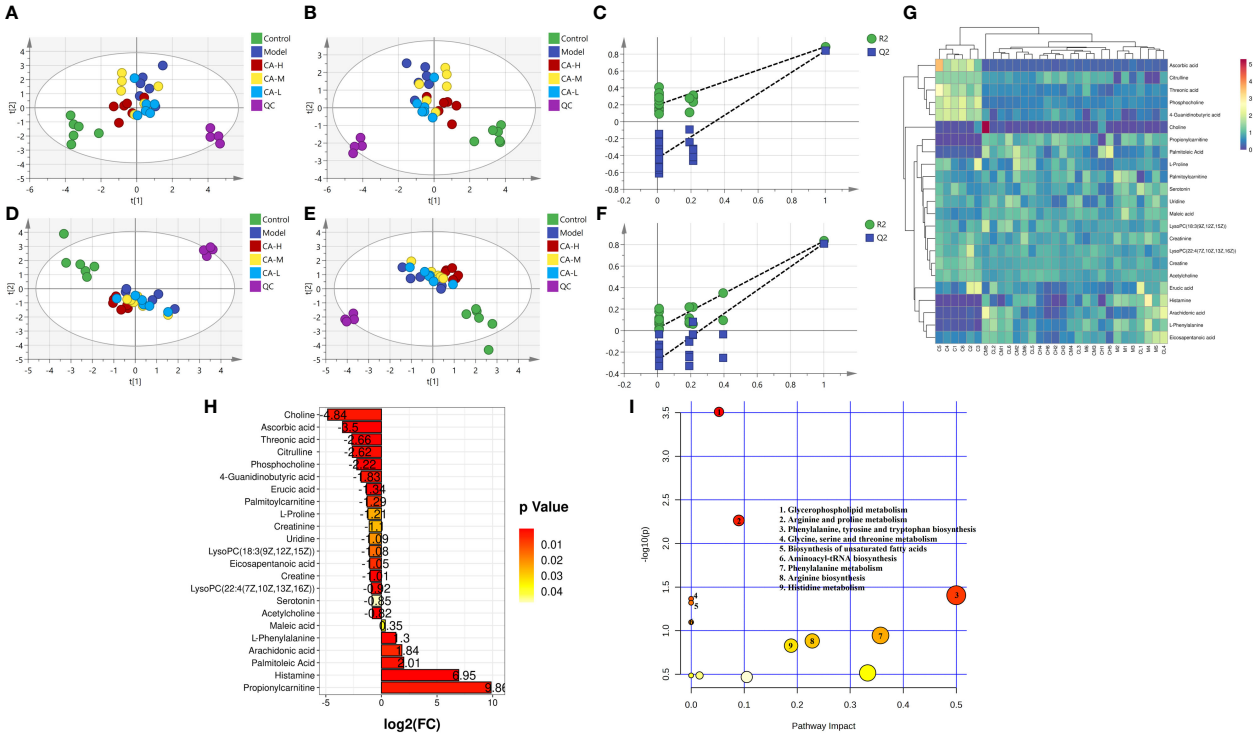

FIGURE 4
Spleen metabolomics. **(A, D)** The PCA analysis represents the positive and negative ion modes of the ESI; **(B, E)** The PLS-DA analysis represents the positive and negative ion modes of the ESI (n = 6); **(C, F)** The permutation test analysis represents the positive and negative ion modes; **(G)** The heatmap and cluster analysis for the potential biomarker; **(H)** Folding change chart; **(I)** Bubble chart.

populations. This method can analyze data from various branches of a microbial community. Due to the extensive dataset, comprehensive statistical analyses were undertaken from the phylum to the genus level, given the abundance of operational taxonomic units (OTUs) detected in this study. The arrangement presented in the cladograms, with an LDA score of 2 or higher, was confirmed to indicate significant differences (Figure 6H). The outcomes showcased in Figure 6I spotlight the bacteria displaying the most pronounced distinctions among the groups: *o_Erysipelotrichales* and *g_unclassified_f:Erysipelotrichaceae* in the control group, *o_Staphylococcales* in the model group, *c_Coriobacteriia* in the CA-H group, and *g_unclassified_f: Lachnospiraceae* in the CA-L group.

3.6.2 Annotation of the gut microbiota function

To elucidate the functional roles of the gut microbiota, we employed PICRUSt2 functional predictions, which involve species-level predictions and their functional relationships based on the KEGG database. We presented the results from a t-test analysis, comparing the predicted functional changes in the gut microbiota across different groups: control vs. model groups and model vs. CA-H groups. There were notable increases in several functional categories compared to the model group. Specifically, functions related to Translation, Metabolism of cofactors and vitamins, Amino acid metabolism, Global and overview maps, and others exhibited significantly higher levels in the control group (Figure 7A). These findings suggest that the control group's gut microbiota may be associated with a more active and diverse functional process in these areas. Turning to the comparison between the model and CA-H groups, the CA-H group displayed considerably elevated levels in various functional categories when contrasted with the model group. Significant alterations were observed in Glycan biosynthesis and metabolism, Replication and repair, Translation, Amino acid metabolism, and other areas (Figure 7B). These results suggest that the CA-H group's gut microbiota may contribute to enhanced functional activities in these pathways compared to the model group.

3.7 Correlation among gut microbiota and metabolic parameters in rat

The research team conducted a Spearman correlation analysis on 22 common serum metabolites and gut microbiota at the genus level, which can further analyze the interaction between gut microbiota and serum metabolites. *Staphylococcus* was positively correlated with Sphinganine, Propionylcarnitine, and Allantoin; Tauroursodeoxycholic acid, Oleic acid, and Cytidine were negatively correlated. *Corynebacterium* was positively correlated with Hippuric acid; Tauroursodeoxycholic acid, Oleic acid, and Eicosapentanoic acid were negatively correlated. Hippuric acid, and Allantoin were negatively correlated. *Bifidobacterium* was positively correlated with Thromboxane B2, Tauroursodeoxycholic acid, Oleic acid, and Cytidine; Sphinganine and propionylcarnitine

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TABLE 2 Detection of BDS related metabolites in spleen metabolomics.

Metabolite	HMDB ID	Adducts	Formula	m/z	RT (min)	a	b	b	b
						Model	CA-H	CA-M	CA-L
Serotonin	259	M+Na	$C_{10}H_{12}N_2O$	159.0693	1.98	↓	↑	↑	↑
Propionylcarnitine	824	M+H	$C_{10}H_{19}NO_4$	217.1332	1.85	↑	↓	↓	↓
Phosphocholine	1565	M+Na	$C_5H_{14}NO_4P$	183.0668	0.61	↓	↓	↓	↓
Palmitoylcarnitine	222	M+H	$C_{23}H_{45}NO_4$	399.3368	10.29	↓	↑	↑	↑
Palmitoleic Acid	3229	M+H	$C_{16}H_{30}O_2$	276.2103	10.71	↑	↓	↓	↓
LysoPC(22:4(7Z,10Z,13Z,16Z))	10401	M+H	$C_{30}H_{54}NO_7P$	571.3669	10.58	↓	↑	↑	↑
LysoPC(18:3(9Z,12Z,15Z))	10388	M+H	$C_{26}H_{48}NO_7P$	517.3185	9.02	↓	↑	↑	↑
L-Proline	162	M+H	$C_5H_9NO_2$	115.0636	5.18	↓	↑	↓	↓
Histamine	870	M+H	$C_5H_9N_3$	111.0807	0.51	↑	↑	↑	↑
Creatinine	562	M+H	$C_4H_7N_3O$	113.0592	0.77	↓	↓	↓	↓
Choline	97	M+H	$C_5H_{13}NO$	103.0998	6.34	↓	↑	↓	↓
Acetylcholine	895	M+H	$C_7H_{15}NO_2$	145.1108	0.66	↓	↓	↓	↓
4-Guanidinobutyric acid	3464	M+H	$C_5H_{11}N_3O_2$	145.0854	0.81	↓	↓	↓	↓
Citrulline	904	M+H	$C_6H_{13}N_3O_3$	175.0962	0.62	↓	↓	↓	↓
Uridine	296	M-H	$C_9H_{12}N_2O_6$	244.0700	1.44	↓	↑	↑	↑
Threonic acid	943	M-H	$C_4H_8O_5$	136.0376	0.69	↓	↑	↑	↑
Maleic acid	176	M-H	$C_4H_4O_4$	116.0105	1.29	↓	↑	↑	↑
L-Phenylalanine	159	M-H	$C_9H_{11}NO_2$	165.0785	3.10	↑	↑	↑	↑
Erucic acid	2068	M-H	$C_{22}H_{42}O_2$	338.3191	15.88	↓	↑	↑	↑
Eicosapentanoic acid	1999	M-H	$C_{20}H_{30}O_2$	302.2249	12.34	↓	↑	↑	↑
Creatine	64	M-H	$C_4H_9N_3O_2$	131.0691	0.70	↓	↓	↓	↓
Ascorbic acid	44	M-Na	$C_6H_8O_6$	116.0107	0.73	↓	↓	↓	↓
Arachidonic acid	1043	M-Na	$C_{20}H_{32}O_2$	304.2410	12.98	↑	↑	↑	↑

a, represents comparison with the Control group; b, represents comparison with the Model group. ↑, represents an increase in content; ↓, represents a decrease in content.

were negatively correlated (Figure 7C). We performed a Spearman correlation analysis of 23 shared spleen metabolites with genus-level gut microbiota. *Psychrobacter* was positively correlated with Citrulline; *Psychrobacter* was negatively correlated with L-Phenylalanine, Histamine, Erucic acid, Eicosapentanoic acid, and Arachidonic acid. *Staphylococcus* was positively correlated with Urine, Maleic acid, L-Phenylalanine, and Histamine; 4-Guanidinobutyric acid was negatively correlated. *Bifidobacterium* was positively correlated with 4-Guanidinobutyric acid; L-Phenylalanine, Histamine, and Eicosapentanoic acid were negatively correlated (Figure 7D).

3.8 Results of expression of key targets

Compared with the control group, the expression of S1P, GAPDH, and PLD in the spleen of the model group was decreased ($p < 0.05$ or $p < 0.01$). Compared with the model group, the CA-H group had increased expression of S1P, GAPDH, and PLD ($p < 0.05$ or $p < 0.01$), and the CA-M and CA-L groups had significantly increased expression of PLD ($p < 0.01$) (Figure 8).

4 Discussion

Chemotherapy, a cornerstone of cancer treatment, employs potent agents such as CP to combat cancer cells (15). While offering promising therapeutic outcomes, this treatment strategy can also introduce a range of potential challenges that demand careful consideration. A significant concern associated with chemotherapy is BDS, which hinders the bone marrow's capacity to produce essential blood cells (16). This suppression often results in anemia, reduced RBC and WBC, leading to fatigue and weakness (17). Chemotherapy can trigger hair loss (18). Additionally, chemotherapy can compromise the immune system, making individuals more susceptible to infections and illnesses. While chemotherapy holds the potential to save lives by targeting and eradicating cancer cells, its adverse effects are an inherent part of the

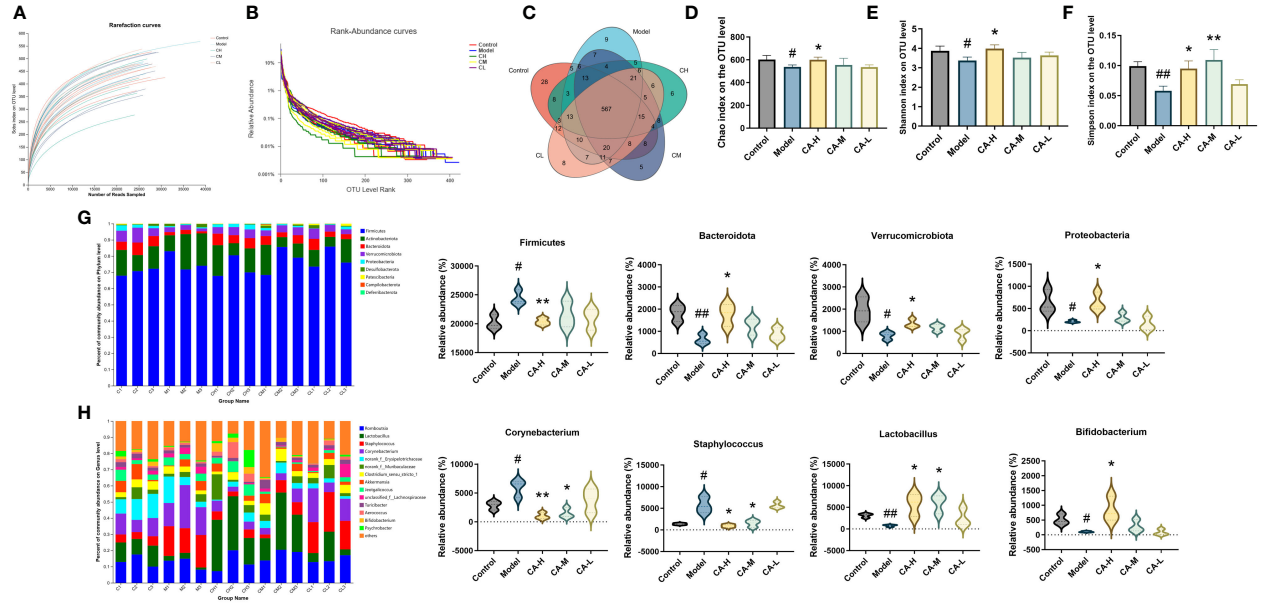

FIGURE 5
Effect of CA on the richness and diversity of colonic contents microbiota in rats. **(A)** Rarefaction curves. **(B)** Rank abundance curves. **(C)** OTU Venn diagram. **(D)** Chao index. **(E)** Simpson index. **(F)** Shannon index. **(G, H)** The colonic contents bacterial community at the phylum, and genus levels. Less than 0.5% abundance of the genus was merged into others. Each value represents the mean ± SD (n = 3). $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$, compared with the control group; $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with the model group.

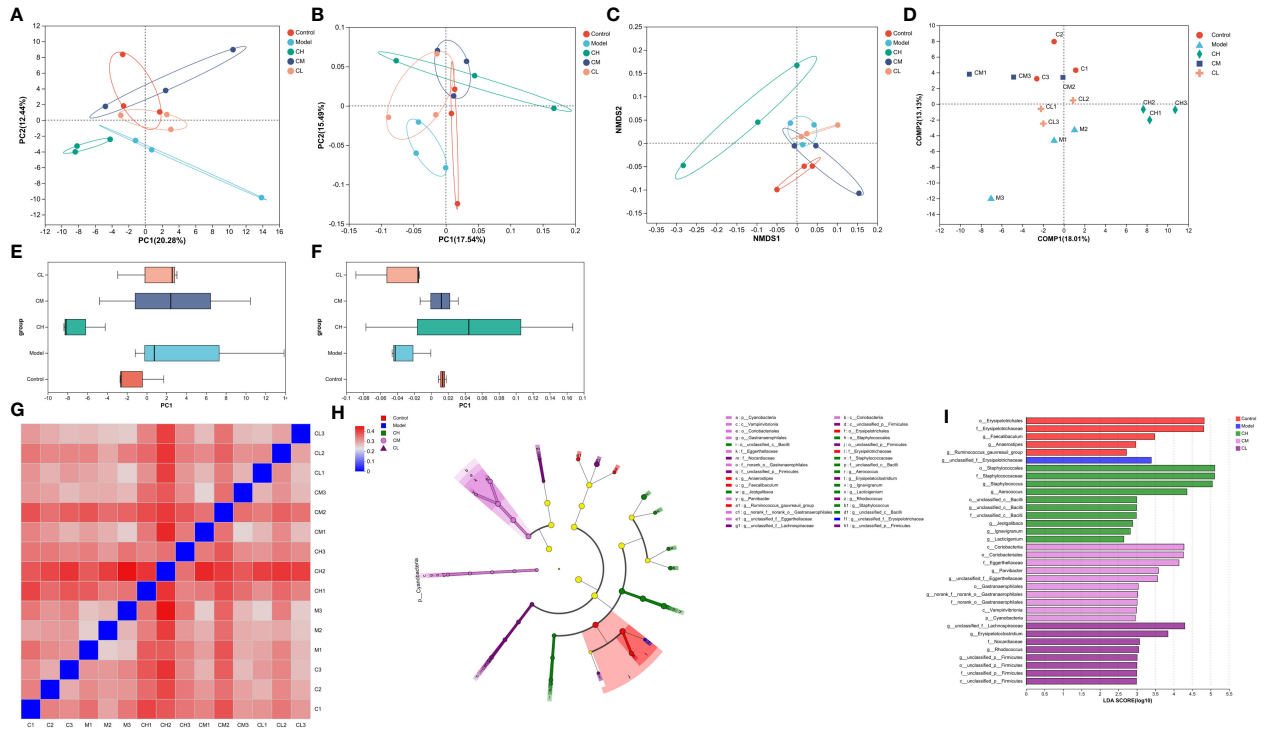

FIGURE 6
β-diversity analysis of gut microbiota. **(A)** PCA analysis; **(B)** PCoA analysis; **(C)** NMDS analysis; **(D)** PLS-DA analysis; **(E)** PCA box diagram; **(F)** PCoA box diagram; **(G)** β-diversity heatmap; **(H)** The indicator bacteria with an LDA score of 2 or higher in the bacterial community were associated with the five groups of rats; **(I)** The areas with different colors represent different components (red, control group; blue, model group; green, CA-H group; pink, CA-M group; purple, CA-L group). The circle indicates the level of phylogeny from phylum to genus.

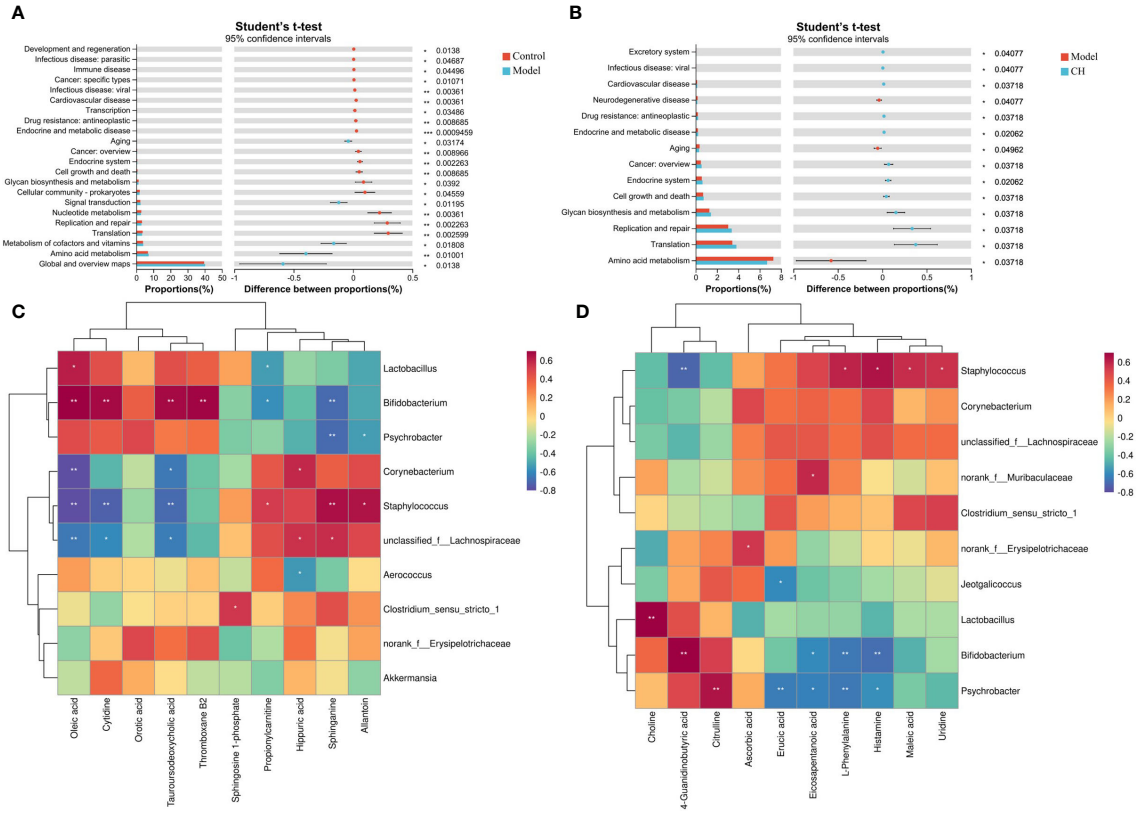

FIGURE 7
Functional prediction of altered gut microbiota by PICRUSt2 analysis based on the rat level II KEGG pathway. **(A)** Control group vs model group; **(B)** Model group vs CA-H group. **(C)** Spearman correlation heatmap between the top 10 important serum metabolite concentrations and the relative abundance of gut microbiota genus levels in the CA-H group; **(D)** The top 10 important spleen essential metabolites and gut microbiota in the CA-H group relative abundance at the genus level. R values are represented by gradient colors, where red and blue cells indicate positive and negative correlations. *$p < 0.05$ or **$p < 0.01$.

treatment process. Therefore, solving the side effects caused by CP and APH is imminent. CA is primarily utilized for specific immune therapy (19). As a result, compared with the model group, upon administering CA to rats, the spleen index of the CA-H group exhibited a notable decrease. The blood routine levels were also observed, revealing that HCT, RBC, WBC, and HGB levels in the CA-H group showed significant improvements. Histopathological analysis indicated that the rats in the CA-H group experienced significant enhancement in spleen morphology. However, no significant changes were observed in the CA-L group compared to the model group.

EPO and G-CSF are two hematopoietic growth factors that play essential roles in regulating blood cell production in the body (20). EPO's prominent role is to stimulate the production of RBC in the

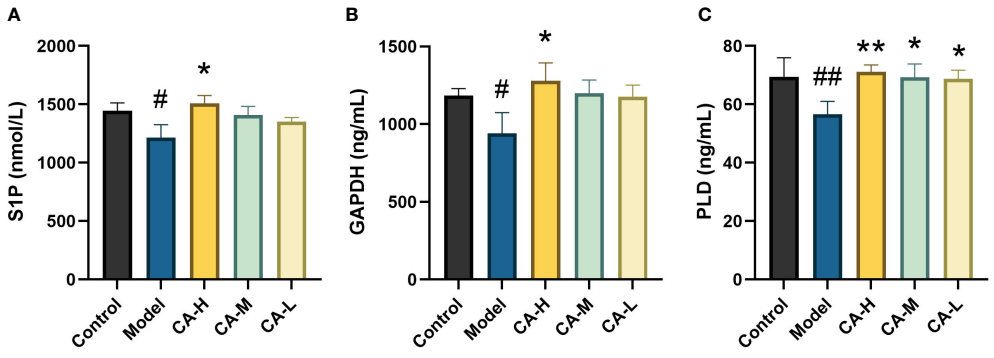

FIGURE 8
Effect of each group on the key target expression. **(A)** The level of S1P; **(B)** The level of GAPDH; **(C)** The level of PLD. $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$, compared with the control group; *$p < 0.05$ and **$p < 0.01$, compared with the model group.

kidney. EPO helps maintain the appropriate level of hemoglobin and RBC, ensuring effective oxygen delivery to tissues and organs (21). G-CSF is another growth factor that stimulates the production of specific WBCs called granulocytes, particularly neutrophils (22). Neutrophils are crucial for the immune response against bacterial infections (23). While G-CSF is not directly related to RBC production, its role in maintaining the immune system is essential. After treatment, the CA-H group showed significant differences from the model group ($p < 0.05$). CA promoted the restoration of RBC production by promoting the synthesis and secretion of EPO in rat bone marrow. The results in Figure 2H showed that APH and CP significantly inhibited G-CSF expression, which was sharply increased after CA administration. It suggested that CA had a specific inhibitory effect on the cytotoxicity of CP and APH. Increase the level of G-CSF and promote the recovery of granulocyte hematopoietic function. Immunocytokines such as TNF-α and IL-6 play an essential role in the coordination of the immune system (24). They enable the communication of lymphatic, inflammatory, and hematopoietic cells. In this study, serum TNF-α and IL-6 (Figures 2G, I) levels were significantly lower in the BDS group compared with the control group. Interestingly, CA administration reversed this inhibitory effect, suggesting its potential to enhance immune function and alleviate BDS.

In recent years, gut microbiota has received extensive attention as an important target for immune system diseases. Under normal conditions, the gut microbiota, consisting of billions of bacteria, maintains a symbiotic relationship with its host and helps regulate the body's metabolism and energy. Many studies have shown that the changes in the gut microbiota of immune system diseases mainly increase the number of *Firmicutes* and reduce the proportion of *Bacteroidetes*. *Firmicutes* to *Bacteroidetes* (F/B) ratio has become one of the most important reference indicators for studying gut microbiota disorders (25). According to 16S rRNA sequencing, CA enhanced gut microbiota composition in BDS model rats, decreased the abundance of *Firmicutes*, and increased the abundance of *Bacteroidetes*. At the genus level, taking CA tended to stimulate the emergence of some potential probiotics, such as *Bifidobacterium* and *Lactobacillus*. Reduce the abundance of potentially harmful bacteria such as *Corynebacterium* and *Staphylococcus*. CA treatment reversed most of the CP and APH induced changes in the gut microbiota, suggesting that CA helps restore CP and APH induced changes in the interplay between the gut microbiota and immunity.

Our observations also demonstrated that the dysregulation of hematopoiesis is associated with Glycerophospholipid metabolism and Sphingolipid metabolism. This includes LysoPC(22:4 (7Z,10Z,13Z,16Z)), LysoPC(18:3(9Z,12Z,15Z)), Choline, Acetylcholine, Sphingosine, Sphinganine, and Sphingosine-1-phosphate (S1P). Orsini's study provides evidence of sphingolipids participating in TNF-α-mediated modulation of the TF/miR network and inhibition of autophagy in HSPCs, affecting erythrocyte formation (26). Sphinganine is a derivative of Sphingosine. The accumulation of these molecules has been confirmed to suppress Bcl-xL expression and downregulate Bcl-2, thereby enhancing apoptosis, accompanied by robust inhibition of the MAPK cascade (27–30). Additionally, Vu's study indicated that a significant decrease in plasma S1P levels in Major facilitator superfamily transporter 2b (Mfsd2b) knockout mice results in damaged erythrocyte membranes and disordered peripheral blood (31). They also discovered that Sphingosine accumulates significantly when the secretion of S1P is blocked due to Mfsd2b knockout in RBC. In contrast, S1P, synthesized through the phosphorylation of Sphingosine in the presence of sphingosine kinase 1 (SphK1), has been shown to possess antiapoptotic properties (30). Our research, along with the work of Wątek, demonstrates a significant up-regulation in the levels of Sphingosine and Sphinganine in the model with immune damage mediated by chemotherapeutic drugs (32). Simultaneously, the concentration of S1P is notably lower ($p < 0.05$). Following CA treatment, there is a substantial decrease in the attention of Sphingosine and Sphinganine compared to the model ($p < 0.05$). Moreover, S1P levels increase significantly after CA treatment ($p < 0.05$). Phospholipase D (PLD) is an enzyme essential for immune receptor signaling and immune cell function. It catalyzes the hydrolysis of phosphatidylcholine, the major phospholipid in the plasma membrane, producing the critical signaling lipid phosphatidic acid (33). In addition to its role in immune cell function, PLD has been shown to play a role in Fcγ-mediated phagocytosis of macrophages and in activating NADPH oxidase in neutrophils (34). Coffman's research reported that tumor growth factor-β (TGF-β) enhances the production of immunoglobulin (Ig) A by lipopolysaccharide-stimulated murine B lymphocytes. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) significantly increased total IgA levels in human peripheral blood mononuclear cells (PBMC), mouse serum, and spleen lymphocytes (35). Additionally, Kinoshita's research revealed that cell surface GAPDH plays a role in the adhesion of lactic acid bacteria to human mucin in the intestine. These findings suggest that GAPDH or its peptides may have immunomodulatory effects on the blood and intestinal immune systems (36). Furthermore, sphingolipids are vital signaling molecules that regulate numerous cellular processes crucial for immunity, inflammation, infection, and cancer. They serve as the fundamental building blocks of eukaryotic cell membranes. These sphingolipid metabolites are ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P; 37, 38).

In this study, significant decreases ($p < 0.05$) were observed in the spleen levels of LysoPC(22:4(7Z,10Z,13Z,16Z)) and LysoPC(18:3 (9Z,12Z,15Z)) 37. However, after CA treatment, these indexes were notably restored ($p < 0.05$). Abundant lysophosphatidylcholine (LPC) in plasma has been confirmed to contribute to the activation of inflammatory responses (39). We speculate that CA might exert its effects by suppressing inflammation and oxidative damage by restoring LPC levels. Additionally, CA could promote Glycerophospholipid metabolism, thus creating a more favorable hematopoietic microenvironment for enhancing hematopoiesis.

5 Conclusion

In summary, our study revealed multiple ameliorative effects of CA on BDS, including improving spleen function, enhancing immune

levels, and pathological changes. The mechanism of CA treating BDS may be related to improving intestinal flora imbalance and regulating sphingolipid metabolism and glycerophospholipid metabolism.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: NCBI, PRJNA1010515.

Ethics statement

The animal study was approved by Ethics Committee of Heilongjiang University of Chinese Medicine (approval number: 2019121101). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Writing – original draft. NC: Writing – review & editing. FS: Validation. YS: Formal analysis. BL: Formal analysis. YR: Validation. PW: Validation. HB: Validation. WG: Validation. BY: Supervision, Project administration. QW: Supervision, Project administration. HK: Supervision, Project administration.

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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/fimmu.2023.1280049/full#supplementary-material

References

1. Wang Y, Li C, Chuo W, Liu Z, Ouyang Y, Li D, et al. Integrated proteomic and metabolomic analysis reveals the NADH-mediated TCA cycle and energy metabolism disorders based on a new model of chronic progressive heart failure. *Mol Biosyst* (2013) 9(12):3135–45. doi: 10.1039/c3mb70263d

2. Li S, Lin H, Qu C, Tang Y, Shen J, Li W, et al. Urine and plasma metabonomics coupled with UHPLC-QTOF/MS and multivariate data analysis on potential biomarkers in anemia and hematinic effects of herb pair Gui-Hong. *J Ethnopharmacology* (2015) 170:175–83. doi: 10.1016/j.jep.2015.05.019

3. Redinus K, Baek JH, Yalamanoglu A, Shin HKH, Moldova R, Harral JW, et al. An Hb-mediated circulating macrophage contributing to pulmonary vascular remodeling in sickle cell disease. *JCI Insight* (2019) 4(15):e127860. doi: 10.1172/jci.insight.127860

4. Xu M, He RR, Zhai YJ, Abe K, Kurihara H. Effects of carnosine on cyclophosphamide-induced hematopoietic suppression in mice. *Am J Chin Med* (2014) 42(1):131–42. doi: 10.1142/S0192415X14500098

5. van Vliet MJ, Tissing WJ, Dun CA, Meessen NE, Kamps WA, de Bont ES, et al. Chemotherapy treatment in pediatric patients with acute myeloid leukemia receiving antimicrobial prophylaxis leads to a relative increase of colonization with potentially pathogenic bacteria in the gut. *Clin Infect Dis: an Off Publ Infect Dis Soc America* (2009) 49(2):262–70. doi: 10.1086/599346

6. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. *PloS One* (2011) 6(12):e28654. doi: 10.1371/journal.pone.0028654

7. Jia L, Yu J, He L, Wang H, Jiang L, Miao X, et al. Nutritional support in the treatment of aplastic anemia. *Nutr (Burbank Los Angeles County Calif.)* (2011) 27(11-12):1194–201. doi: 10.1016/j.nut.2011.01.012

8. Gong W, Zhang N, Cheng G, Zhang Q, He Y, Shen Y, et al. *Rehmannia glutinosa* libosch extracts prevent bone loss and architectural deterioration and enhance osteoblastic bone formation by regulating the IGF-1/PI3K/mTOR pathway in streptozotocin-induced diabetic rats. *Int J Mol Sci* (2019) 20(16):3964. doi: 10.3390/ijms20163964

9. Ou L, Kang W, Liang Z, Gao F, Dong T, Wei P, et al. Investigation of anti-osteoporosis mechanisms of Rehmanniae Radix Preparata based on network pharmacology and experimental verification. *J Orthopaedic Surg Res* (2021) 16 (1):599. doi: 10.1186/s13018-021-02751-5

10. Youn UJ, Gu BS, Kim KH, Ha C, Jung IC. Variation of main components according to the number of steaming and drying of Rehmanniae radix preparata. *J Pharmacopuncture* (2018) 21(2):112–9. doi: 10.3831/KPI.2018.21.014

11. Fu Z, Su X, Zhou Q, Feng H, Ding R, Ye H. Protective effects and possible mechanisms of catalpol against diabetic nephropathy in animal models: a systematic review and meta-analysis. *Front Pharmacol* (2023) 14:1192694. doi: 10.3389/fphar.2023.1192694

12. Wang ZH, Zhan-Sheng H. Catalpol inhibits migration and induces apoptosis in gastric cancer cells and in athymic nude mice. *Biomedicine pharmacotherapy = Biomedecine Pharmacotherapie* (2018) 103:1708–19. doi: 10.1016/j.biopha.2018.03.094

13. Chen H, Deng C, Meng Z, Meng S. Effects of catalpol on Alzheimer's disease and its mechanisms. *Evidence-Based complementary Altern Med eCAM* (2022) 2022:2794243. doi: 10.1155/2022/2794243

14. Wang JH, Li WT, Yu ST, Xie H, Han HR. Catalpol regulates function of hypothalamic-pituitary-adrenocortical-axis in an Alzheimer's disease rat model. *Die Pharmazie* (2014) 69(9):688–93.

15. Chrysostomou D, Roberts LA, Marchesi JR, Kinross JM. Gut microbiota modulation of efficacy and toxicity of cancer chemotherapy and immunotherapy. *Gastroenterology* (2023) 164(2):198–213. doi: 10.1053/j.gastro.2022.10.018

16. Zhang W, Cui N, Su F, Wang Y, Yang B, Sun Y, et al. Comprehensive metabolomics and network pharmacology to explore the mechanism of 5-hydroxymethyl furfural in the treatment of blood deficiency syndrome. *Front Pharmacol* (2022) 12:811331. doi: 10.3389/fphar.2021.811331

17. Pouraboli B, Poodineh Z, Jahani Y. The effect of relaxation techniques on anxiety, fatigue and sleep quality of parents of children with leukemia under chemotherapy in South East Iran. *Asian Pacific J Cancer Prev APJCP* (2019) 20 (10):2903–8. doi: 10.31557/APJCP.2019.20.10.2903

18. Amarillo D, de Boni D, Cuello M. Chemotherapy, Alopecia, and Scalp Cooling Systems. Alopecia, quimioterapia y gorras de frío o «scalp cooling system». *Actas dermo-sifiliograficas* (2022) 113(3):278–83. doi: 10.1016/j.ad.2021.09.003

19. Zou S, Hong J, Liu D, Lai G, Ye J, Song Y. Enhanced effect of catalpol on specific immune therapy in treatment of asthmatic mice. *Am J Trans Res* (2019) 11(4):2463–9.

20. Tabbara IA, Robinson BE. Hematopoietic growth factors. *Anticancer Res* (1991) 11(1):81–90.

21. Turner J, Parsi M, Badireddy M. Anemia. In: *StatPearls*. Island (FL): StatPearls Publishing (2023).

22. Kawashima M, Kondo H. Comparison of therapeutic G-CSF cycles and prophylactic G-CSF cycles in patients receiving paclitaxel and carboplatin combination chemotherapy for ovarian cancer: A retrospective study report. *J Rural Med JRM* (2014) 9(2):86–9. doi: 10.2185/jrm.2889

23. Hidalgo A, Chilvers ER, Summers C, Koenderman L. The neutrophil life cycle. *Trends Immunol* (2019) 40(7):584–97. doi: 10.1016/j.it.2019.04.013

24. Wang T, He C. TNF-α and IL-6: the link between immune and bone system. *Curr Drug Targets* (2020) 21(3):213–27. doi: 10.2174/1389450120666190821161259

25. Yuan Y, He J, Tang M, Chen H, Wei T, Zhang B, et al. Preventive effect of Ya'an Tibetan tea on obesity in rats fed with a hypercaloric high-fat diet revealed by gut microbiology and metabolomics studies. *Food Res Int (Ottawa Ont.)* (2023) 165:112520. doi: 10.1016/j.foodres.2023.112520

26. Orsini M, Chateauvieux S, Rhim J, Gaigneaux A, Cheillan D, Christov C, et al. Sphingolipid-mediated inflammatory signaling leading to autophagy inhibition converts erythropoiesis to myelopoiesis in human hematopoietic stem/progenitor cells. *Cell Death Differentiation* (2019) 26(9):1796–812. doi: 10.1038/s41418-018-0245-x

27. Sakakura C, Sweeney EA, Shirahama T, Hakomori S, Igarashi Y. Suppression of bcl-2 gene expression by sphingosine in the apoptosis of human leukemic HL-60 cells during phorbol ester-induced terminal differentiation. *FEBS Lett* (1996) 379(2):177–80. doi: 10.1016/0014-5793(95)01508-6

28. Jarvis WD, Fornari FA, Auer KL, Freemerman AJ, Szabo E, Birrer MJ, et al. Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol Pharmacol* (1997) 52(6):935–47. doi: 10.1124/mol.52.6.935

29. Shirahama T, Sakakura C, Sweeney EA, Ozawa M, Takemoto M, Nishiyama K, et al. Sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by suppression of bcl-X(L) gene expression. *FEBS Lett* (1997) 407(1):97–100. doi: 10.1016/s0014-5793(97)00304-9

30. Young MM, Kester M, Wang HG. Sphingolipids: regulators of crosstalk between apoptosis and autophagy. *J Lipid Res* (2013) 54(1):5–19. doi: 10.1194/jlr.R031278

31. Vu TM, Ishizu AN, Foo JC, Toh XR, Zhang F, Whee DM, et al. Mfsd2b is essential for the sphingosine-1-phosphate export in erythrocytes and platelets. *Nature* (2017) 550(7677):524–8. doi: 10.1038/nature24053

32. Wątek M, Durnaś B, Wollny T, Pasiarski M, Góźdź S, Marzec M, et al. Unexpected profile of sphingolipid contents in blood and bone marrow plasma collected from patients diagnosed with acute myeloid leukemia. *Lipids Health Dis* (2017) 16(1):235. doi: 10.1186/s12944-017-0624-1

33. Zhu M, Foreman DP, O'Brien SA, Jin Y, Zhang W. Phospholipase D in TCR-mediated signaling and T cell activation. *J Immunol (Baltimore Md.: 1950)* (2018) 200 (6):2165–73. doi: 10.4049/jimmunol.1701291

34. Melendez AJ, Bruetschy L, Floto RA, Harnett MM, Allen JM. Functional coupling of FcgammaRI to nicotinamide adenine dinucleotide phosphate (reduced form) oxidative burst and immune complex trafficking requires the activation of phospholipase D1. *Blood* (2001) 98(12):3421–8. doi: 10.1182/blood.v98.12.3421

35. Coffman RL, Lebman DA, Shrader B. Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J Exp Med* (1989) 170(3):1039–44. doi: 10.1084/jem.170.3.1039

36. Kinoshita H, Wakahara N, Watanabe M, Kawasaki T, Matsuo H, Kawai Y, et al. Cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of Lactobacillus plantarum LA 318 recognizes human A and B blood group antigens. *Res Microbiol* (2008) 159(9-10):685–91. doi: 10.1016/j.resmic.2008.07.005

37. Kinoshita M, Matsumori N. Inimitable impacts of ceramides on lipid rafts formed in artificial and natural cell membranes. *Membranes* (2022) 12(8):727. doi: 10.3390/membranes12080727

38. Takabe K, Paugh SW, Milstien S, Spiegel S. "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol Rev* (2008) 60(2):181–95. doi: 10.1124/pr.107.07113

39. Taylor LA, Arends J, Hodina AK, Unger C, Massing U. Plasma lyso-phosphatidylcholine concentration is decreased in cancer patients with weight loss and activated inflammatory status. *Lipids Health Dis* (2007) 6:17. doi: 10.1186/1476-511X-6-17

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Role of vitamins A, C, D, E in cancer prevention and therapy: therapeutic potentials and mechanisms of action

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Cancer, a leading global cause of mortality, arises from intricate interactions between genetic and environmental factors, fueling uncontrolled cell growth. Amidst existing treatment limitations, vitamins have emerged as promising candidates for cancer prevention and treatment. This review focuses on Vitamins A, C, E, and D because of their protective activity against various types of cancer. They are essential as human metabolic coenzymes. Through a critical exploration of preclinical and clinical studies via PubMed and Google Scholar, the impact of these vitamins on cancer therapy was analyzed, unraveling their complicated mechanisms of action. Interestingly, vitamins impact immune function, antioxidant defense, inflammation, and epigenetic regulation, potentially enhancing outcomes by influencing cell behavior and countering stress and DNA damage. Encouraging clinical trial results have been observed; however, further well-controlled studies are imperative to validate their effectiveness, determine optimal dosages, and formulate comprehensive cancer prevention and treatment strategies. Personalized supplementation strategies, informed by medical expertise, are pivotal for optimal outcomes in both clinical and preclinical contexts. Nevertheless, conclusive evidence regarding the efficacy of vitamins in cancer prevention and treatment is still pending, urging further research and exploration in this compelling area of study.

KEYWORDS

vit-E, vit-A, vit-D, combination anticancer therapy, vit-C

1 Introduction

Cancer is the health problem of the century, being the second leading cause of death after heart disease worldwide. As the world population is increasing, and as humanity have made such huge progress against causes of death that once killed people early in life, the number of cancer deaths has increased from around 5.7 million in 1990 to 8.8 million in 2017. It is true, the number of cancer deaths is increasing, but yet, individual death rates are falling (1). Cancer is defined as an unregulated proliferation and spread of cells in the body and can reach trillions in number. In a normal setting, human cells grow and multiply via cell division to form new needed cells. When this order breaks down, abnormal cells start to grow and multiply to

develop abnormal tissues (2). The complicated prosperous nature of cancer is affected by various elements including genetic and environmental factors such as tobacco smoking, urbanization, and changing diet patterns. The genetic changes that lead to cancer can happen because of several reasons including errors that occur at cell division, DNA damage that may have been caused by substances in the environment, or genetic predispositions that were inherited from the parents. Each cancer is a unique combination of these genetic changes, and as cancer continues to develop, additional changes will occur (3). Studies devoted to establishing measures that focus on environmental factors have been implemented; nevertheless, little progress in favor of reducing cancer incidence has been made (4). In the domain of cancer biology, cancerous cells develop from healthy cells through a complex process known as malignant transformation, involving several key steps. Firstly, it begins by altering the genetic material of the cells, preparing it to become cancerous. The second step, promotion, is induced by agents known as promoters, which can include substances in the environment or certain medications. Promoters allow cells that were primed to become cancerous, and they do not affect cells that have not undergone initiation. The immune system is usually able to recognize cancer cells and destroy them before they duplicate, this explains why certain cancers are more likely to progress in people whose immune system is impaired. The third step is the spreading of cancer cells through the bloodstream or lymphatic system to nearby or distant locations; it can spread and invade surrounding tissues or organs (Figure 1). As it grows, nutrients are provided by their direct diffusion to cells, this further leads to increased cancer volume, destroying adjacent tissues (5). Understanding cancer's cellular kinetics gives important insight when it comes to designing dosing schedules and timing intervals of treatment (6). Prevailed anticancer treatments such as chemotherapy, surgery, and radiotherapy have been challenging in terms of their adverse effects and disease progression, hence the humongous number of efforts to establish an alternative treatment regimen (7). The new approach is associated with vitamins and recently their anticipated anticancer effects have been considerably analyzed. Vitamins are essential nutrients for human metabolism, taking part in an essential function as coenzymes or enzymes in many imperative procedures for the regular functioning of the body, and it has been obvious that nutritional vitamins essentially contribute to human disease and healing (7). In a comprehensive review of 65 clinical trials involving cancer patients, the safety of various dietary supplements, particularly vitamins, was assessed. The findings revealed that vitamins, among other supplements, were generally safe for cancer patients, emphasizing the importance of continued research on the impact of vitamins in cancer treatment (8). On the other hand, recent findings suggest a potential benefit of calcium and vitamin D supplementation in cancer prognosis, particularly in reducing colorectal and breast cancer mortality. Similarly, post-diagnosis intake of specific antioxidants (such as vitamins C, D, and E) has shown correlations with decreased mortality among cancer survivors. However, these associations, primarily derived from observational studies, warrant cautious interpretation. Further research, especially randomized controlled trials, is essential to clarify these relationships, focusing on optimal dosages and the timing of supplementation post-diagnosis. Developing evidence-based recommendations for cancer survivors necessitates a comprehensive understanding of dietary supplement use alongside conventional treatments (9). The rationale for our deliberate choice of vitamins A, C, E, and D for inclusion in our comprehensive review is rooted in their remarkable attributes that make them ideal candidates for exploration in the context of therapeutic applications. First and foremost, these vitamins are readily

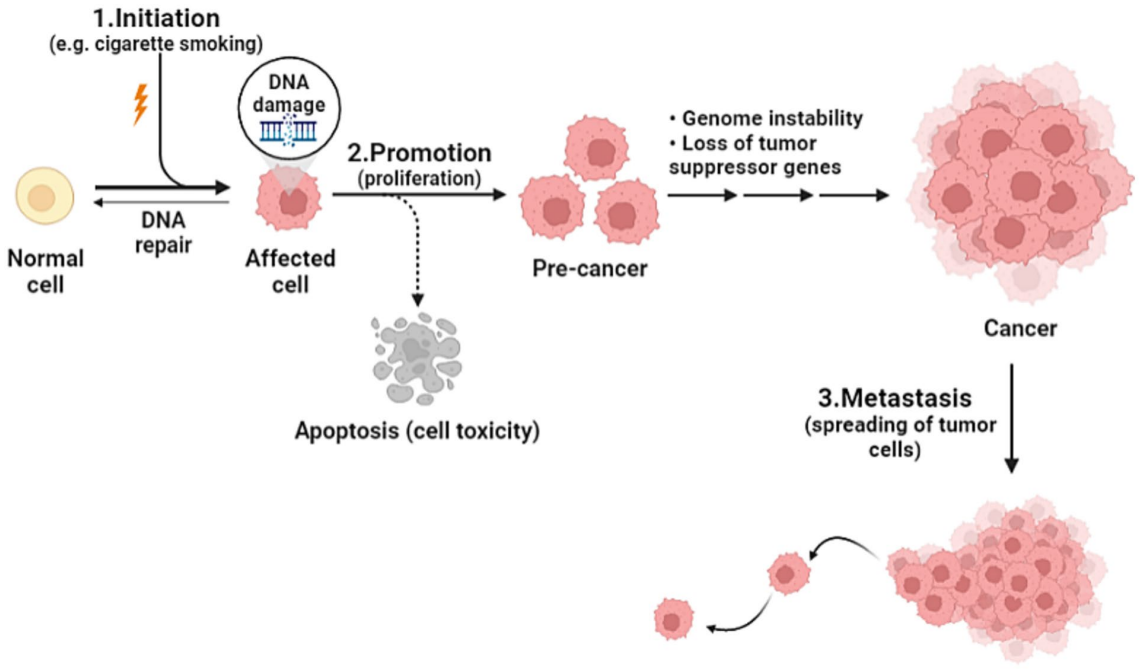

FIGURE 1
Cancer development steps.

available primarily through dietary consumption. This inherent accessibility renders them mainly practical for potential therapeutic interventions. Vitamin A can be found in numerous common foods such as carrots, sweet potatoes, and spinach. Vitamin C is abundantly present in fruits like oranges, strawberries, and kiwi, while vitamin E is found in nuts, seeds, and vegetable oil. Lastly, vitamin D can be synthesized by the skin when exposed to sunlight, and it is also available in fortified dairy products and fatty fish (10–13). Previous reviews of clinical trials, animal models, and *in vitro* studies suggest that dietary vitamins and minerals may contribute to the prevention and treatment of a variety of cancers such as those affecting the breast, lungs, liver, cervix, stomach, brain (glioma), lymphatic system (lymphoma), and prostate (14–26). However, further well-controlled studies are needed for a better understanding of their anticancer potential. In this review, we are going to shed light on the association between pharmacological vitamin doses and cancer and discuss the mechanisms of action. Additionally, we will briefly address how some studies have shown a lower incidence of cancer in vitamin-rich diets, which has received considerable attention in recent years.

2 Methods

Vitamins A, C, E, and D's cancer treatment and prevention effects were examined in a comprehensive literature review. This investigation examined many human and animal scientific studies. Many petri dish and animal experiments were examined. This study examined how vitamins may treat and prevent cancer by reading many research papers. We searched PubMed, Google Scholar, Scopus, Web of Science, and other scientifically correct databases for the information we needed. With search terms like "vitamins," "cancer treatment," "prevention," "chemotherapy," and "immunotherapy," we hoped to find relevant studies and learn from existing research. Researchers looked for English papers on how vitamins A, C, E, and D can treat or prevent cancer. Some studies examined how these vitamins might affect immunotherapy or chemotherapy. After reviewing all literature, 300 studies were found. After that, a strict process selected the best and most relevant studies. After a thorough review, studies that were not directly related to the topic, were not written in English, and did not provide enough experimental details or results were discarded. The best studies to examine and consider were 153. We were able to thoroughly examine the topic because we carefully selected and organized key information.

3 Biological activities of vitamins A, C, D, E and their impact on cancer prevention

Vitamins reflect a concept that involved different groups of bioactive compounds obtaining nutritional benefits for human health (27). Hence, they are one of the main essential nutrients that play a role in enhancing the immune system, improving mental health and the aging process, increasing energy levels, and protecting the cardiovascular system (28). Their biochemical functions in the body are translated by being coenzymes, hormones, antioxidants, promotors of signaling pathways, and modulators of cell and tissue growth and differentiation (29).

Interestingly, vitamin A (VA) showed an indirect impact on regulatory T cell development and cytokines production (30). Due to the chemical structure of VA and carotenoids (the presence of polyene chain), they have exhibited antioxidant activity by protecting cells from the damage of free radicals and singlet oxygen (31, 32). It was reported that dietary VA can improve glutathione peroxidase and superoxide dismutase activities as well as reduce reactive oxygen species (ROS) production (33). Retinol and its derivatives, found in animal-based foods, have essential roles in cell differentiation, proliferation, and apoptosis. They also play a key role in skin and bone growth through retinoic acid. Cytoplasmic binding proteins like CRBP-1 regulate intracellular retinoid levels, affecting retinol uptake and availability. Recent research has linked reduced CRBP-1 levels to increased malignancy in breast, ovarian, and nasopharyngeal cancers. Restoring CRBP-1 could enhance retinol sensitivity and reduce ovarian cancer cell viability, potentially guiding personalized retinoid therapy for cancer patients (34). Moreover, retinoids, when bound to RAR/RXR receptors (RAR: Retinoic Acid Receptor and RXR: Retinoid X Receptor), trigger a series of chromatin structure modifications that can encourage cell differentiation and initiate lasting epigenetic alterations. In the context of cancer cells, these changes hold the potential to induce differentiation toward a less malignant state. Retinoids achieve this by promoting stem cell differentiation and reshaping the gene expression patterns within tumor cells, rendering them more responsive to other therapeutic approaches. Consequently, retinoids are expected to play a significant role in future cancer treatments (35, 36).

On the other hand, Ascorbic acid is a powerful antioxidant in various reactions and metabolic processes. It neutralizes free radicals and toxins, subsequently attenuating oxidative damage and inflammatory responses (37, 38). Acting so, at micromolar concentrations, physiological ascorbate, a fully reduced form of vitamin C (VC), can reduce harmful ROS. Paradoxically, it also functions as a pro-oxidant at millimolar plasma concentrations via intravenous administration of pharmacological ascorbate (39). Besides, several studies have reported the potential of VC in reducing inflammation and modulating inflammatory biomarkers (40–43). A recent study has shown the complicated roles of VC in cancer biology, emphasizing its influence on stem cells, cancer metastasis, and immunotherapy. Vitamin C affects various biochemical reactions in cells, collagen synthesis, and the regulation of hypoxia-inducible factor (HIF), impacting extracellular matrix remodeling and cancer spread. It also displays promise in inhibiting cancer cell glycolysis and enhancing cancer immunotherapy when combined with anti-PD-L1 therapy (44). Beyond its antioxidant properties, VC directly impacts genomic stability, making it relevant in cancer prevention, stem cell therapy, and the treatment of hematological malignancies where TET mutations and aberrant DNA methylation are common (45). In recent years, the role of VC in cancer treatment has gained attention due to its influence on epigenetic regulation. Vitamin C, acting as an antioxidant and a co-factor for crucial enzymes like TET methylcytosine dioxygenases, plays a significant role in cancer therapy. Epigenetic abnormalities are common in cancer, and VC has consistently been shown to enhance DNA demethylation by TET enzymes when combined with DNA methyltransferase inhibitors (46).

As well, strong antioxidant properties are present in all vitamin E (VE) isomers (47). Because their chromanol ring contains phenolic hydrogen, they can scavenge reactive oxygen species (ROS). Vitamin

E's ability to scavenge reactive oxygen species (ROS) and prevent oxidative damage to cells and tissues is due to its phenolic hydrogen (48). Oxidative stress can cause free radical chain reactions that lead to lipid peroxidation in both people and animals used in experiments. Vitamin E is very important for stopping these free radical chain reactions, which stops lipid peroxidation and protects the integrity of biological membranes. As a strong antioxidant, vitamin E helps keep cells and tissues safe from the damage caused by oxidative stress (48). Many cancers, including skin and gastrointestinal cancers, may have oxidative stress as an underlying cause. A transcription factor known as Nrf2 regulates the induction of antioxidant enzymes. Research has demonstrated that specific forms of vitamin E, particularly γ-tocopherol and, to a lesser degree, α-tocopherol, can activate Nrf2. This, in turn, enhances the production of different antioxidant enzymes, such as SOD, catalase, glutathione peroxidase, and phase II detoxifying enzymes. In response to oxidative stress, cells launch an antioxidant response, which aids in cellular homeostasis maintenance (49). One reason why γ-tocopherol and δ-tocopherol are more effective than other tocopherol isoforms is because their chromanol rings have an unmethylated C-5 position. These isoforms are able to effectively combat reactive nitrogen species, such as NO2 and peroxynitrite, due to this structural feature. They can detoxify these reactive nitrogen species more effectively by producing 5-nitro-γ-tocopherol (50). Research has shown that vitamin E metabolites, like CEHCs (carboxyethyl hydroxychromans), can inhibit lipid peroxidation and have strong antioxidant capabilities. The free radical scavenging capabilities of these metabolites are even greater than those of the original vitamin E isoforms. Out of all the metabolites, γ-tocopheryl quinone is the one that triggers the antioxidant response the most effectively. This is accomplished by increasing glutathione levels and activating transcription factor 4 transcription. As a defense mechanism against oxidative stress, this antioxidant response shields cells from harm (51). Tocotrienols, like tocopherols, work as antioxidants by encouraging the production of different antioxidant enzymes, such as catalase and superoxide dismutase (SOD) (52). Tocopherols and tocotrienols are both very good at removing free radicals from membranes and lipoproteins. They can stop fatty acid peroxyl radicals from forming and turn them into tocopheroxyl radicals. These radicals are then broken down to make vitamin E again. This cycle of antioxidants helps keep membranes and lipoproteins intact and protects cell parts from oxidative damage (53, 54). It is widely recognized that eicosanoids, which are generated through the COX-2 and 5-LOX pathways, play a role in the advancement of colon cancer. Inhibiting these pathways is one mechanism by which vitamin E prevents carcinogenesis and reduces inflammation in the colon (55). It is well-established that eicosanoids produced by the COX-2 and 5-LOX pathways contribute to the progression of colon cancer. Therefore, vitamin E's ability to inhibit these pathways contributes to its ability to inhibit colon inflammation and prevent carcinogenesis (55). A group of transcription factors known as peroxisome proliferator-activated receptors (PPARs) include PPAR-α, γ, and δ. These receptors control inflammatory pathways by blocking COX-2. In addition to PI3k/Akt and NF-κB, PPARs are linked to other signaling pathways. The ability of δ-tocopherol to activate PPARγ in various cell lines has been proven in studies conducted in both laboratory and living organism settings. Research has shown that δ-tocopherol inhibits inflammation, slows down cell cycle progression, and triggers cell death by activating PPARγ (56). In addition, research has shown that vitamin E (VE) can inhibit inflammatory markers through various pathways. Important inflammatory factors such as cyclooxygenase 2 (COX-2), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6) are downregulated when prostaglandin E2 (PGE2) is inhibited (57).

Many studies have shown the correlation between vitamin D deficiency and the high incidence of infectious diseases and inflammatory autoimmune diseases (58, 59). Vitamin D reduces inflammation by mediating different pathways such as inhibition of NF-kB, increasing MKP5 expression, and blocking the prostaglandins pathway (60, 61). Regarding antioxidant activity, vitamin D was able to activate the Nrf2-Keap1 antioxidant pathway and improved nephropathy in a diabetic animal model (62). As well, VD supplementation can modulate oxidative stress parameters by reducing nitric oxide (NO), increasing glutathione (GSH) expression, and improving the total antioxidant capacity (63). Moreover, experimental findings indicate that the preventive effects of VD in cancer primarily result from its ability to influence critical biological processes, including cell proliferation, cell differentiation, the expression of growth factor genes, signal transduction, and apoptosis regulation (64). Additionally, VD has been found to interfere with the action of growth factors like insulin-like growth factors (IGF) by promoting the release of IGF binding protein 3 (IGFBP3), which limits the pro-proliferative effects of IGF (65). Vitamin D plays a crucial role in preventing early neoplastic processes through its anti-inflammatory, antioxidant, DNA repair, and cell regulation mechanisms. It also influences the intestinal microbiota, linked to inflammatory bowel diseases and colon cancer (66). It has shown promise in preventing and improving outcomes in colorectal cancer (CRC). Acting as a regulatory prohormone through its widespread vitamin D receptor (VDR) binding, it influences immune modulation and microbial composition in the colonic mucosa, affecting key mechanisms in CRC development (67). Calcitriol, known as bioactive vitamin D, plays a role in regulating numerous biological pathways. Its various actions, such as DNA damage repair and protecting against oxidative stress, can contribute to preservation of somatic stem cells. Additionally, calcitriol may inhibit the growth of cancer stem cells by inducing cell cycle arrest and promoting apoptosis (68). In summary, these vitamins collectively contribute to cancer defense and hold promise for improving cancer-related outcomes.

4 Vitamins as an anticancer agent: preclinical studies

4.1 Vitamin A

Vitamin A is a collection of life-essential, fat-soluble substances with an unsaturated isoprenoid main chain that is derived from both plants and animals. All derivatives of vitamin A share similar structural and physiological functions within the human body. Substances with a common structure of four isoprenoid subunits, whether of synthetic or natural origin, are also categorized as retinoids (69). Nevertheless, the core vitamin A sequence is concealed in their structures, and their activation of retinoid receptors is comparable to most of the other retinoids. Each of these substances is fat-soluble and, unlike water-soluble vitamins, accumulates rapidly in the body, particularly in the adipose tissue and liver. This is advantageous since

temporary vitamin A deficiency is not linked with clinical symptoms, but on the other side, this can lead to accumulation as well as toxic effects (69). Vitamin A can be obtained from the diet either as provitamin A (carotenoids) through plants or as vitamin A (retinol and its near derivatives) through animal-based foods. The most common type of retinoid in the body is retinol. Furthermore, all-trans retinoic acid and 11-cis-retinal are the most active substances within the body (69).

Multiple mechanisms have been studied and proven for vitamin A, including more than gene signaling pathways in terms of anticancer effects (70), the effect of retinoids on mitochondrial functions (71), and other mechanisms and pathways that have been studied and proven (72, 73). All-trans retinoic acid, an endogenous retinoid, has been shown to inhibit the expression of PPARγ and to enhance the expression of DOK1 as it regulates the DOK1/PPARγ pathways thus inhibiting cell proliferation in breast cancer (MCF7 cell line) (74). As well, the synthetic retinoid (ST1926) had a greater effect on MDA-MB-231 and MCF7 cell lines represented by binding to β-catenin and cyclin D1, which regulates the Wnt/−catenin signaling pathway (75). Peretinoin, a synthetic retinoid, revealed a potential to inhibit hepatocarcinogenesis by downregulation of sphingosine kinase 1, which resulted in hindering cancer progression (76). It is also believed that the regulation of microRNAs is linked to retinoid anti-cancer mechanisms. An *in vitro* and *in vivo* study has shown the synergistic effect of all-trans-RA when combined with paclitaxel in A549 cell line. The difference in IC50 values between the combination and all-trans-RA alone was almost three times more efficient, which means that the cytotoxicity of the combination is higher. The researchers went further by testing this combination on solid tumors in mice, and it was proven that the treatment prolonged their survival rate and effectively prevented tumor progression (76). As well, all-trans-RA with a γ-secretase inhibitor (DAPT) as a combination therapy has shown better results than monotherapy in gastric cancer cells (MKN45 cells). Using MTT assay, a significant decrease in cell viability was reported. Additionally, it induced apoptosis by increasing caspases expression (77). An *in vitro* study demonstrated the antiproliferative activity of all-trans-RA-Podophyllotoxin conjugate against human gastric cancer cells (MKN-45 and BGC-823 cell lines). It inhibited cell growth by promoting apoptosis and blocking the ERK1/2 and AKT signaling pathways (78). In addition, all-trans-RA effectively reduced myeloid-derived suppressor cell (MDSC) accumulation and increased the frequency of CD8+ T cells in mice with cervical tumors. Notably, combining all-trans-RA with anti-PD-L1 antibody treatment resulted in further delayed tumor growth, along with an increased proportion of activated T cells and elevated levels of immune-stimulating cytokines in tumors (79). As well, all-trans-RA significantly inhibited hepatocellular carcinoma progression, reduced MDSCs and tumor-associated macrophages, and suppressed the expression of immunosuppressive molecules in the tumor microenvironment. In particular, it reduced the intratumoral infiltration G-MDSCs and the expression of protumor immunosuppressive molecules including arginase 1, iNOS, IDO and S100A8 + A9, which was accompanied by increased cytotoxic cell infiltration (80). Moreover, in a 4T1 mouse model of breast cancer, researchers employed a combination of cryo-thermal therapy and all-trans-RA to address myeloid-derived suppressor cells (MDSCs). The combination significantly increased mouse long-term survival rates by promoting MDSC maturation, reducing suppressive molecule expression, and inhibiting metabolic pathways. Early after treatment, Th2 and Treg subsets decreased, improving Th1-dominant CD4+ T-cell differentiation, while later stages showed enhanced cytotoxicity of CD8+ T cells and natural killer cells. This approach offers promise for breast cancer therapy to overcome MDSC-induced immunosuppression (81). Chuang et al. suggested a synergistic therapeutic effect of polyinosinic-polycytidylic acid combined with 13-*cis*-retinoic acid in neuroblastoma (82). Using this combination in a mouse xenograft model led to suppressing tumor development through stimulating retinoic acid receptors beta (RAR-β), and preventing vessel formation (82).

4.2 Vitamin C

Vitamin C (VC) is a water-soluble natural compound, that has shown multiple mechanisms to target cancer. VC is being widely studied and this may be attributed to its high safety profile. It cannot be synthesized by the human body, and strictly and easily obtained from dietary intake to achieve a daily allowance of 75–90 mg per day. It can be obtained from fruits, like citrus fruits and berries, and vegetables like raw sweet pepper and broccoli, to achieve a plasma ascorbate concentration of 30–80 μM (12). Ascorbic acid is a powerful antioxidant in various reactions and metabolic processes. It neutralizes free radicals and toxins, subsequently attenuating oxidative damage and inflammatory responses (12, 83). VC has shown both preventive and anticancer capacities. As for its preventive capacity, studies have suggested a possible protective role of high VC intake with reduced colorectal cancer (84, 85), stomach cancer (86, 87), and pancreatic cancer (88). In addition, a recent *in vivo* study suggests that oral VC may have a preventive effect on the development of leukemia (89). Determining the adequate amount of oral VC is essential for optimizing human health and preventing chronic diseases including cancer.

Several studies have aimed to demonstrate the anticancer activity of VC alone or as adjunct therapies with conventional treatment methods. A study experimented VC in combination with each of eribulin mesylate, tamoxifen, fulvestrant, and trastuzumab on a variety of breast cancer cell lines (MCF7, SK-BR3, MDA-MB-231, and breast epithelial cells MCF10A), have shown that high-dose VC with each of those anti-cancer drugs had decreased the cell viability of cancer cells more comprehensively than high-dose of VC or chemotherapy alone (90). VC's ability to reduce oxidative stress in order to manage the side effects of different cancer treatments has been explored in multiple studies (91, 92). An *in vitro* study has examined VC along with methotrexate (MTX) on their effectiveness against human glioblastoma multiforme (Human glioblastoma DBTRG cell line) (93). The results showed that VC alone (5 μM) is not cytotoxic to either DBTRG or HK-2 cells; however, in combination with 0.01 μM MTX was significantly able to reduce cell viability. Another *in vitro* study explored VC effects on oral squamous cell carcinoma (OSCC) using human OSCC-derived cell lines CAL27, SCC9, and SCC25 (94). Their finding suggested various mechanisms by which VC can induce anticancer effects. Initially, VC led to a significant rise in the ROS levels, inducing DNA damage and ATP depletion stresses. It also inhibited Bcl-2 expression and promoted Bax expression and caspase-3 cleavage. Additionally, VC induced cell cycle arrest at the G0/G1 phase in OSCC cells. Their data demonstrated a

significant inhibitory impact of VC on the colony-forming ability of OSCC cells, dependent on concentration and time (94).

Another aspect of VC is its ability to modulate anticancer immune responses (39, 95). A study, using several mouse cancer models including colorectal, breast, melanoma, and pancreatic, explored the impact of daily intravenous high-dose VC (4 g/kg) (96). They observed that, in most cases, tumor growth was delayed only in the presence of a fully competent immune system, which suggests that VC antitumor activity is dependent on some immunomodulatory functions and not only on its pro-oxidant effects. Despite this being mostly an uncharted area, this study is evidence of VC's capacity to modulate infiltration of the tumor microenvironment by cells of the immune system, delaying cancer growth in a T-cell-dependent manner. VC enhances the cytotoxic activity of adoptively transferred CD8 T cells, and also co-operates with immune checkpoint therapy (ICT) in many cancer types, proving that a combination of VC and ICT can be curative in models of mismatch repair deficient tumors with high mutational burden. To optimize the antiproliferative impacts of VC in murine tumors such as breast, colorectal, melanoma, and pancreatic cancers, a fully functional immune system is required (96).

Recent studies have provided a better mechanistic understanding of VC anti-cancer effects. VC has been proven to have anti-tumorigenic activity by increasing the reactive oxygen species in cancer cells without major toxicities. It can address three common vulnerabilities shared by many cancer cell, including redox imbalance, epigenetic programming, and oxygen-sensing regulation (39). Cancer cells tend to depict compensation mechanisms to survive. Antioxidants can be both destructive and accelerative of tumor progression. So, alone, it cannot prevent or suppress cancer. Hence the employment of pro-oxidant anticancer therapies, such as radiation, but serious collateral damage can occur causing a narrow therapeutic window. Ascorbate can overcome this issue via two common features of cancer cells: elevated levels of labile iron both extracellular and/or intracellular, and heightened dependence on glucose uptake and glycolysis. In the presence of redox-active transition metals, VC exhibits pro-oxidant effects, inducing oxidative stress through the formation of ROS or by inhibiting the antioxidant system (39, 97). In the process of cellular iron metabolism, the labile iron pool is chelatable and redox-active. When ferrous ion reacts with peroxide in those pools, they can produce the damaging hydroxyl radical •OH via the Fenton reaction. Ascorbate then comes to sustain this reaction by donating electrons to ferric ions generating redox active ferrous ions and perpetuating the generation of ROS in continuation that contributes to cell death. It was shown that Asc•− and peroxide were generated *in vivo* following intravenous ascorbate injections in rats (0.5 g/kg), and the production was ascorbate-dose-dependent (98). In an animal model study, daily high-dose ascorbate (4 g/kg) inhibited neuroblastoma growth, and tumors had increased activity of checkpoint kinase 2 (CHK2) and histone 2AX (H2AX). This implies that pharmacological ascorbate can induce DNA damage in tumors implanted in animals (99). Its selectivity toward cancer cells can be explained by different potential reasons. First, it has been shown in various types of cancer such as breast, prostate, and lymphoma that iron metabolism can be reprogrammed either by the upregulation of several iron intake pathways or the downregulation of iron export and storage pathways (100, 101). For instance, in breast cancer cells, the intracellular pool of labile ions is twice as elevated as in normal breast epithelial cells. Additionally, tumor cells may exhibit greater vulnerability to high-dose ascorbate compared to normal cells as they can generate higher levels of peroxide and •OH than their normal counterparts (39).

Oncogenic KRAS or BRAF mutations are contributors to the Warburg effect by upregulating GLUT1. This exploitation by cancer could serve as a potential therapeutic strategy for targeting the disease. When ascorbate is administered in high doses, it gets oxidized to DHA (Docosahexaenoic acid). DHA is structurally similar to glucose and thus, efficiently taken up via GLUT1 in KRAS or BRAF mutant cells. DHA is rapidly reduced back to ascorbate inside the cell. The decrease in intracellular antioxidants and subsequent rise in ROS levels interferes with glycolytic pathways, resulting in an energy crisis. For instance, recent studies demonstrated the selective elimination of gastric cancers and von Hippel–Lindau-null renal cancers, characterized by high GLUT1 expression, through high-dose ascorbate treatment (102, 103). Ascorbate has a significantly long half-life. It efficiently and continuously generates DHA in the tumors' oxidative microenvironment. In addition, extracellular peroxide generated via the oxidation of ascorbate contributes to increased levels of DHA, as DHA is the main oxidized form of ascorbate. Normal erythrocytes are protected from high-dose ascorbate due to their increased level of antioxidant enzymes like GSH and have an enhanced glucose flux into the pentose phosphate (PP) pathway to generate more NADPH, which is a crucial molecule for the recovery of GSH. Patients who are deficient in G6PD, which is the rate-limiting enzyme in PP pathway, have a lagging in NADPH production, hindering those cells vulnerable to VC-induced oxidative stress, subsequently leading to erythrocytes death, thereby causing anemia (39).

Another way that VC acts to hinder cancer is its ability to activate 10-11 translocation (TET) proteins. Those proteins belong to the αKGDD enzyme family and they demethylate DNA and therefore, liberate the abnormal epigenetic reprogrammed methylated DNA seen in cancer. Ascorbate can donate an electron to a ferric ion and convert it to a ferrous ion, which is critical for TET activity. Ascorbate treatment *in vitro* increased TET activity, which led to DNA methylation, and subsequently liberated the expression of tumor suppressor genes, which increased chemosensitivity (104). An *in vivo* study has shown that daily injection of high-dose ascorbate (4 g/kg) in a leukemic mouse model, promoted DNA demethylation and the expression of genes critical for myeloid cell differentiation, and subsequently recapitulated the TET2 restoration phenotypes (105). Other αKGDDs such as JHDM and ALKB have yet an unclear role in cancer development and to what extent ascorbate influences their activity.

Usually, solid tumors obstruct and compress surrounding blood vessels, resulting in a hypoxic encounter. Tumor cells in their striving to survive, adapt to this hypoxic microenvironment. One of those oxygen-sensing regulations is the tumor cell's activation of HIF1, an evolutionarily conserved transcription factor. HIF1 consists of two subunits, the O2-regulated HIF1α and a constitutively expressed HIF1β. Oxygen regulates HIF1α activity through "Proline hydroxylase domain proteins" (PHD) and HIF hydroxylases. Under normal oxygen concentration, HIF1 transcription is inhibited. Under hypoxic conditions, PHDs and HIF hydroxylases are inactive, leading to HIF1 activation. So, cells that lack adequate amounts of ascorbate can have increased HIF1α function, which may potentially play a role in tumor progression. HIF1 is an important target in cancer therapy via HIF

hydroxylase that is enhanced by ascorbate treatment, thus suppressing tumor growth. Two studies support this hypothesis, indicating a mounting body of evidence that suggests inhibition of HIF1α-dependent tumor growth by ascorbate (106, 107). An *in vitro* study demonstrated that pharmacological ascorbate led to a reduction in the expression of HIF1α and its downstream target GLUT1 in thyroid cancer cells (108).

The previously mentioned findings have renewed people's scope and vision of the pharmacological antitumor effects of VC, putting it in the spotlight for further elucidation of its function and mechanism.

4.3 Vitamin E

The compound known as α-tocopherol, often termed the "classic" form of vitamin E, has shown significant efficacy in traditional fertility restoration tests compared to other tocopherols. It is primarily present in wheat germ, almonds, and sunflower oil. On the other hand, γ-tocopherol is more commonly found in the American diet and is frequently present in vegetable oils like soybean, corn, and cottonseed (109). δ-Tocopherol is predominantly present in soybean and castor oils, with a comparatively lower concentration in wheat germ oil (52). The production of β-tocopherol (γ-TmT) frequently occurs as an incidental outcome in the course of vegetable oil refinement (110). Tocotrienols are commonly consumed in diets prevalent in East-South Asian regions, with palm and annatto oils serving as their primary sources (111).

A plethora of epidemiological research have investigated the association between the risk of cancer and vitamin E. Emerging studies indicate that vitamin E exhibits potential as a viable contender for cancer adjuvant therapy, primarily due to its anticarcinogenic characteristics. The initial hypothesis regarding the chemopreventive effects of vitamin E emerged due to the observation that individuals residing in the Mediterranean region, whose diets are rich in various isoforms of vitamin E, demonstrate a decreased susceptibility to colon cancer in comparison to individuals residing in Northern Europe and the United States (49, 112). Vitamin E exhibits an inhibitory effect on cell proliferation through the induction of apoptosis and cell cycle arrest. Apoptosis occurs via two distinct pathways: the extrinsic pathway, which is mediated by death receptor signaling, and the intrinsic pathway, which is impacted by mitochondrial rupture leading to the release of cytochrome c into the cytosol. In the end, these routes converge to trigger the activation of execution caspases, particularly caspase-3, resulting in the fragmentation of poly-ADP-ribose-polymerase (PARP) (52). Angiogenesis plays a crucial role in both the growth and spread of tumors, involving the rapid proliferation and migration of endothelial cells. Studies conducted in both laboratory settings (*in vitro*) and living organisms (*in vivo*) have provided evidence that certain isoforms of vitamin E, particularly tocotrienols, possess the capability to hinder the angiogenesis process (113). Additionally, it has been found that tocotrienols have the ability to decrease the expression of VEGF receptors. In a study conducted by Shibata et al., it was observed that tocotrienol administration led to the suppression of hypoxia-inducible factor (HIF-1), a transcription factor known to induce the expression of vascular endothelial growth factor (VEGF) and initiate angiogenesis in the presence of low oxygen levels. The study focused on human colorectal adenocarcinoma cells in an *in vitro* setting. The suppression of HIF-1 resulted in the blocking of the release of angiogenic factors (114). The proangiogenic cytokines interleukin-6 and interleukin-8 are well-known to elevate VEFG levels. Tocotrienol therapy resulted in a reduction of IL-6 and IL-8 levels in human umbilical vein endothelial cells (HUVEC cells) (115). Tocotrienols have demonstrated inhibitory effects on the phosphorylation of the PI3k/Akt signaling pathway, resulting in the downregulation of key signaling molecules including endothelial nitric oxide synthase (eNOS), glycogen synthase kinase 3 (GSK3), and extracellular signal-regulated kinase (ERK). This pathway plays a role in angiogenesis by promoting the binding interaction between vascular endothelial growth factor (VEGF) and its corresponding receptor (116).

In a comprehensive study investigating the anticancer effects of vitamin E variants, including natural (αT) and synthetic (γT) forms, on breast cancer models, γT emerged as particularly promising. *In vitro* experiments demonstrated γT's efficacy in inhibiting colony formation and inducing apoptosis in various breast cancer cell types. The underlying mechanisms involved activation of caspases-8 and -9, coupled with downregulation of c-FLIP and survivin. Concurrently, assessments in a BALB/c mouse mammary cancer model revealed that both all-rac-αT and all-rac-αTAc significantly reduced tumor development and lung metastasis, highlighting their potential as anti-tumor and anti-metastatic agents in breast cancer. The study also compared αT, all-rac-αT, and all-rac-αTAc, with αT and all-rac-αT exhibiting lesser efficacy. The findings emphasize γT's persistent and robust anticancer effects across *in vitro* and *in vivo* models, positioning it as a noteworthy candidate for breast cancer treatment. Further research is needed to unravel the molecular mechanisms and assess efficacy in clinical settings (117).

Research on β-tocotrienol (β-T3) showed its ability to lower PD-L1, a key immune checkpoint ligand, in both *in vitro* and *in vivo* settings. This decrease was caused by JAK2/STAT3 pathway inactivation. Results showed improved immune response and reduced PD-L1-induced tumor-intrinsic signaling, highlighting β-T3's anticancer potential (118).

δ-Tocotrienol (δ-T3) has been widely recognized for its remarkable anticancer properties (119). In accordance with prior investigations, it has been observed that the administration of the antioxidant δ-tocotrienol (δ-T3) elicits apoptotic, paraptotic, and autophagic responses in castration-resistant prostate cancer (CRPC) cells (120). Recent findings show that δ-T3, a vitamin E variation, increases ROS and mitochondrial Ca2+ levels in PC3 and DU145 cell lines. This study aims to understand the mechanisms behind δ-T3's anticancer effects on PC3 cells, focusing on the role of Ca2+ and ROS. The study examines how δ-T3 affects biological processes such as cell viability, apoptosis, paraptosis, autophagy, and mitophagy (121). Consistent with our earlier hypothesis, an elevated concentration of Ca2+ ions was identified as a contributor to the pro-death effects triggered by δ-T3. Interestingly, this excess Ca2+ did not influence autophagy and mitophagy processes in autophagy-defective DU145 cells. Additionally, our study reveals that δ-T3 retains its anticancer properties in this cell line, and notably, these properties persist despite an excessive accumulation of reactive oxygen species (ROS). This observation highlights the limited vulnerability of these cells to oxidative stress induced by ROS (121). Collectively, these discoveries demonstrate that δ-T3 induces structural and functional abnormalities in the mitochondria of CRPC cells, leading to cell death. These

insights provide novel mechanistic understanding into the anticancer effects of this compound (121).

In a previous investigation, the utilization of mice as experimental subjects was undertaken to examine the levels of tocotrienol (T3) in plasma. T3, a member of the vitamin E family renowned for its anti-cancer attributes, was analyzed alongside a recently developed succinate ether derivative of T3 known as 6-O-carboxypropyl-α-tocotrienol (T3E). The primary objective of this study was to discern any potential disparities in pharmacokinetics between these two compounds (122). A mouse xenograft model was employed to evaluate the impact of oral administration of T3E on tumor growth in human malignant mesothelioma MM cells. Specifically, the H2052 cell line was utilized for this assessment (122). Tumor volume was significantly decreased in mice given 150 mg/kg T3E orally once every 2 days for 10 days without any reduction in body weight, indicating that T3E might have anti-MM effects (122). An *in vitro* study was undertaken to investigate the anticancer effects and mechanisms of action of methotrexate (MTX) in conjunction with vitamin E variants (tocopherol) and derivatives (tocopherol succinate) on triple-negative breast cancer (TNBC) (123). The study evaluated cell survival rates and protein levels using the MTT test and western blot analysis. Results indicated that combined therapy with MTX and tocopherol inhibited the growth of triple-negative breast cancer (TNBC) cells. Interestingly, varying doses of MTX exhibited distinct lethal effects on cells treated with tocopherol succinate. Higher MTX doses enhanced the anticancer activity of tocopherol succinate, while lower doses impeded it. Furthermore, the combined treatment demonstrated caspase-3 activation and poly (adenosine diphosphate ribose) polymerase cleavage in the treated cells (123). Against chondrosarcoma cells, a form of cartilage cell malignant primary bone tumor (124), Annatto tocotrienol (AnTT), -T3, and -T3 have anticancer properties. AnTT, -T3 and -T3 produced G1 arrest in SW1353 cells after 24 h of treatment, which escalated to apoptosis after 48 h. Furthermore, tocotrienol treatment caused considerable cytoplasmic vacuolation in SW1353 cells. The transcriptome analysis after tocotrienol therapy revealed enhanced signaling pathways in the endoplasmic reticulum stress, unfolded protein response, autophagy, and transcription (125).

4.4 Vitamin D

Vitamin D (VD) is a lipid-soluble vitamin that belongs to steroidal hormones (126). Vitamin D is found in two forms: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) (127). Yeast and plants can produce ergocalciferol while humans obtain it from their diet. The human body can make vitamin D endogenously by exposing the skin to UV radiation from the sun and then converting cholesterol to vitamin D (128). Vitamin D can undergo different metabolism processes in the liver and kidney through the hydroxylation process. Vitamin D3 25-Hydroxyvitamin is produced by hydroxylation in the liver, whereas calcitriol (1,25-dihydroxycholecalciferol), the active form of vitamin D, is produced in the kidney (129). Calcitriol can bind to a special receptor called vitamin D receptor (VDR) and bind to DNA promoter sequences then control the gene expression. In addition, Calcitriol can play a major role in triggering Intracellular signaling such as Ca2+ and Cl− channels (130). In recent years, scientists conducted many studies and research to show either the effect of VD on cancer or enhancing the chemotherapy response. Vitamin D low levels significantly increased tumor growth in solid and non-solid types (131).

Most studies have found that the status of VD in serum can be a risk factor in various kinds of tumors for instance gastric, breast, colon, and prostate tumor (132). Furthermore, VD is important in controlling the tumorigenesis pathway, from admission to metastasis and microenvironment responses (64). Through a variety of mechanisms, including the regulation of cell behaviors like proliferation, differentiation, increased maturation and apoptosis, autophagy, and epithelial-mesenchymal transition (EMT), as well as the modulation of interactions between cells and their microenvironment, such as the suppression of inflammation, the immune system, angiogenesis, and anti-oxidants, vitamin D can control tumor cells (64). Furthermore, VD had been showed to activate apoptosis in cancer cells such as breast and carcinomas of the colon but not in a variety of prostate carcinoma cells, showing that various tumor cells react differently to vitamin D (133).

The animal model preclinical studies revealed that VD treatment is essential in controlling tumor development (134). A significant variable linked to tumor aggression in prostatic cancer (PCa) is the expression of Lysine-specific demethylase 1A (LSD1), which controls the expression of both estrogen and androgen receptors (ER) (135). Lysine-specific demethylase 1A (LSD1) regulates the transcriptional process of vitamin D receptors (VDR). They evaluated the role of LSD1 in VDR-mediated gene transcription for Cdkn1a, E2f1, Cyp24a1, and S100g using CWR22 cells transplanted in athymic nude mice via qRT-PCR-TaqMan. The cancer progression in xenograft mouse models correlated to the elevation of LSD1 and VDR protein levels (135). The findings showed that lowering LSD1 lowers PCa survival, and information on gene expression suggests that LSD1 has a dual co-regulatory function for VDR. LSD1 is regulating the 1, 25(OH)2D effects brought on by target genes by functioning as a coactivator and corepressor of VDR action. Translation (135).

In an *in vivo* examination, they used a non-immunodeficient MMTV-PyMT mice model for metastatic breast cancer to examine the effect of VD expression on breast tumor development. The study demonstrated that using exogenic of 25(OH)D slow down cancer growth with no hypercalcemic was observed (136). When compared to a normal diet (1,000 IU/kg), the non-immunodeficient MMTV-PyMT (mouse mammary tumor virus-polyoma middle tumor-antigen) mouse model of metastatic breast cancer with a low VD diet (25 IU/kg) demonstrated a significant advancement of mammary neoplasia (136). Moreover, systemic perfusion containing 1,25(OH)2D or 25(OH)D delayed the development of tumors and reduced lung metastasis. Equally metabolites decreased Ki-67, cyclin D1, and ErbB2 levels in cancer. In constant, the profusion of D 25(OH)obtained increasing levels of vitamin D up to 50% in tumor cells, which indicates effective activation and good cancer invasion. The obtained results in this study revealed that neoplasia, tumor growth, and metastasis can be delayed by vitamin D (136).

To determine if vitamin D-facilitated reduction of cell growth is associated with JNK1 (c-Jun NH2-terminal kinases), an *in vitro* study was carried out utilizing colorectal cells (137). The HT29 and Caco-2 human colon cancer cell lines as well as the HEK 293 T human embryonic kidney cell line were treated with calcitriol (1,25(OH)2-D3). Calcitriol was able to reduce cell creation in HT29 colon tumors and was linked with cell cycle stop. The phosphorylation of JNK1 and elevated expression of the cell cycle regulators p21 and p27 may

be connected to the inhibitory effects of calcitriol (137). It has been shown unequivocally that JNK1 interacted with VDR both physically and functionally, gradually regulating both transcriptional and translational levels of VDR expression. The JNK1/VDR connection generated calcitriol-mediated suppression of cancer cell growth through JNK1 activation (137).

1,25-dihydroxyvitamin D (1,25(OH)2D) was discovered to predominantly modulate ROS and mitochondrial activity in an *in vitro* investigation employing osteosarcoma cells, which led to anticancer effects. The treatment led to reduced mitochondrial ROS and altered mitochondrial dynamics, while enhancing mitophagy and ROS defense mechanisms. Key molecular changes included the activation of tumor-suppressing pathways and the relocalization of the mTORC1 inhibitor DDIT4/REDD1, indicating a shift toward non-oxidative energy metabolism and reduce of tumor cell growth. This highlights the role of 1,25(OH)2D in controlling osteosarcoma cell creation through mitochondrial and redox balance regulation (138).

Moreover, it was found that effective vitamin D [(1,25(OH)2D3)] significantly hindered the growth of CD44-expressing human gastric cancer cells. Researchers linked the vitamin D impact to the Wnt/β-catenin signaling pathway and CD44 downregulation. Positive correlations were observed between CD44 and Vitamin D Receptor gene expressions in gastric tumor cells and patient tissues. In mouse model, both oral vitamin D intake and injections of 1,25(OH)2D3 overcame gastric cancer growing and CD44 expression, suggesting a potential therapeutic role for vitamin D in gastric cancer management and prevention (139).

Table 1 summarizes the preclinical studies of the mentioned vitamins.

5 Clinical trials

Numerous *in vitro* studies determined the importance of vitamins in preventing cancer initiation and empowering the immune system to defeat tumorigenesis. To support the preclinical results several clinical trials have been conducted using vitamins as adjuvant treatment with chemotherapy or immunotherapy.

5.1 Vitamin A

In a randomized, controlled, phase 3 trial, arsenic acid, and all-trans retinoic acid (45 mg/m^2) treatment was applied in 235 patients with acute promyelocytic leukemia (140). The outcomes of this trial have shown that using this combination led to an increase in the cure rate and reduce relapse chances (140).

Another clinical trial demonstrated the effect of oral vitamin A (6,000 IU/day) in patients (n = 32) with low-risk gestational trophoblastic neoplasia who receive methotrexate chemotherapy (141). The intervention group exhibited a reduction of chemoresistance as well as a downregulation of the tumor marker β-hCG level (141). It was reported in a randomized double-blind study that using vitamin A supplementation (8,000 IU/8 h) with neoadjuvant chemotherapy (64 weeks) in 15 patients (total sample size is 30) has significantly improved the therapeutic response in the treatment of an advanced cervical carcinoma (142).

5.2 Vitamin C

Vitamin C is well-supported with studies that prove its direct potential to hinder cancer cell growth in preclinical models, yet there is limited clinical evidence regarding its anti-tumoral efficacy. Many studies have scrutinized the effects of pharmacological VC's mechanisms on targeting vulnerable survival utilities of cancer cells that are essential for their perpetuating growth; however, the outcomes of high-dose intravenous VC in terms of its anti-cancer effects remain controversial. In the 1970s, clinical trials on patients with terminal cancer showed that intravenous high-dose ascorbate treatment, that achieved a peak plasma concentration of 6 mM, extended their survival (143, 144), whereas in the 1980s they failed to confirm these findings because ascorbate was given at the same dose orally and the peak plasma concentration was of less than 200 μM (145, 146). This was revealed with follow-up studies which concluded that the route of administration strongly affects VC pharmacokinetics, hence the discrepant results (147). A recent clinical study evaluated the addition of pharmacological ascorbate to platinum-based chemotherapy in patients with advanced-stage non-small cell lung cancer (NSCLC). This study was conducted on 38 patients who were administered 75 g ascorbate twice per week intravenously with carboplatin and paclitaxel every 3 weeks for four cycles. Reported results have shown that the addition of pharmacological ascorbate to platinum-based chemotherapy significantly improves tumor deterioration in the advanced stage of NSCLC, by meeting its primary endpoint with an objective response rate of 34.2% and reaching a disease-control rate of 84.2%. cytokine and chemokine levels propose that the studied combination induces an immune response by altering the host immune defenses (148). Another clinical study investigated the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D) of pharmacological ascorbic acid when it is combined with mFOLFOX6 or FOLFIRI with or without bevacizumab regimens in patients with metastatic colorectal cancer or gastric cancer. Thirty-six patients received 0.2–1.5 g/kg ascorbic acid infusion, in the dose-escalation phase, once daily (days 1–3) with mFOLFOX6 or FOLFIRI in a 14-day cycle until the MTD was reached. Followed by the speed-expansion phase in which pharmacological ascorbic acid was administered at the MTD, but the MTD was not reached and the RP2D was established to be 1.5 g/kg/day, days 1–3, with mFOLFOX6 or FOLFIRI. Results have reported 58.3% objective response rate, and 95.8% disease-control rate. This study has shown that combining pharmacological ascorbic acid with mFOLFOX6 or FOLFIRI has a significant decrease effect in all-grade and grade ≥ 3 bone marrow and gastrointestinal toxic effects (149).

5.3 Vitamin E

Furthermore, within the confines of a randomized controlled study, a total of 89 individuals who had been clinically diagnosed with differentiated thyroid cancer were subjected to I-131 radiation therapy. In the subsequent phase of the study, participants were assigned to receive various interventions, namely vitamin E ($n = 30$), vitamin C ($n = 30$), and supragingival scaling with vitamin C ($n = 29$). The present study aimed to investigate the potential protective effect of vitamin E on salivary glands, with a specific focus on enhancing parotid excretion function. The results obtained from the experimental

TABLE 1 Preclinical studies of vitamins A, C, E, and D.

	Form of the vitamin	Type of cancer	Model of the study	Mechanism of action	References
Vitamin A	All-trans retinoic acid	Breast cancer	*In vitro* (MCF-7 cells)	↓expression of PPARγ	(74)
				↑stimulation of DOK1/PPARγ pathways	
				↓ cell proliferation	
	Synthetic retinoid	Breast cancer	*In vitro* (MCF-7 and MDA-MB-231)	↓ Wnt/−catenin signaling pathway	(75)
				↓ cell proliferation and differentiation	
	All-trans retinoic acid	Lung cancer	*In vitro* (Huh-7 cells)	↑ survival rate	(76)
			In vivo (SPHK1 knockout mice and SPHK1 transgenic mice)	↓ tumor progression	
	All-trans retinoic acid	Gastric cancer	*In vitro* (Human GC cell lines, AGS and MKN-45)	↓ cell viability	(77)
				↑ caspases	
				↑ apoptosis	
	All-trans-RA-Podophyllotoxin	Gastric cancer	*In vitro* (MKN-45 and BGC-823)	↓ cell growth	(78)
				↓ ERK1/2 and AKT signaling pathways	
				↑ apoptosis	
	Polyinosinic-polycytidylic acid/ 13-*cis*-retinoic acid	Neuroblastoma	*In vitro* [(SK-N-AS) (CRL-2137), SK-N-FI (CRL-2142), SK-N-DZ (CRL-2149), and BE(2)-M17 (CRL-2267)]	↑ stimulation of retinoic acid receptors beta (RAR-β)	(82)
			In vivo (nonobese diabetic/SCID) (NOD/SCID, NOD.CB17-Prkdcscid/NcrCrl) mice	↓ vessel formation	
	all-trans-RA	Cervical cancer	*In vivo* (BALB/c mice model)	↓ MDSC	(79)
				↑ CD + 8 T cells	
				↓ tumor growth	
	all-trans-RA	Hepatocellular carcinoma	*In vitro* (HepG2, Hepa1-6 and H22 hepatoma carcinoma cell line)	↓ MDSC	(80)
			In vivo (C57BL/6 N mice with Hepa1-6 tumor)	↓ tumor-associated macrophages	
				↓ arginase 1, iNOS, IDO, S100A8 + A9	
	all-trans-RA	Breast cancer	*In vivo* (4 T1 mouse model of breast cancer)	↑ mouse long-term survival rates	(81)
				↑ cytotoxicity of CD8+ T cells and natural killer cells	
Vitamin C		Breast cancer	*In vitro* (MCF10A, MDA-MB-231, MCF-7, and SK-BR-3)	↓ cell viability	(90)
		Brain cancer	*In vitro* [human glioblastoma DBTRG and human renal tubular epithelial (HK-2)]	↓ cell viability	(93)
		Oral cancer	*In vitro* (human OSCC-derived cell lines CAL27, SCC9, and SCC25)	↑ ROS level	(94)
				↓ Bcl-2	
				↑ Bax expression, caspases	
				↑ cell cycle arrest at the G0/G1 phase	
		Colorectal cancer, breast cancer, melanoma, pancreatic cancer	*In vivo* (C57BL/6 J, BALB/c, FVB, and NOD-SCID mice)	↓ tumor growth	(96)
				↑ CD8 T cells activity	

(Continued)

TABLE 1 (Continued)

	Form of the vitamin	Type of cancer	Model of the study	Mechanism of action	References
	Ascorbate	Lymphoma	*In vitro* (DLBCL cell line OC-LY1)	↑ TET activity ↑ expression of tumor suppressor genes ↑ chemosensitivity	(104)
	Ascorbate	Leukemia	*In vivo* (Vav-tTA (VTA) transgenic mice)	↑ DNA demethylation ↑ TET2 restoration phenotypes	(105)
	Ascorbate	Thyroid cancer	*In vitro* (FTC133 and 8305c)	↓ expression of HIF1α and GLUT1	(108)
Vitamin E	β-tocotrienol	Prostate cancer and lung cancer	*In vitro* (Mouse Lewis lung cancer (LLC) cells, DU145 human prostate cancer cells and Jurkat T cells) *In vivo*	↓ PD-L1 expression ↓ JAK2/STAT3 pathway	(118)
	δ-tocotrienol	Prostate cancer	*In vitro* (DU145 and PC3)	↑ ROS ↑ apoptosis	(120)
	Tocotrienol/6-*O*-carboxypropyl-α-tocotrienol	Malignant mesothelioma	*In vitro* (H2052 cell line) *In vivo* (ICR mice)	↓ tumor size	(122)
	Tocopherol/ tocopherol succinate	Breast cancer	*In vitro* (MDA-MB-231 and MDA-MB-468)	↓ cell growth	(123)
	Annatto tocotrienol/γ-tocotrinol/ δ-tocotrienol	Chondrosarcoma	*In vitro* (Human chondrosarcoma SW1353 cells)	↑ G1 arrest ↑ apoptosis	(125)
	(RRR-α-tocopherol [αT], RRR-γ-tocopherol [γT])	Breast cancer	*In vitro* (human MDA-MB-435, MCF-7, and murine 66 cL-4 mammary cancer cells)	↑ caspases-8 and -9 ↓ c-FLIP and surviving ↑ apoptosis ↓ colony formation	(117)
	Synthetic forms (all-rac-α-tocopherol [all-rac-αT], all-rac-α-tocopheryl acetate [all-rac-αTAc])		*In vivo* (BALB/c mouse mammary cancer model)	↓ tumor growth ↓ metastasis	
Vitamin D	1,25$(OH)_2$-D_3	Prostate cancer	*In vitro* (C4-2 cells and CWR22 cells) *in vivo* (athymic nude (Hsd:Athymic Nude-Foxn1nu) mice)	↑ tumor regression ↓ LSD1	(135)
	1,25-dihydroxycholecalciferol	Breast cancer	*In vitro* *In vivo* (MMTV-polyoma middle T antigen (PyMT) transgenic mice)	↑ tumor regression ↓ lung metastasis ↓ Ki-67, cyclin D1, and ErbB2 levels	(136)
	Calcitriol	Colon cancer	*In vitro* (HT29 and Caco-2 cells)	↑ cell cycle arrest ↑ expression of cell cycle regulators p21 and p27 ↑ activation of JNK1	(137)
	1,25-dihydroxyvitamin D (1,25(OH)2D)	Bone cancer	*In vitro* (Human MG-63 osteosarcoma cells)	↓ mTORC1 ↓ ROS	(138)
	1,25(OH)2D3	Gastric cancer	*In vitro* (Human GC cells MKN45, MKN28, and KATO III) *In vivo* (orthotopic GC nude mice model)	↓ CD44 ↓ Wnt/β-catenin signaling pathway	(139)

analysis provide evidence supporting the notion that vitamin E indeed possesses a beneficial impact on salivary glands, particularly in terms of augmenting parotid excretion function (150). Vitamin E's impact on lowering prostate cancer incidence has been demonstrated by two randomized controlled trials' use of serum metabolomics analysis (151). Serum C22 lactone sulfate, a molecule strongly linked to the modulation of androgenic steroid metabolites, was found to be substantially reduced by a high-dose of vitamin E (400 IU/day) (151). On the other hand, the utilization of α-tocopherol and β-carotene as dietary supplements did not demonstrate a significant reduction in the likelihood of developing liver cancer or chronic liver disease (152).

5.4 Vitamin D

High serum levels of 25(OH)D were found to be associated with improved early-stage survival in non-small-cell lung cancer (NSCLC) in a randomized, double-blinded trial (153). The outcomes were classified into RFS and OS as primary and secondary, respectively. Referred to RFS was the period of time between the start date of the supplement and the earlier date of the cancer relapse or death from any cause. They referred to this period of time as OS, which is the start date of the supplement and the date of death from any cause. The overall results were no improvements in RFS and OS in the total study population, better OS in patients with high Vitamin D before taking the supplement (≥20 ng/mL) than the low level (<20 ng/mL) (153). When the study was limited to patients with early-stage adenocarcinoma and low 25(OH)D, the vitamin D group had significantly better 5-year RFS (86% vs. 50%, $p = 0.04$) and OS (91% vs. 48%, $p = 0.02$) than the placebo group. Among the polymorphisms studied, vitamin D binding protein (DBP1-rs7041) TT and CDX2 (rs11568820) AA/AG genetic constitution were associated with a well prediction, even after multivariable adjustment. Finally, vitamin D dietary supplementation may improve the prognosis of patients with lower 25(OH)D levels who have early-stage lung adenocarcinoma (153). Table 2 reviews the clinical studies of the mentioned vitamins.

6 Critical assessment of vitamins safety and potential toxicity

In this section, we will assess the safety and potential toxicity of vitamins A, C, E, and D. By examining their toxicity profiles, we aim to provide a holistic perspective on their applications and shed light on the fine balance between their potential anticancer effects and the risk of toxicity when taken in excess.

Hypervitaminosis is a rapid-onset pathological condition caused by the excessive accumulation of any vitamin in the body. Specifically concerning vitamin A, hypervitaminosis is considered when the plasma concentration of retinol surpasses 2.09 μM. Toxicity is commonly associated with the improper use of dietary supplements but may also manifest following an elevated consumption of foods rich in preformed vitamin A, such as liver and eggs (154, 155). Hypervitaminosis A can occur in two forms: acute and chronic. Acute toxicity manifests within days to weeks after ingesting a few very high doses, typically more than 100 times the recommended dietary allowance (RDA). Symptoms include severe headache, blurred vision, nausea, dizziness, muscle aches, and coordination problems. Chronic hypervitaminosis A can result from regular consumption of high doses and may lead to dry skin, joint pain, fatigue, depression, and abnormal liver test results (10). Beta-carotene, a provitamin A, is generally regarded as safe when consumed through a balanced diet rich in fruits and vegetables. However, taking high-dose beta-carotene supplements may pose certain risks (156). Long-term, excessive beta-carotene intake, particularly in supplement form, has been associated with a higher risk of adverse health effects. Some studies suggest that high-dose beta-carotene supplements may be linked to an increased risk of lung cancer, particularly in individuals who smoke (157). Additionally, excessive beta-carotene intake can cause a harmless but noticeable condition called carotenemia, characterized by yellowish or orange discoloration of the skin (155).

Vitamin C has low toxicity, and excessive consumption is unlikely to cause serious adverse effects. The most commonly reported adverse effects include diarrhea, nausea, abdominal cramps, and other gastrointestinal disturbances due to the osmotic effect of unabsorbed vitamin C in the gastrointestinal tract. There is a theoretical concern that high vitamin C intake might lead to excess iron absorption, as vitamin C enhances nonheme iron absorption. Additionally, there is a possibility that vitamin C could act as a pro-oxidant, contributing to oxidative damage. Long-term intakes of vitamin C exceeding the tolerable upper intake level (UL) of 2,000 mg may increase the risk of adverse health effects (12). High doses of intravenous vitamin C or oral vitamin C can trigger hemolysis in individuals with glucose-6-phosphate deficiency. For those with paroxysmal nocturnal hemoglobinuria, oral ascorbic acid can exacerbate hemolysis. *In vitro* studies show that the impact of vitamin C on red blood cell lysis is concentration-dependent, worsening at low concentrations and inhibiting at high concentrations (158).

There is no evidence that high consumption of vitamin E in food can cause any adverse effects. However, it has been shown in animals that high doses of vitamin E supplements can cause hemorrhage and interrupt blood coagulation, and platelet aggregation inhibition. Consumption of 1,000 mg/day in adults appears to be safe, but yet, data are limited and based on small groups of people for short periods of time (11).

Excess consumption of vitamin D can be toxic, leading to marked hypercalcemia, hypercalciuria, and elevated serum 25(OH)D levels. Symptoms of vitamin D toxicity include nausea, vomiting, muscle weakness, neuropsychiatric disturbances, pain, loss of appetite, dehydration, polyuria, excessive thirst, and kidney stones. Toxicity is unlikely at daily intakes below 250 mcg, but even intakes below the UL may have adverse health effects over time. It is recommended to avoid serum 25(OH)D levels above 125–150 nmol/L (13).

7 Conclusion

In conclusion, the potential responsibility of vitamins A, C, E, and D in preventing and treating tumor is a subject of growing interest, supported by a combination of preclinical, clinical studies, and considerations of safety. Vitamins play complex roles in cancer prevention, influencing immune job, antioxidant resistance, inflammatory modulation, and epigenetic directive, which could enhance outcomes by affecting cell differentiation, proliferation, and apoptosis, and combating oxidative stress and DNA damage. Particularly, vitamin A with its

TABLE 2 Clinical studies of vitamins A, C, E, and D.

Vitamins	Cancer type	Sample size	Study type	Type of vitamins and doses	The outcome of the study	References
Vitamin A	Acute promyelocytic leukemia	235	A randomized, controlled, open-label multicenter trial, phase 3 trial	All-trans retinoic acid (45 mg/m^2)	Using all-trans retinoic acid with arsenic acid has increased the cure rate and reduced relapse chances	(140)
	Gestational trophoblastic neoplasia	32	A randomized, controlled clinical trial	oral vitamin A (6,000 IU/day)	The intervention group exhibited a reduction of chemoresistance as well as a downregulation of the tumor marker β-hCG level	(141)
	Advanced cervical carcinoma	30	A randomized double-blind study	Vitamin A supplementation (8,000 IU/8 h)	Improved therapeutic response of the chemotherapy	(142)
Vitamin C	Advanced-stage non-small cell lung cancer	38	Open-label, single-arm, non-randomized phase II study	Ascorbate (75 g twice per week) intravenously	Improved tumor deterioration and enhance immune response	(148)
	Metastatic colorectal cancer or gastric cancer	36	Phase 1 open-label, single-center, dose-escalation, and speed-expansion study	Ascorbic acid (0.2–1.5 g/kg) infusion	Improved therapeutic response of the chemotherapy and significantly decreased the side effects	(149)
Vitamin E	Thyroid carcinoma	89	A randomized, controlled clinical trial	NA	The results revealed the protective impact of vitamin E on salivary glands by enhancing parotid excretion function	(150)
	Prostate cancer	154	A randomized, placebo-controlled, double-blinded clinical trial	Vitamin E (400 IU/day)	Significantly decreased serum C_{22} lactone sulfate which is highly associated with modulating androgenic steroid metabolites	(151)
Vitamin D	non-small-cell lung cancer	155	A randomized, double-blind, placebo-controlled, parallel group trial	Vitamin D supplements (1,200 IU/day)	Patients with lower 25(OH)D levels who have early-stage lung adenocarcinoma may have a better chance of surviving if they take vitamin D supplements.	(153)

regulatory role in cell growth and differentiation, impacts several signaling pathways linked to cancer. Vitamin C functions as an antioxidant, reducing oxidative stress and possibly enhancing immune responses against cancer cells. High-dose intravenous applications of vitamin C have shown potential in selectively targeting cancer cells. The antioxidative properties of vitamin E protect cell membranes from damage, with certain forms exhibiting anti-inflammatory effects. Vitamin D, through its role in cell differentiation and immune function, shows links to reduced risks in certain cancers like colorectal cancer. Interestingly, combining vitamins A, C, D, and E with chemotherapy and radiotherapy offers a customized strategy for cancer treatment. These vitamins enhance immune response, alleviate oxidative stress, inhibit angiogenesis, and induce apoptosis. The synergy between vitamins and conventional treatments provides promising paths for more effective and personalized therapies. Further research is essential to unlock their complete potential in cancer treatments.

Nevertheless, the current studies present limitations that must be acknowledged. Using different dosage regimens in human and animal studies may impact the mechanisms underlying the medicinal prospective of vitamins in tumors, often with complex, unpredictable outcomes. Although animal models offer preliminary insights, they do not always replicate human metabolic processes, potentially leading to different bioavailability and efficacy in humans. Additionally, *in vitro* findings, such as the observed toxicity of vitamins A and E to cancer cells, may not translate directly into therapeutic benefits *in vivo*, where complex biological systems can alter the vitamins' impact on cancer cells.

The integration of preclinical and clinical research highlights the need for particular approaches based on cancer type, stage, and patient characteristics. However, there are significant gaps in our understanding, including determining optimal dosages for efficacy and safety, elucidating precise mechanisms of action, establishing clinical efficacy through more large-scale trials, developing personalized treatment approaches, and studying the longstanding effects of vitamin supplementation in cancer patients. Addressing these gaps through comprehensive, multidisciplinary research is crucial for fully realizing the potential of these vitamins in oncology.

Author contributions

WT: Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – review & editing. DA: Data curation, Software, Writing – original draft. ZA: Data curation, Methodology, Software, Writing – original draft. MJ: Data curation, Investigation, Software, Writing – original draft. LA: Conceptualization, Project administration, Writing – review & editing. RH: Data curation, Methodology, Software, Visualization, Writing – original draft. AM: Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing.

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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.

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References

1. Roser M, Ritchie H. Cancer. Our World in Data (2015); Available at: https://ourworldindata.org/cancer.

2. MSD, *Overview of Cancer*. Merck Sharp & Dohme Corp. (MSD) (2022).

3. MSD, *Risk factors for Cancer*. Merck Sharp & Dohme Corp. (MSD) (2022).

4. You W, Henneberg M. Cancer incidence increasing globally: the role of relaxed natural selection. *Evol Appl*. (2018) 11:140–52. doi: 10.1111/eva.12523

5. MSD, *Development and spread of Cancer*. Merck Sharp & Dohme Corp. (MSD) (2022).

6. Manuals. TM *Cellular and molecular basis of Cancer*. Rahway, NJ, USA: Merck & Co, Inc. (2022).

7. Behranvand N, Nasri F, Emameh RZ, Khani P, Hosseini A, Garssen J, et al. Correction to: chemotherapy: a double-edged sword in cancer treatment. *Cancer Immunol Immunother*. (2021):1. doi: 10.1007/s00262-021-03013-3

8. Zeng Z, Mishuk AU, Qian J. Safety of dietary supplements use among patients with cancer: a systematic review. *Crit Rev Oncol Hematol*. (2020) 152:103013. doi: 10.1016/j.critrevonc.2020.103013

9. Kanellopoulou A, Riza E, Samoli E, Benetou V. Dietary supplement use after cancer diagnosis in relation to total mortality, cancer mortality and recurrence: a systematic review and meta-analysis. *Nutr Cancer*. (2021) 73:16–30. doi: 10.1080/01635581.2020.1734215

10. National Institute of Health. Vitamin A and carotenoids: Fact sheet for health professionals. (2022); Available at: https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/.

11. National Institute of Health. Vitamin E: Fact sheet for health professionals. (2021); Available at: https://ods.od.nih.gov/factsheets/VitaminE-HealthProfessional/

12. National Institute of Health, *Vitamin C: Fact sheet for health professionals*. (2021). Available at: https://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional/

13. National Institute of Health, *Vitamin D: Fact sheet for health professionals*. (2023). Available at: (https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/)

14. O'Connor EA, Evans CV, Ivlev I, Rushkin MC, Thomas RG, Martin A, et al. Vitamin and mineral supplements for the primary prevention of cardiovascular disease and Cancer: updated evidence report and systematic review for the US preventive services task force. *JAMA*. (2022) 327:2334–47. doi: 10.1001/jama.2021.15650

15. Cheema HA, Fatima M, Shahid A, Bouaddi O, Elgenidy A, Rehman AU, et al. Vitamin D supplementation for the prevention of total cancer incidence and mortality: an updated systematic review and meta-analysis. *Heliyon*. (2022) 8:e11290. doi: 10.1016/j.heliyon.2022.e11290

16. Mohseni S, Tabatabaei-Malazy O, Ejtahed HS, Qorbani M, Azadbakht L, Khashayar P, et al. Effect of vitamins C and E on cancer survival; a systematic review. *DARU J Pharmaceut Sci*. (2022) 30:427–41. doi: 10.1007/s40199-022-00451-x

17. Barnhart AS, Anthony AL, Conaway KR, Sibbitt BG, Delaney E, Haluschak J, et al. Safety and efficacy of Vitamin C, Vitamin E, and selenium supplementation in the oncology setting: a systematic review. *J Oncol Pharm Pract*. (2023) 15:10781552231182362. doi: 10.1177/10781552231182362

18. de Oliveira VA, Oliveira IKF, Pereira IC, Mendes LKF, Carneiro da Silva FC, Torres–Leal FL, et al. Consumption and supplementation of Vitamin E in breast cancer risk, treatment, and outcomes: a systematic review with meta-analysis. *Clin Nutri ESPEN*. (2023) 54:215–26. doi: 10.1016/j.clnesp.2023.01.032

19. Goulão B, Stewart F, Ford JA, MacLennan G, Avenell A. Cancer and vitamin D supplementation: a systematic review and meta-analysis. *Am J Clin Nutr*. (2018) 107:652–63. doi: 10.1093/ajcn/nqx047

20. Leelakanok N, D'Cunha RR, Sutamtewagul G, Schweizer ML. A systematic review and meta-analysis of the association between vitamin a intake, serum vitamin a, and risk of liver cancer. *Nutr Health*. (2018) 24:121–31. doi: 10.1177/0260106018777170

21. Alsharairi NA. Dietary Antioxidants and Lung Cancer Risk in Smokers and Non-Smokers. *Healthcare (Basel)* (2022) 10:2501. doi: 10.3390/healthcare10122501

22. He J, Gu Y, Zhang S. Vitamin a and breast cancer survival: a systematic review and meta-analysis. *Clin Breast Cancer*. (2018) 18:e1389–400. doi: 10.1016/j.clbc.2018.07.025

23. van Gorkom GNY, Lookermans EL, van Elssen CHMJ, Bos GMJ. The effect of vitamin C (ascorbic acid) in the treatment of patients with cancer: a systematic review. *Nutrients*. (2019) 11:977. doi: 10.3390/nu11050977

24. Hossain S, Beydoun MA, Beydoun HA, Chen X, Zonderman AB, Wood RJ. Vitamin D and breast cancer: a systematic review and meta-analysis of observational studies. *Clin Nutr ESPEN*. (2019) 30:170–84. doi: 10.1016/j.clnesp.2018.12.085

25. Chen Z, Huang Y, Cao D, Qiu S, Chen B, Li J, et al. Vitamin C intake and cancers: an umbrella review. *Front Nutr*. (2022) 8:812394. doi: 10.3389/fnut.2021.812394

26. Xu Y, Qian M, Hong J, Ng DM, Yang T, Xu L, et al. The effect of vitamin D on the occurrence and development of colorectal cancer: a systematic review and meta-analysis. *Int J Color Dis*. (2021) 36:1329–44. doi: 10.1007/s00384-021-03879-w

27. Kraemer K, Semba RD, Eggersdorfer M, Schaumberg DA. Introduction: the diverse and essential biological functions of vitamins. *Ann Nutr Metab*. (2012) 61:185–91. doi: 10.1159/000343103

28. Zhang FF, Barr SI, McNulty H, Li D, Blumberg JB. Health effects of vitamin and mineral supplements. *BMJ*. (2020) 369:m2511. doi: 10.1136/bmj.m2511

29. Bender DA. *Nutritional biochemistry of the vitamins*. New York: Cambridge University Press (2003).

30. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med*. (2007) 204:1765–74. doi: 10.1084/jem.20070719

31. Xavier AA, Pérez-Gálvez A. Carotenoids as a source of antioxidants in the diet. *Subcell Biochem*. (2016) 79:359–75. doi: 10.1007/978-3-319-39126-7_14

32. Palace VP, Khaper N, Qin Q, Singal PK. Antioxidant potentials of vitamin a and carotenoids and their relevance to heart disease. *Free Radic Biol Med*. (1999) 26:746–61. doi: 10.1016/S0891-5849(98)00266-4

33. Zhang T, Wang Z, Wang X, Sun W, Cui X, Li R, et al. Effects of vitamin a on antioxidant functions, immune functions and production performance in male sika deer (Cervus nippon) during the first antler growth period. *Ital J Anim Sci*. (2019) 18:98–104. doi: 10.1080/1828051X.2018.1456978

34. Doldo E, Costanza G, Agostinelli S, Tarquini C, Ferlosio A, Arcuri G, et al. Vitamin a, cancer treatment and prevention: the new role of cellular retinol binding proteins. *Biomed Res Int*. (2015) 2015:1–14. doi: 10.1155/2015/624627

35. Gudas LJ, Wagner JA. Retinoids regulate stem cell differentiation. *J Cell Physiol*. (2011) 226:322–30. doi: 10.1002/jcp.22417

36. Bar-El Dadon S, Reifen R. Vitamin a and the epigenome. *Crit Rev Food Sci Nutr*. (2017) 57:2404–11. doi: 10.1080/10408398.2015.1060940

37. Abdullah M, Jamil RT, Attia FN. Vitamin C (ascorbic acid) In: *StatPearls*. NA, Treasure Island (FL): StatPearls Publishing (2022)

38. Vitamin D, Fact sheet for health professionals. National Institutes of Health. Office of Dietary Supplements. Available at: https://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional, (2017).

39. Ngo B, van Riper JM, Cantley LC, Yun J. Targeting cancer vulnerabilities with high-dose vitamin C. *Nat Rev Cancer*. (2019) 19:271–82. doi: 10.1038/s41568-019-0135-7

40. Ellulu MS, Rahmat A, Patimah I, Khaza'ai H, Abed Y. Effect of vitamin C on inflammation and metabolic markers in hypertensive and/or diabetic obese adults: a randomized controlled trial. *Drug Des Devel Ther*. (2015) 9:3405–12. doi: 10.2147/DDDT.S83144

41. Yussif NM, Abdul Aziz MA, Abdel Rahman AR. Evaluation of the anti-inflammatory effect of locally delivered Vitamin C in the treatment of persistent gingival inflammation: clinical and histopathological study. *J Nutr Metab*. (2016) 2016:2978741. doi: 10.1155/2016/2978741

42. Kwak SG, Choo YJ, Chang MC. The effectiveness of high-dose intravenous vitamin C for patients with coronavirus disease 2019: a systematic review and meta-analysis. *Complement Ther Med*. (2022) 64:102797. doi: 10.1016/j.ctim.2021.102797

43. du YT, Long Y, Tang W, Liu XF, Dai F, Zhou B. Prooxidative inhibition against NF-κB-mediated inflammation by pharmacological vitamin C. *Free Radic Biol Med*. (2022) 180:85–94. doi: 10.1016/j.freeradbiomed.2022.01.007

44. Fu J, Wu Z, Liu J, Wu T. Vitamin C: a stem cell promoter in cancer metastasis and immunotherapy. *Biomed Pharmacother*. (2020) 131:110588. doi: 10.1016/j.biopha.2020.110588

45. Brabson JP, Leesang T, Mohammad S, Cimmino L. Epigenetic regulation of genomic stability by vitamin C. *Front Genet*. (2021) 12:675780. doi: 10.3389/fgene.2021.675780

46. Mikkelsen SU, Gillberg L, Lykkesfeldt J, Grønbæk K. The role of vitamin C in epigenetic cancer therapy. *Free Radic Biol Med*. (2021) 170:179–93. doi: 10.1016/j.freeradbiomed.2021.03.017

47. Khan Z, Ahmed S, Marya F, Ullah H, Khan H. Vitamin E (tocopherols and tocotrienols) (natural-occurring antioxidant; bright and dark side) In: Nabavi, SM and Silva, AS editors. *Antioxidants effects in health*. United States, Cambridge: Elsevier (2022). 547–60.

48. Jiang Q. Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. *Free Radic Biol Med*. (2014) 72:76–90. doi: 10.1016/j.freeradbiomed.2014.03.035

49. Smolarek AK, So JY, Thomas PE, Lee HJ, Paul S, Dombrowski A, et al. Dietary tocopherols inhibit cell proliferation, regulate expression of ERα, PPARγ, and Nrf2, and decrease serum inflammatory markers during the development of mammary hyperplasia. *Mol Carcinog*. (2013) 52:514–25. doi: 10.1002/mc.21886

50. Smolarek AK, Suh N. Chemopreventive activity of vitamin E in breast cancer: a focus on γ-and δ-tocopherol. *Nutrients*. (2011) 3:962–86. doi: 10.3390/nu3110962

51. Cornwell DG, Williams MV, Wani AA, Wani G, Shen E, Jones KH. Mutagenicity of tocopheryl quinones: evolutionary advantage of selective accumulation of dietary a-tocopherol. *Nutr Cancer*. (2002) 43:111–8. doi: 10.1207/S15327914NC431_13

52. Aggarwal BB, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem Pharmacol*. (2010) 80:1613–31. doi: 10.1016/j.bcp.2010.07.043

53. Powers SK, Sollanek KJ, Wiggs MP. Endurance exercise and antioxidant supplementation: sense or nonsense?-Part 1. *Sport Sci Exch*. (2014) 27:1–4.

54. Higgins MR, Izadi A, Kaviani M. Antioxidants and exercise performance: with a focus on vitamin E and C supplementation. *Int J Environ Res Public Health*. (2020) 17:8452. doi: 10.3390/ijerph17228452

55. Ju J, Picinich SC, Yang Z, Zhao Y, Suh N, Kong AN, et al. Cancer-preventive activities of tocopherols and tocotrienols. *Carcinogenesis*. (2010) 31:533–42. doi: 10.1093/carcin/bgp205

56. Campbell SE, Stone WL, Whaley SG, Qui M, Krishnan K. Gamma (γ) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (γ) expression in SW 480 human colon cancer cell lines. *BMC Cancer*. (2003) 3:1–13. doi: 10.1186/1471-2407-3-25

57. Lewis ED, Meydani SN, Wu D. Regulatory role of vitamin E in the immune system and inflammation. *IUBMB Life*. (2019) 71:487–94. doi: 10.1002/iub.1976

58. Ao T, Kikuta J, Ishii M. The effects of vitamin D on immune system and inflammatory diseases. *Biomol Ther*. (2021) 11:1624. doi: 10.3390/biom11111624

59. L Bishop E, Ismailova A, Dimeloe S, Hewison M, White JH. Vitamin D and immune regulation: antibacterial, antiviral, anti-inflammatory. *JBMR plus*. (2021) 5:e10405. doi: 10.1002/jbm4.10405

60. Raymond E, Dalgleish A, Damber JE, Smith M, Pili R. Mechanisms of action of tasquinimod on the tumour microenvironment. *Cancer Chemother Pharmacol*. (2014) 73:1–8. doi: 10.1007/s00280-013-2321-8

61. Liu W, Zhang L, Xu HJ, Li Y, Hu CM, Yang JY, et al. The anti-inflammatory effects of vitamin D in tumorigenesis. *Int J Mol Sci*. (2018) 19:2736. doi: 10.3390/ijms19092736

62. Nakai K, Fujii H, Kono K, Goto S, Kitazawa R, Kitazawa S, et al. Vitamin D activates the Nrf2-Keap1 antioxidant pathway and ameliorates nephropathy in diabetic rats. *Am J Hypertens*. (2014) 27:586–95. doi: 10.1093/ajh/hpt160

63. Sepidarkish M, Farsi F, Akbari-Fakhrabadi M, Namazi N, Almasi-Hashiani A, Maleki Hagiagha A, et al. The effect of vitamin D supplementation on oxidative stress parameters: a systematic review and meta-analysis of clinical trials. *Pharmacol Res*. (2019) 139:141–52. doi: 10.1016/j.phrs.2018.11.011

64. Giammanco M, di Majo D, la Guardia M, Aiello S, Crescimannno M, Flandina C, et al. Vitamin D in cancer chemoprevention. *Pharm Biol*. (2015) 53:1399–434. doi: 10.3109/13880209.2014.988274

65. Fleet JC. Molecular actions of vitamin D contributing to cancer prevention. *Mol Asp Med*. (2008) 29:388–96. doi: 10.1016/j.mam.2008.07.003

66. Antonio V, Francesca F, Grazia BM, Federica G, Anna C, Giovanni S, et al. Vitamin D in the prevention, development and therapy of oncological diseases. *Pharmacologyonline*. (2021) 2:267–76.

67. Rinninella E, Mele MC, Raoul P, Cintoni M, Gasbarrini A. Vitamin D and colorectal cancer: Chemopreventive perspectives through the gut microbiota and the immune system. *Biofactors*. (2022) 48:285–93. doi: 10.1002/biof.1786

68. Marigoudar JB, Sarkar D, Yuguda YM, Abutayeh RF, Kaur A, Pati A, et al. Role of vitamin D in targeting cancer and cancer stem cell populations and its therapeutic implications. *Med Oncol*. (2022) 40:2. doi: 10.1007/s12032-022-01855-0

69. Sajovic J, Meglič A, Glavač D, Markelj Š, Hawlina M, Fakin A. The role of vitamin a in retinal diseases. *Int J Mol Sci*. (2022) 23:1014. doi: 10.3390/ijms23031014

70. Gui Y, Duan S, Xiao L, Tang J, Li A. Bexarotent attenuated chronic constriction injury-induced spinal neuroinflammation and neuropathic pain by targeting mitogen-activated protein kinase phosphatase-1. *J Pain*. (2020) 21:1149–59. doi: 10.1016/j.jpain.2019.01.007

71. Terao M, Goracci L, Celestini V, Kurosaki M, Bolis M, di Veroli A, et al. Role of mitochondria and cardiolipins in growth inhibition of breast cancer cells by retinoic acid. *J Exp Clin Cancer Res*. (2019) 38:1–20. doi: 10.1186/s13046-019-1438-y

72. Cui J, Gong M, Fang S, Hu C, Wang Y, Zhang J, et al. All-trans retinoic acid reverses malignant biological behavior of hepatocarcinoma cells by regulating miR-200 family members. *Genes Dis*. (2021) 8:509–20. doi: 10.1016/j.gendis.2019.12.012

73. Nguyen PH, Giraud J, Staedel C, Chambonnier L, Dubus P, Chevret E, et al. All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. *Oncogene*. (2016) 35:5619–28. doi: 10.1038/onc.2016.87

74. Ding X, Wang W, Wang M, Wu J, Yao F. DOK1/PPARgamma pathway mediates anti-tumor ability of all-trans retinoic acid in breast cancer MCF-7 cells. *Biochem Biophys Res Commun*. (2017) 487:189–93. doi: 10.1016/j.bbrc.2017.04.018

75. Aouad P, Saikali M, Abdel-Samad R, Fostok S, el-Houjeiri L, Pisano C, et al. Antitumor activities of the synthetic retinoid ST1926 in two-dimensional and three-dimensional human breast cancer models. *Anti-Cancer Drugs*. (2017) 28:757–70. doi: 10.1097/CAD.0000000000000511

76. Funaki M, Kitabayashi J, Shimakami T, Nagata N, Sakai Y, Takegoshi K, et al. Peretinoin, an acyclic retinoid, inhibits hepatocarcinogenesis by suppressing sphingosine kinase 1 expression in vitro and in vivo. *Sci Rep*. (2017) 7:16978. doi: 10.1038/s41598-017-17285-2

77. Patrad E, Niapour A, Farassati F, Amani M. Combination treatment of all-trans retinoic acid (ATRA) and γ-secretase inhibitor (DAPT) cause growth inhibition and apoptosis induction in the human gastric cancer cell line. *Cytotechnology*. (2018) 70:865–77. doi: 10.1007/s10616-018-0199-3

78. Zhang L, Wang J, Liu L, Zheng C, Wang Y. Synthesis and antiproliferative activity of novel all-trans-retinoic acid-podophyllotoxin conjugate towards human gastric cancer cells. *Molecules*. (2017) 22:628. doi: 10.3390/molecules22040628

79. Liang Y, Wang W, Zhu X, Yu M, Zhou C. Inhibition of myeloid-derived suppressive cell function with all-trans retinoic acid enhanced anti-PD-L1 efficacy in cervical cancer. *Sci Rep*. (2022) 12:9619. doi: 10.1038/s41598-022-13855-1

80. Li X, Luo X, Chen S, Chen J, Deng X, Zhong J, et al. All-trans-retinoic acid inhibits hepatocellular carcinoma progression by targeting myeloid-derived suppressor cells and inhibiting angiogenesis. *Int Immunopharmacol*. (2023) 121:110413. doi: 10.1016/j.intimp.2023.110413

81. Lou Y, Peng P, Wang S, Wang J, du P, Zhang Z, et al. Combining all-trans retinoid acid treatment targeting myeloid-derived suppressive cells with cryo-thermal therapy enhances antitumor immunity in breast cancer. *Front Immunol*. (2022) 13:1016776. doi: 10.3389/fimmu.2022.1016776

82. Chuang H-C, Lin HY, Liao PL, Huang CC, Lin LL, Hsu WM, et al. Immunomodulator polyinosinic-polycytidylic acid enhances the inhibitory effect of 13-cis-retinoic acid on neuroblastoma through a TLR3-related immunogenic-apoptotic response. *Lab Investig*. (2020) 100:606–18. doi: 10.1038/s41374-019-0356-0

83. Abdullah M, Jamil RT, Attia FN. *Vitamin C (Ascorbic Acid)*. Treasure Island (FL): National Library of Medicine NIH (2022).

84. Fu Y, Xu F, Jiang L, Miao Z, Liang X, Yang J, et al. Circulating vitamin C concentration and risk of cancers: a mendelian randomization study. *BMC Med*. (2021) 19:1–14. doi: 10.1186/s12916-021-02041-1

85. Park Y, Spiegelman D, Hunter DJ, Albanes D, Bergkvist L, Buring JE, et al. Intakes of vitamins a, C, and E and use of multiple vitamin supplements and risk of colon cancer: a pooled analysis of prospective cohort studies. *Cancer Causes Control*. (2010) 21:1745–57. doi: 10.1007/s10552-010-9549-y

86. Jacobs EJ, Connell CJ, McCullough ML, Chao A, Jonas CR, Rodriguez C, et al. Vitamin C, vitamin E, and multivitamin supplement use and stomach cancer mortality in the Cancer prevention study II cohort. *Cancer Epidemiol Biomark Prev*. (2002) 11:35–41.

87. Kong P, Cai Q, Geng Q, Wang J, Lan Y, Zhan Y, et al. Vitamin intake reduce the risk of gastric cancer: meta-analysis and systematic review of randomized and observational studies. *PLoS One*. (2014) 9:e116060. doi: 10.1371/journal.pone.0116060

88. Hua Y-F, Wang GQ, Jiang W, Huang J, Chen GC, Lu CD. Vitamin C intake and pancreatic cancer risk: a meta-analysis of published case-control and cohort studies. *PLoS One*. (2016) 11:e0148816. doi: 10.1371/journal.pone.0148816

89. Agathocleous M, Meacham CE, Burgess RJ, Piskounova E, Zhao Z, Crane GM, et al. Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature*. (2017) 549:476–81. doi: 10.1038/nature23876

90. LEE SJ, JEONG JH, LEE IH, LEE J, JUNG JH, PARK HY, et al. Effect of high-dose vitamin C combined with anti-cancer treatment on breast cancer cells. *Anticancer Res*. (2019) 39:751–8. doi: 10.21873/anticanres.13172

91. Jafari E, Alavi M, Zal F. The evaluation of protective and mitigating effects of vitamin C against side effects induced by radioiodine therapy. *Radiat Environ Biophys*. (2018) 57:233–40. doi: 10.1007/s00411-018-0744-7

92. Klimant E, Wright H, Rubin D, Seely D, Markman M. Intravenous vitamin C in the supportive care of cancer patients: a review and rational approach. *Curr Oncol*. (2018) 25:139–48. doi: 10.3747/co.25.3790

93. Yiang GT, Chen TY, Chen C, Hung YT, Hsueh KC, Wu TK, et al. Antioxidant vitamins promote anticancer effects on low-concentration methotrexate-treated glioblastoma cells via enhancing the caspase-3 death pathway. *Food Sci Nutr*. (2021) 9:3308–16. doi: 10.1002/fsn3.2298

94. Zhou J, Chen C, Chen X, Fei Y, Jiang L, Wang G. Vitamin C promotes apoptosis and cell cycle arrest in oral squamous cell carcinoma. *Front Oncol*. (2020) 10:976. doi: 10.3389/fonc.2020.00976

95. Shenoy N, Creagan E, Witzig T, Levine M. Ascorbic acid in cancer treatment: let the phoenix fly. *Cancer Cell*. (2018) 34:700–6. doi: 10.1016/j.ccell.2018.07.014

96. Magrì A, Germano G, Lorenzato A, Lamba S, Chilà R, Montone M, et al. High-dose vitamin C enhances cancer immunotherapy. *Sci Transl Med*. (2020) 12:eaay8707. doi: 10.1126/scitranslmed.aay8707

97. Sotler R, Poljšak B, Dahmane R, Jukić T, Pavan Jukić D, Rotim C, et al. Prooxidant activities of antioxidants and their impact on health. *Acta Clin Croat*. (2019) 58:726–36. doi: 10.20471/acc.2019.58.04.20

98. Chen Q, Espey MG, Sun AY, Lee JH, Krishna MC, Shacter E, et al. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci*. (2007) 104:8749–54. doi: 10.1073/pnas.0702854104

99. Ma E, Chen P, Wilkins HM, Wang T, Swerdlow RH, Chen Q. Pharmacologic ascorbate induces neuroblastoma cell death by hydrogen peroxide mediated DNA damage and reduction in cancer cell glycolysis. *Free Radic Biol Med*. (2017) 113:36–47. doi: 10.1016/j.freeradbiomed.2017.09.008

100. Kiessling MK, Klemke CD, Kamiński MM, Galani IE, Krammer PH, Gülow K. Inhibition of constitutively activated nuclear factor-κB induces reactive oxygen species- and iron-dependent cell death in cutaneous T-cell lymphoma. *Cancer Res*. (2009) 69:2365–74. doi: 10.1158/0008-5472.CAN-08-3221

101. Rychtarcikova Z, Lettlova S, Tomkova V, Korenkova V, Langerova L, Simonova E, et al. Tumor-initiating cells of breast and prostate origin show alterations in the expression of genes related to iron metabolism. *Oncotarget*. (2017) 8:6376–98. doi: 10.18632/oncotarget.14093

102. Lu Y-X, Wu QN, Chen DL, Chen LZ, Wang ZX, Ren C, et al. Pharmacological ascorbate suppresses growth of gastric cancer cells with GLUT1 overexpression and enhances the efficacy of oxaliplatin through redox modulation. *Theranostics*. (2018) 8:1312–26. doi: 10.7150/thno.21745

103. Tian W, Wang Y, Xu Y, Guo X, Wang B, Sun L, et al. The hypoxia-inducible factor renders cancer cells more sensitive to vitamin C-induced toxicity. *J Biol Chem*. (2014) 289:3339–51. doi: 10.1074/jbc.M113.538157

104. Shenoy N, Bhagat T, Nieves E, Stenson M, Lawson J, Choudhary GS, et al. Upregulation of TET activity with ascorbic acid induces epigenetic modulation of lymphoma cells. *Blood Cancer J*. (2017) 7:e587–7. doi: 10.1038/bcj.2017.65

105. Cimmino L, Dolgalev I, Wang Y, Yoshimi A, Martin GH, Wang J, et al. Restoration of TET2 function blocks aberrant self-renewal and leukemia progression. *Cells*. (2017) 170:1079–1095.e20. e20. doi: 10.1016/j.cell.2017.07.032

106. Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, et al. HIF-dependent antitumorigenic effect of antioxidants in vivo. *Cancer Cell*. (2007) 12:230–8. doi: 10.1016/j.ccr.2007.08.004

107. Wilkes JG, O'Leary BR, du J, Klinger AR, Sibenaller ZA, Doskey CM, et al. Pharmacologic ascorbate (P-AscH−) suppresses hypoxia-inducible factor-1α (HIF-1α) in pancreatic adenocarcinoma. *Clin Exp Metastasis*. (2018) 35:37–51. doi: 10.1007/s10585-018-9876-z

108. Jóźwiak P, Ciesielski P, Zaczek A, Lipińska A, Pomorski L, Wieczorek M, et al. Expression of hypoxia inducible factor 1α and 2α and its association with vitamin C level in thyroid lesions. *J Biomed Sci*. (2017) 24:1–10. doi: 10.1186/s12929-017-0388-y

109. Traber MG. Vitamin E regulatory mechanisms. *Annu Rev Nutr*. (2007) 27:347–62. doi: 10.1146/annurev.nutr.27.061406.093819

110. Lee HJ, Ju J, Paul S, So JY, DeCastro A, Smolarek A, et al. Mixed tocopherols prevent mammary tumorigenesis by inhibiting estrogen action and activating PPAR-γ. *Clin Cancer Res*. (2009) 15:4242–9. doi: 10.1158/1078-0432.CCR-08-3028

111. Sen CK, Khanna S, Roy S. Tocotrienols in health and disease: the other half of the natural vitamin E family. *Mol Asp Med*. (2007) 28:692–728. doi: 10.1016/j.mam.2007.03.001

112. Meganathan P, Fu J-Y. Biological properties of tocotrienols: evidence in human studies. *Int J Mol Sci*. (2016) 17:1682. doi: 10.3390/ijms17111682

113. Samant G, Sylvester P. γ-Tocotrienol inhibits ErbB3-dependent PI3K/Akt mitogenic signalling in neoplastic mammary epithelial cells. *Cell Prolif*. (2006) 39:563–74. doi: 10.1111/j.1365-2184.2006.00412.x

114. Shibata A, Nakagawa K, Sookwong P, Tsuduki T, Tomita S, Shirakawa H, et al. Tocotrienol inhibits secretion of angiogenic factors from human colorectal adenocarcinoma cells by suppressing hypoxia-inducible Factor-1 α. *J Nutr*. (2008) 138:2136–42. doi: 10.3945/jn.108.093237

115. Selvaduray KR, Radhakrishnan AK, Kutty MK, Nesaretnam K. Palm tocotrienols decrease levels of pro-angiogenic markers in human umbilical vein endothelial cells (HUVEC) and murine mammary cancer cells. *Genes Nutr*. (2012) 7:53–61. doi: 10.1007/s12263-011-0223-0

116. Liu H-K, Wang Q, Li Y, Sun WG, Liu JR, Yang YM, et al. Inhibitory effects of γ-tocotrienol on invasion and metastasis of human gastric adenocarcinoma SGC-7901 cells. *J Nutr Biochem*. (2010) 21:206–13. doi: 10.1016/j.jnutbio.2008.11.004

117. Yu W, Jia L, Wang P, Lawson KA, Simmons-Menchaca M, Park SK, et al. In vitro and in vivo evaluation of anticancer actions of natural and synthetic vitamin E forms. *Mol Nutr Food Res*. (2008) 52:447–56. doi: 10.1002/mnfr.200700254

118. Sun Z, Yin S, Zhao C, Fan L, Hu H. Inhibition of PD-L1-mediated tumor-promoting signaling is involved in the anti-cancer activity of β-tocotrienol. *Biochem Biophys Res Commun*. (2022) 617:33–40. doi: 10.1016/j.bbrc.2022.05.082

119. Montagnani Marelli M, Marzagalli M, Fontana F, Raimondi M, Moretti RM, Limonta P. Anticancer properties of tocotrienols: a review of cellular mechanisms and molecular targets. *J Cell Physiol*. (2019) 234:1147–64. doi: 10.1002/jcp.27075

120. Fontana F, Moretti RM, Raimondi M, Marzagalli M, Beretta G, Procacci P, et al. δ-Tocotrienol induces apoptosis, involving endoplasmic reticulum stress and autophagy, and paraptosis in prostate cancer cells. *Cell Prolif*. (2019) 52:e12576. doi: 10.1111/cpr.12576

121. Fontana F, Raimondi M, Marzagalli M, Audano M, Beretta G, Procacci P, et al. Mitochondrial functional and structural impairment is involved in the antitumor activity of δ-tocotrienol in prostate cancer cells. *Free Radic Biol Med*. (2020) 160:376–90. doi: 10.1016/j.freeradbiomed.2020.07.009

122. Sato A, Arai T, Fusegi M, Ando A, Yano T. A redox-inactive derivative of tocotrienol suppresses tumor growth of mesothelioma cells in a xenograft model. *Biol Pharm Bull*. (2019) 42:1034–7. doi: 10.1248/bpb.b18-00924

123. Wei CW, Yu YL, Chen YH, Hung YT, Yiang GT. Anticancer effects of methotrexate in combination with α-tocopherol and α-tocopherol succinate on triple-negative breast cancer. *Oncol Rep*. (2019) 41:2060–6. doi: 10.3892/or.2019.6958

124. Leddy LR, Holmes RE. Chondrosarcoma of bone. *Orthopaed Oncol*. (2014) 2014:117–30. doi: 10.1007/978-3-319-07323-1_6

125. Pang K-L, Foong LC, Abd Ghafar N, Soelaiman IN, Law JX, Leong LM, et al. Transcriptomic analysis of the anticancer effects of annatto tocotrienol, Delta-tocotrienol and gamma-tocotrienol on chondrosarcoma cells. *Nutrients*. (2022) 14:4277. doi: 10.3390/nu14204277

126. Zhang R, Naughton DP. Vitamin D in health and disease: current perspectives. *Nutr J*. (2010) 9:1–13. doi: 10.1186/1475-2891-9-65

127. Bikle DD. *Vitamin D: production, metabolism and mechanisms of action*. South Dartmouth, Massachusetts, USA: Endotext (2021).

128. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol*. (2014) 21:319–29. doi: 10.1016/j.chembiol.2013.12.016

129. Trump DL, Aragon-Ching JB. Vitamin D in prostate cancer. *Asian J Androl*. (2018) 20:244–52. doi: 10.4103/aja.aja_14_18

130. Hii CS, Ferrante A. The non-genomic actions of vitamin D. *Nutrients*. (2016) 8:135. doi: 10.3390/nu8030135

131. Jiang L, Zhang X, Chen Y, Huo X, Deng S, Yang X, et al. Alteration of serum 25 (OH) Vitamin D, Vitamin D binding protein, and C-reactive protein levels in acute leukemia patients. *Clin Lab*. (2018) 64:1553–9. doi: 10.7754/Clin.Lab.2018.180412

132. Wu X, Hu W, Lu L, Zhao Y, Zhou Y, Xiao Z, et al. Repurposing vitamin D for treatment of human malignancies via targeting tumor microenvironment. *Acta Pharm Sin B*. (2019) 9:203–19. doi: 10.1016/j.apsb.2018.09.002

133. Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev*. (2005) 26:662–87. doi: 10.1210/er.2004-0002

134. Negri M, Gentile A, de Angelis C, Montò T, Patalano R, Colao A, et al. Vitamin D-induced molecular mechanisms to potentiate cancer therapy and to reverse drug-resistance in cancer cells. *Nutrients*. (2020) 12:1798. doi: 10.3390/nu12061798

135. Battaglia S, Karasik E, Gillard B, Williams J, Winchester T, Moser MT, et al. LSD1 dual function in mediating epigenetic corruption of the vitamin D signaling in prostate cancer. *Clin Epigenetics*. (2017) 9:1–15. doi: 10.1186/s13148-017-0382-y

136. Rossdeutscher L, Li J, Luco AL, Fadhil I, Ochietti B, Camirand A, et al. Chemoprevention activity of 25-hydroxyvitamin D in the MMTV-PyMT mouse model of breast CancerChemopreventive action of 25 (OH) Vitamin D in breast Cancer. *Cancer Prev Res*. (2015) 8:120–8. doi: 10.1158/1940-6207.CAPR-14-0110

137. Bi X, Shi Q, Zhang H, Bao Y, Hu D, Pohl N, et al. C-Jun NH2-teminal kinase 1 interacts with vitamin D receptor and affects vitamin D-mediated inhibition of cancer cell proliferation. *J Steroid Biochem Mol Biol*. (2016) 163:164–72. doi: 10.1016/j.jsbmb.2016.05.009

138. Quigley M, Rieger S, Capobianco E, Wang Z, Zhao H, Hewison M, et al. Vitamin d modulation of mitochondrial oxidative metabolism and mTOR enforces stress adaptations and anticancer responses. *JBMR plus*. (2022) 6:e10572. doi: 10.1002/jbm4.10572

139. Li Q, Li Y, Jiang H, Xiao Z, Wu X, Zhang H, et al. Vitamin D suppressed gastric cancer cell growth through downregulating CD44 expression in vitro and in vivo. *Nutrition*. (2021) 91:111413. doi: 10.1016/j.nut.2021.111413

140. Burnett AK, Russell NH, Hills RK, Bowen D, Kell J, Knapper S, et al. Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): results of a randomised, controlled, phase 3 trial. *Lancet Oncol*. (2015) 16:1295–305. doi: 10.1016/S1470-2045(15)00193-X

141. Hidayat YM, Darmadi E, Rachmayati S, Kusumah WP, Djuwantono T, Pramatirta AY, et al. Efficacy of Oral Vitamin a in reducing β-hCG levels in low-risk gestational trophoblastic neoplasia patients. *Asian Pac J Cancer Prev*. (2020) 21:3325–9. doi: 10.31557/APJCP.2020.21.11.3325

142. Sanusi RS. Outcome of combined neoadjuvant chemotherapy and Vitamin a in advanced cervical carcinoma: a randomized double-blind clinical trial. *Asian Pac J Cancer Prev*. (2019) 20:2213–8. doi: 10.31557/APJCP.2019.20.7.2213

143. Cameron E. Ascorbic acid and cancer: a review. *Proc Natl Acad Sci USA*. (1987) 75:4538–42. doi: 10.1073/pnas.75.9.4538

144. CameronPauling E. Supplemental ascorbate in the supportive treatment of cancer: prolongation of survival times in terminal human cancer. *Proc Natl Acad Sci U S A*. (1976) 73:3685–9. doi: 10.1073/pnas.73.10.3685

145. Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J, et al. Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. *N Engl J Med*. (1979) 301:687–90. doi: 10.1056/NEJM197909273011303

146. Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ, Ames MM. High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy: a randomized double-blind comparison. *N Engl J Med*. (1985) 312:137–41. doi: 10.1056/NEJM198501173120301

147. Padayatty SJ, Sun AY, Chen Q, Espey MG, Drisko J, Levine M. Vitamin C: intravenous use by complementary and alternative medicine practitioners and adverse effects. *PLoS One*. (2010) 5:e11414. doi: 10.1371/journal.pone.0011414

148. Furqan M, Abu-Hejleh T, Stephens LM, Hartwig SM, Mott SL, Pulliam CF, et al. Pharmacological ascorbate improves the response to platinum-based chemotherapy in advanced stage non-small cell lung cancer. *Redox Biol*. (2022) 53:102318. doi: 10.1016/j.redox.2022.102318

149. Wang F, He MM, Wang ZX, Li S, Jin Y, Ren C, et al. Phase I study of high-dose ascorbic acid with mFOLFOX6 or FOLFIRI in patients with metastatic colorectal cancer or gastric cancer. *BMC Cancer*. (2019) 19:460–10. doi: 10.1186/s12885-019-5696-z

150. Cheng Y, Tong H, Li X, Zhang X, Fang J, Yue R, et al. Effect of vitamin E and supragingival scaling on salivary gland function in patients with differentiated thyroid cancer treated with 131I. *Nucl Med Commun*. (2022) 43:995–1003. doi: 10.1097/MNM.0000000000001605

151. Huang J, Hodis HN, Weinstein SJ, Mack WJ, Sampson JN, Mondul AM, et al. Serum metabolomic response to low-and high-dose Vitamin E supplementation in two randomized controlled TrialsSerum metabolomic response to Vitamin E supplementation. *Cancer Epidemiol Biomark Prev*. (2020) 29:1329–34. doi: 10.1158/1055-9965.EPI-20-0187

152. Lai GY, Weinstein SJ, Taylor PR, McGlynn KA, Virtamo J, Gail MH, et al. Effects of α-tocopherol and β-carotene supplementation on liver cancer incidence and chronic liver disease mortality in the ATBC study. *Br J Cancer*. (2014) 111:2220–3. doi: 10.1038/bjc.2014.514

153. Akiba T, Morikawa T, Odaka M, Nakada T, Kamiya N, Yamashita M, et al. Vitamin D supplementation and survival of patients with non–small cell lung Cancer: a randomized, double-blind, placebo-controlled TrialRCT of Vitamin D for lung Cancer. *Clin Cancer Res*. (2018) 24:4089–97. doi: 10.1158/1078-0432.CCR-18-0483

154. Beltrán-de-Miguel B, Estévez-Santiago R, Olmedilla-Alonso B. Assessment of dietary vitamin a intake (retinol, α-carotene, β-carotene, β-cryptoxanthin) and its sources in the National Survey of dietary intake in Spain (2009–2010). *Int J Food Sci Nutr*. (2015) 66:706–12. doi: 10.3109/09637486.2015.1077787

155. Carazo A, Macáková K, Matoušová K, Krčmová LK, Protti M, Mladěnka P. Vitamin a update: forms, sources, kinetics, detection, function, deficiency, therapeutic use and toxicity. *Nutrients*. (2021) 13:1703. doi: 10.3390/nu13051703

156. Roop JK. Hypervitaminosis-an emerging pathological condition. *Int J Heal Sci Res*. (2018) 8:280.

157. Yang J, Zhang Y, Na X. β-Carotene supplementation and risk of cardiovascular disease: a systematic review and Meta-analysis of randomized controlled trials. *Nutrients*. (2022) 14:1284. doi: 10.3390/nu14061284

158. Doseděl M, Jirkovský E, Macáková K, Krčmová L, Javorská L, Pourová J, et al. Vitamin C—sources, physiological role, kinetics, deficiency, use, toxicity, and determination. *Nutrients*. (2021) 13:615. doi: 10.3390/nu13020615

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Glossary

DNA	Deoxyribonucleic acid
VA	Vitamin A
VC	Vitamin C
VE	Vitamin E
VD	Vitamin D
SOD	Superoxide dismutase
ROS	Reactive oxygen species
PCa	Prostatic cancer
LSD1	Lysine-specific demethylase 1A
VDR	Vitamin D receptors
AR	Androgen receptor
ER	Estrogen receptors
JNK1	c-Jun NH2-terminal kinases
HEK	Human embryonic kidney
DOK1	Docking protein 1
PPARγ	Peroxisome proliferator-activated receptor gamma
ERK1/2	The extracellular signal-regulated kinase 1/2
akt	Protein kinase B
RAR-beta	Retinoic acid receptor beta
HIF-1-alpha	Hypoxia-inducible factor 1-alpha
GLUT1	Glucose transporter 1,
PD-L1	Programmed death-ligand 1
JAK2	Janus kinase 2
STAT3	Signal transducer and activator of transcription 3
Ki-67	Antigen Kiel 67
MTD	Maximum tolerated dose
RP2D	Recommended phase 2 dose
OS	Overall survival
RFS	Recurrence-free survival
CRBP-1	Cellular Retinol-Binding Protein 1
RAR	Retinoic Acid Receptor
RXR	Retinoid X Receptor
IGF	Insulin-like growth factors
IGFBP3	Insulin-like growth factors binding protein 3
MDSCs	Myeloid-derived suppressor cell
RDA	Recommended dietary allowance
UL	Upper intake level

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Fenugreek derived diosgenin as an emerging source for diabetic therapy

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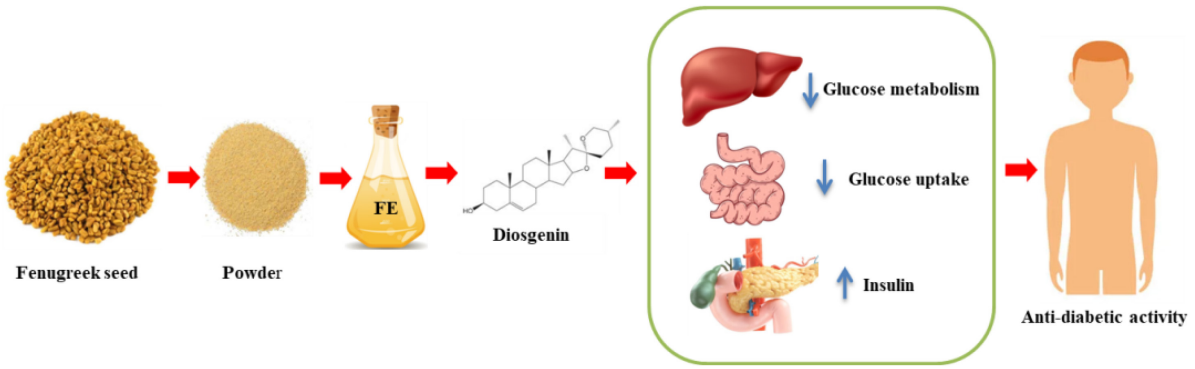
Diabetes is a chronic metabolic disease that endangers the entire body's tissues and organs. Diabetes impairs glucose and insulin regulation in the human body by causing pancreatic cell damage. Diabetes modifies pathways such as serine/threonine protein kinase (Akt) and Protein kinase C (PKC)/- glucose transporter 4 (GLUT4), peroxisome proliferator-activated receptor (PPAR) glucose absorption, and inhibits α-amylase and α-glucosidase, Sodium/glucose cotransporter 1 (SGLT-1), and Na^+-K^+-ATPase activity. Diabetes may also be caused by a decrease in the expression of sterol regulatory element binding protein 1 (SREBP-1) and its target genes, fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and acetyl-CoA carboxylase α (ACC), as well as a decrease in the levels of C/EBP homologous protein (CHOP), Caspase12, and Caspase3 proteins. Diabetes has long been linked to diseases of the cardiovascular, nervous, skeletal, reproductive, hepatic, ocular, and renal systems. Diosgenin, a steroidal compound derived from fenugreek, aids in the prevention of diabetes by altering cellular pathways in favor of healthy bodily functions. Diosgenin is a new nutraceutical on the market that claims to cure diabetes in particular. This article focuses on diosgenin extraction and purification, fenugreek bioactive compounds, pharmacological properties of diosgenin, mode of action of diosgenin to cure diabetes, and dosages.

KEYWORDS

bioactive, diabetes, diosgenin, extraction, fenugreek

Introduction

Due to promising results and rare side effects, medicinal herbs have recently received a lot of attention across the world for their use in the treatment of various ailments. Phytochemicals found in herbal medicines, such as phenolic acids, saponins, flavonoids, tannins, alkaloids, and terpenoids, aid in the treatment of human disease. Fenugreek (*Trigonella foenum-graecum* L.) is an ancient or traditional remedial plant is one of the evidence-based herbal treatments (1). Fenugreek, also known as Methi, Chandrika, Alholva, Bird's Foot, Bockshornsame, and Greek Clover, is a member of the Fabaceae family that originated in India and Northern Africa and is now commercially grown in



GRAPHICAL ABSTRACT

Mediterranean Europe, China, Southeast Asia, Australia, the United States, Argentina, and Canada. India is one of the world's greatest fenugreek growers, yet it does not have a significant proportion of the worldwide fenugreek trade due to high internal consumption. Fenugreek seeds and leaves have been used for millennia in Indian, Tibetan, and Chinese medicine to treat a variety of ailments, including diabetes, obesity, polycystic ovarian syndrome, atherosclerosis, cancer, inflammation, and blood cholesterol (2, 3). The excellent nutritional profile and bioactive components in fenugreek give it medicinal and pharmacological properties including antibacterial, anticholesterolemic, carminative, restorative, uterine tonic, anti-carcinogenic, anti-inflammatory, antiviral, antioxidant, and hypotensive effects (4). Essential oil, coumarins, alkaloids (trigonelline, isoorientin), polyphenols (rhaponticin, isovitexin), and steroidal saponins (diosgenin) are all found in fenugreek seed extract (5, 6). The most active antidiabetic bioactive found in fenugreek is diosgenin, which possesses antioxidative properties (7).

According to recent data from the United States, prediabetes affects 34.6% of the adult population, whereas impaired fasting glucose (IFG) affects 19.4%, impaired glucose tolerance (IGT) affects 5.4%, and IFG plus IGT affect 9.8% (8). In India, according to ICMR 11.4% population living with diabetes whereas, 15.3% of the population is prediabetic. IGT is believed to have 316 million users worldwide, with that number expected to climb to 471 million by 2035 (9). Persons with diabetes have a 7-year reduction in life expectancy than the general non-diabetic population, an effect that is intimately linked to the most serious diabetic outcomes, which include heart disease, limb amputations, End-Stage Renal Disease, and blindness (10). Currently used chemotherapeutic mediators have shown to be helpful in the treatment of diabetes, but they come with a slew of unpleasant side effects, including appetite loss, stomach discomfort, muscle cramps, and weakness. Diosgenin has antioxidative properties and aids in the treatment of diabetes through a variety of mechanisms, including β-cell renewal and insulin secretion stimulation by increasing CCAAT/enhancer-binding protein (C/EBP δ) and peroxisome proliferator-activated receptor- γ (PPAR- γ) mRNA transcription levels (11). Chemotherapeutic mediators used today have shown to be quite helpful in the treatment of diabetes; however, they have a number of unfavorable side effects such as loss of appetite, abdominal discomfort, muscle cramping, and weakness. This review article seeks to make the most of the information available to highlight the topic's scientific standing and the requirement for new research in order to improve current knowledge since more study is currently needed in this area.

Diabetes types and issues

Diabetes is a chronic disease in which the pancreas is unable to produce insulin or the human body is unable to use the insulin produced effectively, resulting in an imbalance in glucose metabolism or an increase in blood sugar levels. According to American Diabetes Association 2009 (12), there are mainly diabetes types are: Type 1 diabetes, type 2 diabetes, type 3 diabetes, and gestational diabetes. Diabetes also causes other serious health problems and is a significant financial burden for a vast number of people around the world. Poor nutritional diets and unhealthy or contemporary lifestyles contribute to weight gain and obesity, which can be a contributing factor to diabetes around the world. According to the World Health Organization (WHO), diabetes affects around 422 million people globally, the majority of whom live in low- and middle-income countries. Diabetes or higher-than-optimal blood glucose caused 1.5 million individuals to die directly from cardiovascular disease, chronic renal disease, or tuberculosis in 2019. According to the American Diabetes Association (ADA) (13), almost 1.9 million Americans, including roughly 2.44 lakh children and adolescents, have type 1 diabetes. In 2017, the entire cost of diabetes was $327 billion, with $237 billion in direct medical costs and $90 billion in indirect costs due to lost productivity. Type 1 diabetes is a polygenic inherited disease caused by a complicated interplay between the pancreatic β-cell and the innate and adaptive immune systems. Insulin is not generated as a result of destructed-β cell and the human body is unable to store excess glucose, resulting in an increase in blood sugar levels. Hypoglycemia is a complication of Type 1 diabetes that causes confusion, convulsions, or coma in the patient. Individuals with Type 1 diabetes can benefit from continuous subcutaneous insulin infusions, oral medication, and a nutritious diet combined with a healthy lifestyle, thyroid dysfunction treatment, and glucose self-monitoring (14, 15).

Globally, Diabetes mellitus affects approximately one in every eleven adults, with Type 2 diabetes accounting for 90% of cases. Asia is a chief region of the world's rapidly spreading Type 2 diabetes mellitus epidemic, with China and India serving as the top two epicenters (16). Type 2 diabetes is caused by high blood sugar levels. Blood glucose is your primary source of energy, and it is derived primarily from the foods we consume. In Type 2 diabetes, the body either does not produce enough insulin or does not use insulin effectively. Type 2 diabetes affects more than 90% of diabetic patients, causing microvascular and macrovascular complications that cause profound psychological and physical distress in patients, as well as a significant burden on health-care systems. Overweight and obesity, a lack of physical activity, insulin resistance, and genetic factors all contribute to Type 2 diabetes (17). Insulin resistance is linked to a slew of metabolic issues, including glucose intolerance, hypertension, a distinct dyslipidemia, a procoagulant state, and an upsurge in macrovascular disease (18). Cardiovascular disease is the leading cause of illness and death in Type 2 diabetes and necessitates close monitoring of glucose and lipid levels, as well as blood pressure, to reduce the risk of complications and disease progression (19). Increased thirst, frequent urination, increased hunger, weight loss, blurred vision, slow-healing sores, and frequent infections are common symptoms of Type 2 diabetes.

Gestational diabetes is a type of glucose intolerance that develops during pregnancy as a result of insufficient insulin supply to meet tissue demands for standard blood glucose regulation (20). Gestational diabetes appears to be caused by the same wide range of physiological and genetic abnormalities that characterize diabetes in general. Maternal overweight and obesity, later childbearing age, previous history of Gestational diabetes, family history of type 2 diabetes mellitus, and ethnicity are all major risk factors for Gestational diabetes (21). In the United States, African American, Hispanic American, Native American, Pacific Islander, and South or East Asian women have a higher prevalence than Caucasian women (22). According to a recent International Diabetes Federation report, 16% of live births worldwide in 2013 were complicated by hyperglycemia during pregnancy (23). According to the most recent meta-analysis by Saeedi et al. (24), the global prevalence of GDM is 14.7% based on the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria, which is the most widely used screening method worldwide. Pregnancy diabetes can result in problems such as the baby growing larger than usual, polyhydramnios, premature birth, pre-eclampsia, and infant jaundice.

Fenugreek's cultural and historical importance

Fenugreek is culturally significant Ayurvedic medicine around the world, including India, Egypt, and the Middle East. Fenugreek is a spice that is widely used in India and the Mediterranean region and is recognized to have a variety of medicinal properties. Fenugreek seeds are widely used in Indian cuisine and are an essential ingredient in curry powders. They are also used to enhance the flavour of pickles and chutneys (25). Fenugreek leaves are commonly used in salads and traditional Egyptian dishes such as Ful medames. Fenugreek seeds are used as a spice in many Middle Eastern dishes, including meat and vegetable dishes. In recent years, selected genotypes of this species have developed a niche crop that produces high yields of bloat-free forage, which can boost both beef and milk production in semiarid regions of western Canada. Fenugreek has cultural significance in Jewish tradition, where it is used during the Sukkot festival. The herb is one of four plant species that are gathered and waved during prayers. Fenugreek is also important in Islamic culture, where it is used in cooking and medicine.

Fenugreek has a long history dating back to antiquity. The herb was used for its medicinal properties in ancient Egypt and was thought to have been used by Cleopatra to enhance her beauty. Fenugreek was used to treat a variety of ailments in ancient Greece, including digestive issues, respiratory problems, and skin inflammation. Fenugreek was also used extensively in ancient Ayurvedic medicine in India. The herb was thought to have healing properties that could be used to treat a variety of health issues, including diabetes, inflammation, and digestive disorders (26). Fenugreek was also used in traditional Chinese medicine to treat various conditions, including asthma and digestive problems. In addition to its medicinal uses, Fenugreek was also used for religious and cultural purposes in ancient times. In ancient Egypt, Fenugreek was used in religious ceremonies and was believed to have healing properties. In ancient Rome, Fenugreek was used as a flavouring agent in food and was also used in perfumes and cosmetics. Fenugreek is still used for its medicinal and culinary properties today. It is thought to have a variety of health benefits, including lowering blood sugar levels, reducing inflammation (27), and increasing milk production in breastfeeding mothers National Institute of Child Health and Human Development (28). Fenugreek is also a popular ingredient in bodybuilding supplements because it is thought to boost testosterone levels (29).

Bioactive compounds of fenugreek seeds and their pharmacological property

Fenugreek seeds are high in bioactive compounds such as saponins, alkaloids, flavonoids, and phenolic compounds. At this point, we will look at fenugreek's bioactive compounds and their potential health benefits (**Table 1**). Secondary metabolites found in fenugreek seed include saponin (4.8%), flavonoids (100°mg/gm), alkaloids (35%), and diosgenin (0.2−0.9%) (30). Among these, alkaloids are primarily responsible for the distinctive taste and aroma. Saponins, which are glycosides with strong foam-forming properties, are abundant in fenugreek seeds. Saponins are thought to have a variety of health benefits, including cholesterol-lowering and anticancer properties. Saponins have been shown in studies to bind to bile acids in the intestine and prevent their reabsorption, resulting in lower cholesterol levels. Saponins have also been shown *in vitro* to inhibit cancer cell growth and induce apoptosis (programmed cell death) (31).

Trigonelline, gentianine, and carpaine are among the alkaloids found in fenugreek seeds. Trigonelline is an extremely powerful antioxidant that has been shown to protect against oxidative stress and DNA damage (32). Gentianine has anti-inflammatory and analgesic properties, whereas carpaine has hypotensive (blood pressure-lowering) properties. Flavonoids, which are polyphenolic compounds with antioxidant properties, are abundant in fenugreek

TABLE 1 Bioactive compounds of fenugreek (leaves and seed).

Class	Bioactive compound	Bioactivity	References
Sapogenin	3,5-Spirostadiene derivative, Smilagenin, Sarsasapogenin, Diosgenin (0.1–0.9%), Tigogenin, Yamogenin, Neotigogenin, Yuccagenin, Gitogenin, Neogitogenin	Antinflammatory, antidiabetic, neuroprotectiver, anticancerous, hypolipidemic effect	(37–39)
Saponin	Graecunins, Fenugrin B, Fenugreekine, Trigofoenosides A–G	–	(39)
Flavonoids	Naringenin, Quercetin, Tricin, Kaempferol, Luteolin, Quercitrin, Afroside, Isovitexin, Vitexin, Vicenin-1, Vicenin-2	antidiabetic, antiatherogenic, antidepressant, immunomodulatory, antitumor, anti-inflammatory, anticancer	(40–42)
Phenolics and their derivatives	p-Coumaric acid, Caffeic acid, Chlorogenic acid, Hymecromone, Trigoforin, Trigocoumarin, Scopoletin, gamma-Schizandrin, Zingerone, Vanillin, Gingerol, Eugenol	antidiabetic, antihyperlipidemic, antiobesity, anticancer, anti-inflammatory, antioxidant, antifungal, antibacterial	(2, 42)
Alkaloid	Trigonelline, N-methylnicotinic acid, Trimethylamine, Neurin, Choline, Gentianine, Carpaine, and Betain	hypoglycaemic, neuroprotective, anti-cancer, estrogenic, and antibacterial activities	(2, 43)
Lipids	Oleic, Linoleic, Linolenic acids, Phosphatidylcholine, Phosphatidyl ethanolamine	anti-cancer, anti-inflammatory	(2, 42)
Vitamin and minerals	Vitamins: A, B_1, B_2, C, niacin, nicotinic acid, β-carotene, Folic acid Minerals: Fe, Ca, P, S, Mg, Co, Cu, Mn, Zn, Br	–	(25)
Dietary fiber	Galactomannan	Reduce obesity and improve gut health	(44)

seeds (33). The flavonoids vitexin and isovitexin are the most abundant in fenugreek seeds. Vitexin has been shown to have anti-diabetic properties by increasing insulin sensitivity and decreasing blood glucose levels. Isovitexin has been shown to have neuroprotective properties and may aid in the prevention of cognitive decline (34). Fenugreek seeds contain phenolic compounds such as coumarins and lignans. Coumarins have anticoagulant properties and may aid in the prevention of blood clots. Lignans have anticancer properties and may help reduce the risk of breast cancer (35).

Other bioactive compounds found in fenugreek seeds include galactomannans, mucilages, and phytosterols. Galactomannans are complex carbohydrates that have been shown to lower cholesterol levels. Mucilages are water-soluble fibres that have been shown to have prebiotic properties and may aid in the improvement of digestive health (36). Phytosterols are plant-based compounds that have been shown to lower cholesterol levels. It contains a lot of dietary fibre, protein, amino acids, iron, silica, and vitamin B1. Furthermore, fenugreek seed contains an unusual amino acid called hydroxy isoleucine, which increases insulin secretion and helps to prevent diabetes (11).

Diosgenin extraction, purification, and characterization

The most active bioactive ingredient in fenugreek seed extract is diosgenin [0.113—0.135% (w/w)], which has anti-diabetic properties. The melavonate route, which comprises of a hydrophilic sugar moiety attached to a hydrophobic steroid aglycone, is used to produce diosgenin. When fenugreek seed was extracted using 70% (v/v) 2-propanol in water and sulphuric acid, the extract included a mixture of diosgenin with steroidal saponins, and other slight sapogenins (11). Diosgenin can be extracted from *Dioscorea nipponica* Makino using a magnetic sulfonated solid composite and hydrolyzing it with 2.5°M hydrochloric acid at 110°C for 5°h (45). Green extraction technologies such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) can extract the most diosgenin from fenugreek seeds, which can then be used to treat diabetes patients. For diosgenin extraction, the MAE method used a sample-to-solvent ratio is 1:5 (w/v) with solvents (acetone, ethanol, hexane, and petroleum ether) and extraction time (1.5, 3.0, 4.5, and 6.0°min) at 180 W power to yield 7.83% diosgenin content. In the UAE approach, 21.48% diosgenin was obtained when the sample-to-solvent ratio was 1:5 (w/v) with different solvents, treatment times were varied (30, 40, 50, and 60 min), and the ultrasonic bath temperature was prolonged at 30°C throughout the procedure (46). Likewise, deoiled fenugreek seed powder (20 g) was extracted with 100 ml ethanol (20—100%,v/v), extraction time 40, 50, and 60 min, and the temperature of the ultrasonic bath throughout the extraction process was 35°C to detect diosgenin (0.041—1.294 geq/100 g) (47). UAE method showed highest diosgenin from fenugreek seeds with 80% ethanol for 5 min, with α-amylase inhibition (IC_{50} crude = 371.7°μg/ml, IC_{50} defat = 370.5°μg/ml) and pancreatic lipase (IC_{50} crude = 550.0°μg/ml and IC_{50} defat = 497.6°μg/ml). For purification dehydrated fenugreek seed extract was dissolved in distilled water (50 ml), flushed twice with diethyl ether (50 mL), and extract liquid layer was extracted with water-saturated n-butanol (50 mL). Diosgenin profiles were constructed for seed samples of various fenugreek varieties, and the results revealed 200 and 480 mg/100 g FW diosgenin concentrations (48).

Chemical structure, health benefits, and worldwide status of diosgenin

Diosgenin is a major bioactive constituent of many edible pulses and roots, particularly in the seeds of fenugreek and the root tubers of wild yams. Diosgenin is found in 137 different Dioscorea species. A total of 41 of them contain more than 1% diosgenin. It is derived from the roots of the Dioscorea wild

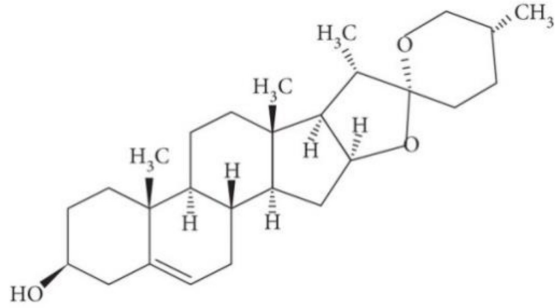


FIGURE 1
Chemical structure of diosgenin (53).

yam that is commonly used as a precursor in the production of synthetic steroid chemicals such as progesterone and cortisol (49). According to the National Center for Biotechnology Information 2023 (50), Diosgenin (25*R*-spirost-en-3β-ol) is a C27 triterpenoid spiroketal steroid sapogenin with a molecular weight of 414.62 and its formula is $C_{27}H_{42}O_3$ (**Figure 1**). It is described as a spirostan with a hydroxyl group at the β position in terms of molecular structure. It also contains a double bond at 5,6 position and has an R configuration at position 25. It has a hydroxyl group in the third position; hydroxyl groups are typically found in combination with sugars, making the compounds water-soluble and highly saponaceous (51). In aqueous medium, it has a solubility of about 0.7°ng/ml. It is a white crystalline powder that dissolves in organic solvents such as ethanol, DMSA, and dimethylformamide. Cholesterol is a precursor in the biosynthesis of diosgenin, which is catalysed by two P450 enzymes: C-16,22-dihydroxylase and C-26 hydroxylase (52).

In addition to being an important starting material for the preparation of several steroidal drugs in the pharmaceutical industry, diosgenin has shown high potential and interest in the treatment of various disorders such as cancer, diabetes, arthritis, asthma, and cardiovascular disease (54). Diosgenin supplementation is thought to be an excellent way to promote women's health because it slows the decline of estrogen and progesterone levels and aids in the prevention of hormonal imbalances. In essence, it may reduce the risk of osteoporosis, mood swings, irritability, and other symptoms associated with fluctuating hormone levels (55). Furthermore, research suggests that Diosgenin supplementation may help gastroprotection against stomach mucosa damage by inhibiting certain enzyme activity (56).

Because of the COVID-19 pandemic, the global Diosgenin market is estimated to be worth USD 99.5 million in 2022 and is expected to grow to USD 142.3 million by 2030, with a CAGR of 6.1% from 2022 to 2030. According to a Press Release of Diosgenin Market Size by 2030, Diosgenin accounts for 60% of the world's steroidal products out of all steroid drug precursors. With a market share of roughly 60%, China is the world's largest diosgenin consumer market. Sabinsa, Himachal Pharmaceuticals, Namiex Chemicals, Zhenhua Biology, and Shaanxi Jiahe Biotechnology are the global top five diosgenin manufacturers, with a combined market share of more than 85%, with Zhenhua Bio being the largest manufacturer, with a market share of more than 55%.

Modes of action of fenugreek derived diosgenin in diabetes

Numerous researches have looked into the effects of fenugreek extracts and diosgenin in the treatment of diabetes and their mechanisms of action with possible advantages for diabetics (**Table 2**). These consist of clinical trials, *in vitro* and *in vivo* studies (**Figure 2**). Further explanations of the processes, outcomes, and diosgenin's mode of action are provided in this section.

Diminish glucose absorption in intestine

The pancreatic β-cells' ability to secrete insulin is reduced by hyperglycemia in the toxicity cycle, which is then followed by a rise in insulin resistance, which worsens hyperglycemia and renders β -cells completely ineffective. The long-lasting and serious health repercussions of hyperglycemia take time to manifest (57). The small intestine has the ability to absorb carbohydrates, which allows glucose to enter the bloodstream and easily raises postprandial hyperglycemia in diabetics. Polysaccharides are primarily broken down into oligosaccharides in the luminal bulk fluid by secreted enzymes such as α-Glucosidase, disaccharidases, Na^+-K^+-ATPase, and additional hydrolysis is carried out by an array of carbohydrases in the brush border of the mature enterocytes (58). The Na^+-glucose cotransporter SGLT1 actively transports glucose and galactose into the enterocyte via the transmembrane electrochemical Na^+ gradient, whereas the glucose transporter GLUT2 actively transports glucose and galactose out across the basolateral membrane (59). Diosgenin showed a considerable amount of α -amylase and α -glucosidase inhibitory impact, supporting its involvement in lowering high blood glucose levels. Only one catalytic residue from α-amylase is involved in hydrogen bonding contact with diosgenin, while diosgenin interacts with two catalytic residues from α-glucosidase (Asp352 and Glu411) to generate the lowest energy inhibitor complex (60). Other hydrophobic interactions, in addition to hydrogen bonding interactions, contributed to the maximum binding affinity of diosgenin toward α-glucosidase. 33 steroidal saponins and sapogenins were extracted from fenugreek, and their *in vitro* α -glucosidase inhibitory action was assessed (61). There were five 25R and 25S isomer combinations of spirostanol saponins or sapogenins among them, and these were compounds 10 (25R/S)-5α-spirostane-2α,3β-diol 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, 12 diosgenin/yamogenin, 17 (25R/S)-5-en-spirostane-3β-ol 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, 22 (25R/S)-5-enspirostane-2α,3β-diol 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, and 29 sarsasapogeninn/smilagenin. Saponins 18 (25R)-5-en-spirostane-3β-ol 3-O-β-Dglucopyranosyl-(1 → 4)-β-D-glucopyranoside, 23 (25R)-5-en-spirostane-2α,3β-diol 3-O-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, 26 soyasapogenol B, 27 3-O-β-D-glucuronopyranosyl soyasapogenol B methyl ester, and 14 isonarthogenin significantly inhibited α-glucosidase at IC_{50} values of 15.16, 8.98, 7.26, 5.49, and 14.01°M, respectively, as compared to the positive control. Lactase and

TABLE 2 *In vitro, in vivo* studies and mechanism of action for treating diabetes with fenugreek and diosgenin.

Plant/Part and Location	Bioactive compound/Extract	Study	Clinical trial	Mode of action/Inference	Reference
Trillium govanianum, India	Sitagliptin, Diosgenin, Borassoside, Protodioscin	*In vitro*	–	Borassoside E (IC50 7.15 ± 1.78°μM), protodioscin (IC50 6.72 ± 0.04°μM), and diosgenin (IC50 12.75 ± 2.70°μM) inhibiting the activity of α-amylase, α-glucosidase, and dipeptidyl peptidase IV, respectively.	(100)
Gnidia glauca (Leaves, stems and flowers), *Dioscorea bulbifera* (Bulbs) Maharashtra, India	Petroleum ether, ethyl acetate and methanol extracts of plant	*In vitro*	–	Petroleum ether extract of *G. glauca* flower inhibit α-amylase by 78.56% and ethyl acetate extract of *D. bulbifera* bulbs inhibit α-amylase by 73.39%. Can use to treat type II diabetes mellitus.	(101)
Fenugreek (*Trigonella foenum-graecum* L. seed), London	Crude ethanolic extract B, further sub-fractions of B (saponin-free C, saponin D and sapogenin E) and a gum fiber fraction F	*In vitro*	–	Inhibited glucose-uptake at 0.33 and/or 3.3°mg/mL ($p < 0.001$).	(63)
Fenugreek (*Trigonella foenumgraecum*) seeds, India	80% ethanol by UAE method for 5 min produced 13.78 g diosgenin equivalent/100 g	*In vitro*	–	Inhibit α-amylase (IC50 crude = 371.7°μg/ml, IC50 defat = 370.5°μg/ml). Diosgenin assist in delaying glucose diffusion.	(48)
Fenugreek seed and Balanites fruit, Egypt	Methanolic extract	α-amylase (*in vitro*), starch absorption (*in vivo*)	STZ-diabetic rats (50 mg/kg) normal ($n = 10$) and diabetic groups ($n = 45$)	Fenugreek extract administrated STZ-diabetic mice, and decreased liver glucose-6-phosphatase activity, restored liver glycogen content, cut blood glucose levels by 58% and inhibit intestinal α-amylase activity.	(102)
Trigonella foenum-graecum	Saponin fraction contains diosgenin	*In vivo*	Four-week-old male KK-Ay/Ta Jcl mice administrated high-fat diet supplemented with 0.5 or 2.0% fenugreek	In HepG2 cells, diosgenin (5 and 10°μmol/L) reduced TG accumulation and the expression of genes related to lipogenesis and helpful for the controlling of diabetes-related hepatic dyslipidemias.	(103)
Fenugreek seed, Hungary	Diosgenin	*In vivo*	Diosgenin in three different doses (1°mg/bw kg, 10°mg/bw kg, and 50 mg/bw kg) and fenugreek seed (0.2 g/bw kg) were administered orally for 6 weeks to Male Wistar rats.	Rats given 1 mg/kg diosgenin with fenugreek seed showed better peripheral insulin sensitivity as evidenced by higher insulin sensitivity index and high metabolic clearance rates of insulin. Fenugreek seed regulates hormones in synchronised action with IGF-1, which is crucial for maintaining appropriate blood sugar levels.	(95)
Fenugreek seed, Puducherry, India	Methanolic extract contain trigonelline and diosgenin	*In vivo*	Male Sprague–Dawley rats (8—10 weeks with 150—200 g BW) administering STZ (dose of 35 mg/kg BW). Group 1 = control (NPD), Group 2 = T2DM model rats (HFD followed by STZ administration), Group 3, 4, 5 T2DM rats were given 300 mg FSE/kg BW, 40 mg of trigonelline/kg BW, 60 mg of diosgenin/kg BW, respectively.	Rats treated with FSE (217.11 ± 2.36 mg/dL), trigonelline (275.13 ± 24.88 mg/dL), and diosgenin (218.32 ± 37.9 mg/dL) showed significant reduction in glucose levels as compared to T2DM group (506.94 ± 7.06 mg/dL). T2DM rats showed two- to threefold increase in ER chaperones Bip, protein disulfide isomerase (PDI), as well as ER stress-related proapoptotic markers CHOP, Caspase12, and Caspase3 in the liver, and increased lipid peroxidation (LPO) and decreased antioxidant levels.	(104)

(*Continued*)

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TABLE 2 (Continued)

Plant/Part and Location	Bioactive compound/Extract	Study	Clinical trial	Mode of action/Inference	Reference
India	Diosgenin	*In vivo*	Male albino Wistar rats (BW = 150−180g) fed with HFD and administered with STZ (35 mg kg^{-1}), Group 1: Control, Group 2: Diabetic control, Group 3: Diabetic rats administered with diosgenin (60 mg $kg^{-1}b.w^{-1}$), Group 4: Diabetic rats administered with glyclazide (5 mg kg^{-1} $b.w^{-1}$).	Administration of diosgenin to HFD-STZ diabetic rats depicted a decrease in body weight gain, blood glucose (118.9 ± 9.92 mg/dL), insulin (14.65 ± 2.4°μU ml^{-1}), insulin resistance (2.01 ± 0.32) and modulated lipid profile in plasma and tissues as compared to HFD-STZ Diabetic control rats.	(105)
Fenugreek, India	Diosgenin	*In vivo*	Male Wistar rats (BW = 150−180 g) administrated 55 mg/kg streptozotocin with three different doses (15, 30, and 60 mg/kg BW) of diosgenin	Rats given 30 mg/kg BW diosgenin had lower serum Glucose 6-phosphatase levels (106.1 ± 2.9) compared to diabetic rats (155.0 ± 6.3) and higher hexokinase levels (15.8 ± 2.03) compared to diabetic rats (8.09 ± 2.1), which improved glycogen metabolism and lowered blood glucose levels.	(84)
China	Diosgenin	*In vivo*	Female mice were administered orally with DSG (10 mg/kg b.w. and 20 mg/kg b.w) using an intra-gastric tube	Diosgenin decreases gestational diabetes in db/ + pregnant mice, by improvements in glucose and insulin resistance, a decline in fasting blood glucose and insulin levels, and an increase in hepatic glycogen content.	(106)
Fenugreek seed, India	–	*In vivo*	Sixty patients having Type 2 diabetes divided into two groups. Group 1 = 30 patients received 10°gm of fenugreek seeds soaked in hot water every day, Group 2 = 30 patients didn't received fenugreek seeds.	Reduction in fasting blood glucose levels in the 5th month in the study group ($P = 0.0421$) while significant reduction in HbA_1C in the 6th month ($P = 0.0201$)	(107)

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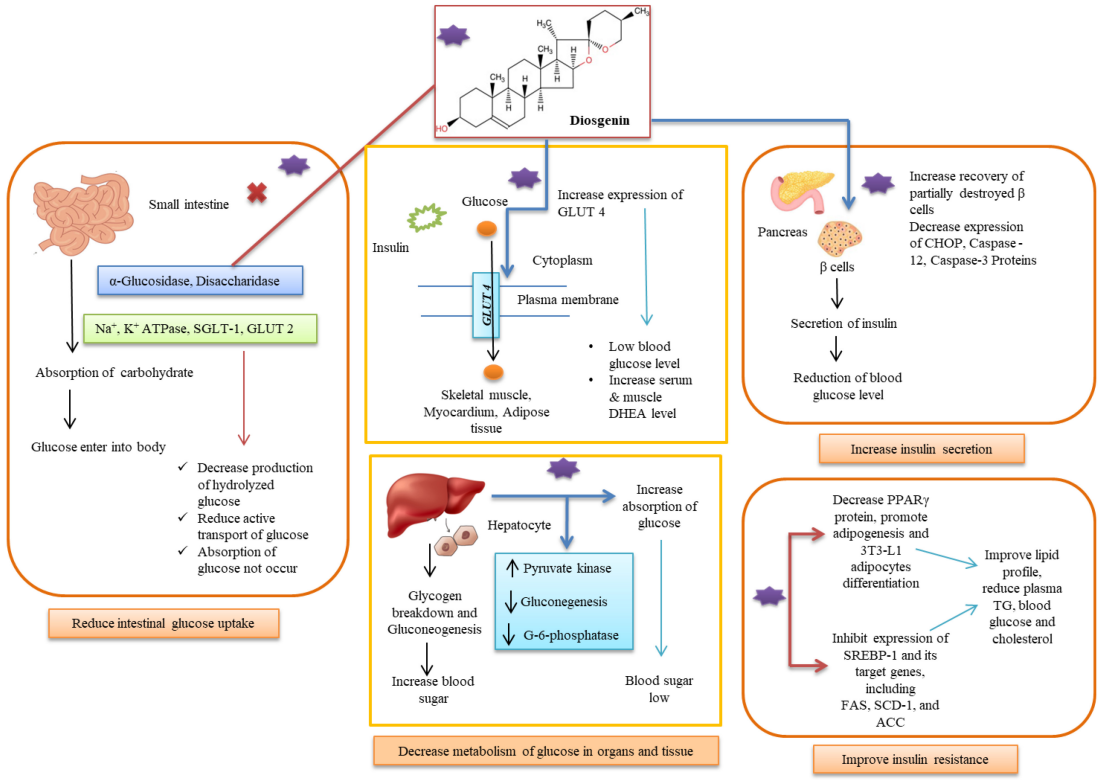

FIGURE 2
Diosgenin for diabetes treatment by multiple pathways in different organs.

maltase activity in the gut of diabetic rats was significantly reduced by sapogenin extract or diosgenin supplementation. Diosgenin consumption revealed hypoglycemic qualities that are advantageous in diabetes by lowering intestinal disaccharidases activity. Diabetic rats' Na^{+}-K^{+}-ATPase activity was shown to be drastically decreased when diosgenin was added to their diet. Supplementation of the diet with the 1% commercial diosgenin significantly reduced Na^{+}-K^{+}-ATPase activity in all three regions proximal (12.8 ± 0.2°nmol Pi/min/mg protein), mid (9.5 ± 0.1°nmol Pi/min/mg protein), distal (7.3 ± 0.1°nmol Pi/min/mg protein) of the intestine of the diabetic control rats when compared to the normal control group compared to the diabetic control group (62). Comparing the diabetes control to the diosgenin-supplemented group, the proximal area of the body showed a significantly higher level of Ca^{2+} ATPase activity. Sapogenin found in fenugreek extract reduced glucose absorption at concentrations of 0.33 and/or 3.3 mg/mL ($p < 0.001$). A total of 1 kg Fenugreek seed was ground and continuously extracted with light petroleum for 16 h and dried by rotary evaporation to yield an oil fraction (A; 45°g). Fenugreek seed powder (895°g), which had been defatted, was dried at room temperature and subjected to a 24-hour continuous extraction with 100% ethanol. The crude ethanolic extract (B; 65°g) was obtained after drying as before. A portion of B was redissolved in water and further extracted by n-butanol (C; 16.9°g) by evaporating the n-butanol layer, leaving the water layer containing saponin-free extract (D; 13.7°g on freeze drying). Diosgenin and trigonelline both prevented the absorption of glucose, with IC_{50} values of nearly 8 and 19 mM, respectively. Diosgenin (1.65 mg/ml) was more efficient than trigonelline (9.4% non-significant inhibition at 1.65 mg/ml) in inhibiting glucagon-induced HGPa activity (63). Diosgenin, which shares structural similarities with dehydroepiandrosterone (DHEA), reduces hyperglycemia in streptozotocin (STZ)-induced type 1 diabetes mellitus mice through increasing muscle GLUT4 signalling. After receiving diosgenin injection for 120 min, serum DHEA levels dramatically increased; concurrently, blood glucose levels significantly dropped (64).

Inhibit glucose uptake

It's crucial to comprehend the mechanism through which organs and tissues absorb glucose. Inhibiting hyperglycemia and its associated consequences can be prevented in the ideal way thanks to this information. Since most live cells in the body require the transportation of glucose, it is a primary source of energy for mammalian cells. Every cell's internal structure is protected by a phospholipid bilayer (65). Glucose molecules must cross lipid bilayers in the cell membrane as part of the transportation mechanism. Passive diffusion is used to allow hydrophobic species to travel across the hydrophobic lipid bilayers, which are permeable to them. Contrarily, glucose is hydrophilic and travels through the bilayer via enhanced diffusion, which is made possible by the career protein. Although the precise structure of the career protein for glucose update is unclear, conformational changes are known to trigger transport (66). The direction of glucose molecule

migration during facilitated diffusion is governed by the relative concentration on the two sides of the lipid bilayer. In facilitated diffusion, the molecules are moved across the membrane with the aid of channel proteins and carrier proteins rather than dissolving in the lipid bilayer (67). When the concentration of glucose outside the cell declines, such as in liver cells where glucose is synthesized when blood sugar levels are low, the transportation of glucose via the lipid bilayer can also occur in the opposite direction. The largest superfamily of membrane transporters, which includes 74 families and more than 10,000 members, includes glucose transporters. The facilitative glucose transporters (GLUTs) and sodium glucose co-transporters (SGLTs) are two possible types of glucose transporters (68). The rate of pancreatic insulin production is inversely correlated with the rate of glucose diffusion. Glucose-6-phosphate is produced after diffusion into the cell. By doing this, a cell is equipped with an energy reserve that may be tapped into as needed by the body (69).

HepG2 cells were treated in media containing insulin in a study to determine the action and mechanism of diosgenin and 5-methoxypsorlen (5-MOP). The findings showed that glucose consumption decreased at high insulin concentrations (10^{-6}°μmol/L) (70). However, the intake of glucose increased in a group of cells treated with diosgenin. The intracellular glycogen content also rose, showing that diosgenin was having a positive effect. During study related to action mechanism of diosgenin, it was revealed that the action of diosgenin significantly increased the phosphorylated expression of estrogen receptor-α (ERα), sarcoma (Src), Akt/protein kinase B, glycogen synthase kinase-3β (GSK3β), and the p85 regulatory subunit of phosphatidylinositol 3-kinase p85 (PI3Kp85x). In a group of cells that had received diosgenin treatment, the expression of GLUT-4 had significantly increased. Thus, it was possible to draw the following conclusions from the studies: diosgenin could reduce insulin resistance, increase glucose uptake, and hasten intracellular glycogen production. By encouraging the expression of adipocyte development in 3T3-L1 cells, diosgenin improves glucose uptake. The increased expression of mRNA also demonstrated its therapeutic potential (11, 49, 71). Diosgenin's therapeutic potential was also examined in groups of 10 male Wister rats that had been given STZ. Sesame oil, diosgenin (10 mg), and diosgenin combined with a 5α-reductase (1 mg) inhibitor were administered to each diabetic group. Within 90−180 min, it was seen that the diosgenin-injected group's blood glucose level had dropped. Diosgenin elevated serum dehydroepiandrosterone, which caused hyperglycemia in type 1 diabetic individuals. Diosgenin caused a 28% drop in glucose levels. The process is happening through the activation of AkT and PKC ζ/λ - GLUT4 signalling pathways. Both muscular AkT and PKC ζ/λ phosphorylation and GLUT4 translocation increased by 30% as compared to the untreated type 1 diabetic mice (64, 72).

Hepatic functions including gluconeogenesis and decomposition of glycogens also contribute to high blood sugar levels. These hepatic processes have been successfully controlled by diosgenin dose (73). The activity of glucose-6-phosphate dehydrogenase was dramatically decreased in the diet supplemented with 1% steroidal saponins from bitter yam or commercial diosgenin (74). Steroidal saponins possess a hexacyclic aglycone, such as diosgenin or tigogenin, in which the 3-OH group is decorated with an oligosaccharide chain. Also, diosgenin is a steroid saponin that is present in many plant species and is thought to provide a variety of essential therapeutic qualities. It is well recognized that glucose-6-phosphate plays a crucial function in controlling blood sugar levels while fasting. In glycolysis, glucose-6-phosphate is used to generate energy in place of ATP and NADH. Hyperglycemia has also been connected to a deficit in glucose-6-phosphate dehydrogenase (75).

Insulin, insulin analogues, improved delivery, and insulin resistance

Insulin is a natural hormone playing a critical role in blood sugar level regulation and is formed by beta cells of Islets of Langerhans in the pancreas. Though the main function of insulin is the regulation of blood sugar levels, besides it is essential for certain metabolic regulations in the body including glucose storage control, protein and fat metabolism, and appetite regulation. In persons suffering from diabetes, either insulin is not produced by the body which is Type 1 diabetes, or the produced insulin is not used effectively by the body which is Type 2 diabetes (76). Insulin helps in the regulation of blood sugar levels by permitting sugar (glucose) from the blood to pass into the cells, where it is utilized as an energy source. During diabetes, the absence of insulin or its inability consequences in raised sugar levels in the bloodstream.

The artificial sorts of insulin being altered to enhance their pharmacokinetic activities are called Insulin analogs. They are produced to impersonate the normal insulin release from the pancreas resulting in improved glucose control in the blood in diabetic patients (77). Insulin analogs are produced to have advantages than traditional insulins including better absorption, extended action-time, and better flexibility. Insulin analogs are of a number of types including rapid-acting, short-acting, intermediate-acting, and long-acting analogs (78). Insulin which is rapid-acting works swiftly and should primarily be taken prior to meals in order to resistor sugar spikes post-meal blood. These insulins work within 15 min post administration and showed a crowning effect between 0.5−3°h and last for around 3−5 h. On the other hand, short-acting insulin also known as regular insulin (such as human insulin) works slowly comparatively and likewise is also given formerly meals. This starts working within 0.5°h after administration, peaks in 2−4 h, and lasts for 6−8 h. Insulin that is intermediate-acting shows an extended interval and is taken once or twice a day, having a listless action onset, typically 1−2 h after taking. Its peak effect is between 4−12 h which lasts for 12−18 h. Neutral Protamine Hagedorn is this type of insulin analogue (79). While long-lasting insulin delivers a sturdy insulin release for a prolonged time and is frequently administered as basal insulin. These insulins provide a basal level of insulin release over an extended period, usually covering 24 h or longer. They have a relatively stable effect with no pronounced peaks. However, considering an individual's requirements, an amalgamation of diverse types of insulin might be given.

Maintaining sugar (glucose) levels in the blood, insulin therapy lessens the menace of severe snags and recovers long-term consequences in diabetic patients. By providing exogenic insulin, it recompenses for the insufficient formation or insulin utilization in the body. This assistance averts hyperglycemia and its related problems. Type 1 diabetes, a disorder identified

as diabetic ketoacidosis may happen during severe insulin lack (80). Insulin therapy improves essential in diabetic ketoacidosis prevention. Giving sufficient insulin, energy, or fat breakdown can be prevented, which is usually responsible for ketones accumulation and blood acidification. Insulin enables glucose uptake by cells, letting it be cast off for the production of energy.

There are numerous approaches to the improvement of insulin delivery that aim to augment the absorption, administration, and glycemic control of insulin. Insulin pens are expedient devices using one-use insulin-filled cartridges and deliver insulin better than outdated vials and syringes. Insulin pumps deliver insulin continuously by using a catheter located underneath the skin, provide a stable basal insulin rate, and permit bolus dosages prior to meals (81). Jet injectors distribute insulin by using a high-pressure stream that infiltrates the skin, eradicating the use of needles. Inhalable insulin is a newfangled insulin delivery method using the device to deliver insulin in a powdered form and inhaled it into the lungs. Insulin patches stick to the skin and deliver insulin over microneedles or a permeable membrane and offer continuous insulin delivery (82). Although the abdomen is the furthermost site for the injection of insulin, alternate injection sites *viz.,* thighs, upper arms, and buttocks. Insulin resistance is a disorder where the cells of the body especially fat, muscle and liver turn out to be less receptive to the insulin effects (83). As an outcome, the pancreas produces more insulin is being produced to recompense for the diminished sensitivity which leads to advanced insulin levels in the bloodstream called hyperinsulinemia.

Diosgenin has been explored for its anti-diabetic activities and its latent to advance insulin sensitivity. Some reports have indicated that diosgenin enhances the uptake of glucose in the cells, improves insulin signaling, and ameliorates diabetes effects. It has been reported that administration of different diosgenin doses (15, 30, and 60 mg/kg body weight) daily to diabetic rats for 45 days caused a significant ($p < 0.05$) decrease in glucose levels in the bloodstream and an upsurge in plasma insulin. The transformed actions of key enzymes of carbohydrate metabolism in the muscle and kidneys were regressed significantly ($p < 0.05$) to almost normal levels (84) and the found outcomes were related to a standard oral hypoglycaemic drug. Diosgenin effect on the skeletal disarrays persuaded by experimental type 1 diabetes in 3-month-old female rats induced by single streptozotocin injection (60 mg/kg *i.p.*) was studied (85). Diosgenin (50 mg/kg/day) was given after 2 weeks till 4 weeks and found that diosgenin countered the diabetes effect on the growth and cancellate bone in the distal femur signifying positive impact on the skeleton. Recently, it has been studied that diosgenin attenuates non-alcoholic fatty liver disease in type 2 diabetes by regulating SIRT6-related fatty acid uptake in spontaneous diabetic db/db mice *in vitro* and *in vivo* (86). Fenugreek extract and diosgenin protected the liver against Non-alcoholic steatohepatitis, and diosgenin showed a dose-dependent impact. Though, the activation of the AMPK cascade was believed to be the mechanism for hepatoprotective effects (87). However, most of the investigation of diosgenin's effect on insulin role is led in animal models or cell cultures. Partial clinical research is done in humans, and the consequences are not yet decisive. Additional examination is required to elucidate the exact actions or mechanisms and therapeutic potential.

Promote adipocyte differentiation

Adipocyte differentiation is a process in which preadipocytes (precursor cells) progress into mature adipocytes having a tendency to store and release fat. In the milieu of diabetes, lessened differentiation of adipocytes may result in insulin resistance, a key indicator of type 2 diabetes, and led to raised sugar levels in the bloodstream and the pancreas generating more insulin in an effort to reimburse, ultimately causing dysfunction of pancreatic beta-cells. Numerous aspects including chronic inflammation, obesity, hormonal imbalance, and genetic malfunctioning can interrupt the normal adipocyte process differentiation leading to insulin resistance during diabetes (88). Visceral fat in the abdominal cavity is further active metabolically and related to a sophisticated risk of insulin resistance and diabetes related to subcutaneous fat under the skin. In diabetes, the study of molecular mechanisms and dysregulation of adipocyte differentiation is an active extent of research. By detecting vital aspects intricated in the process, investigators aim to advance new beneficial approaches for controlling diabetes and its linked difficulties. Despite the fact that diminished differentiation of adipocytes and insulin resistance are strictly associated with type 2 diabetes (89), type 1 diabetes is chiefly an autoimmune disorder when the immune system erroneously outbreaks and abolishes insulin-generating beta cells. Differentiation of adipocytes is not as much directly linked to type 1 diabetes, nonetheless, metabolic dysregulation and resistance of insulin may still happen in persons with this illness.

Diosgenin improves the metabolism of glucose by endorsing the differentiation of adipocytes and hindering inflammation in adipose tissues as reported in the literature. Diosgenin reduced the adipocytes and improved the expression levels of mRNA differentiation-related genes in adipose tissues, repressed penetration of macrophage into adipose tissues, and hindered expressions of numerous molecular components linked with inflammation in 3T3-L1 cells (90). Diosgenin weakened metabolic dysfunction in high-fat diet-fed mice, as demonstrated by declined glucose levels in the blood and improvement of glucose and insulin intolerance. Diosgenin repressed 3T3-L1 adipocyte differentiation, declined the size of adipocytes, inhibited PPARγ, and increased nuclear expression of Erβ which significantly suppressed diosgenin-exerted suppression of adipocyte differentiation and PPARγ expression indicating the repressive effect of diosgenin on adipocyte differentiation and validated that ERβ-exerted regulation of PPARγ expression and action is critical for diosgenin-inhibited adipocyte differentiation (91). 3T3-L1 adipocytes and RAW 264 macrophages evidently heightened tumor necrosis factor-α production, chemoattractant protein-1 monocyte, and nitric oxide, however, diosgenin treatment repressed the formation of these proinflammatory mediators and also blocked the inflammation macrophages persuaded from 3T3-L1 adipocytes. Also, diosgenin repressed the degradation of inhibitor κB and c-jun N-terminal kinase phosphorylation in macrophages and might be useful for amending the seditious changes in obese adipose tissues (92). Type 2 diabetes was induced in experimental animals by feeding high-fat diet (HFD) for 8 weeks followed by streptozotocin (STZ) injection (sub-diabetogenic dose; 35 mg/kg body weight). Oral administration of diosgenin at two doses (40 and 80 mg/kg body weight) for 14 days abridged hyperglycemia, hypercholesterolemia

and hypertriglyceridemia and lipid accumulation in 3T3-L1 preadipocytes inveterate its adipogenic activity prejudiced by PPAR γ and PPAR α (93). Diosgenin lowers the damage of diabetes by altering cellular pathways for pancreatic β cell renewal for improved secretion of insulin and modifying ER-α-mediated PI3K/Akt pathways (94).

Safety dosage of diosgenin

Diosgenin is the main component of fenugreek saponins and a secondary metabolite. At dosages of 1125 mg/kg and higher, steroidal saponins, which include diosgenin, exhibited deleterious effects and even death. Interestingly, the traditional steroidal saponins dosage is 510 mg/kg/day, implying that steroidal saponins, in combination with diosgenin, have no significant toxicity at this dosage. Male Wistar rats were given diosgenin in three different doses (1, 10, and 50 mg/bw kg, respectively) and fenugreek seed (0.2 g/bwkg) orally for 6 weeks. The rats given 1 mg/kg diosgenin and fenugreek seed had a higher insulin sensitivity index and a higher metabolic clearance rate (95). For 1°week, male F344 rats were fed 0 or 1% fenugreek seed powder (FSP) or 0.05% or 0.1% diosgenin before receiving azoxymethane (15 mg/kg body weight). Bioactive substances found in fenugreek seeds, including protodioscin, trigoneoside, diosgenin, and yamogenin, affect a number of enzymes, including those involved in glucose and lipid metabolism. Dietary FSP at 1% and diosgenin at 0.1% inhibited total aberrant crypt foci by up to 33 and 39%, respectively, during the promotional stage (96). The viability and growth of HCT-116 cells were reduced by diosgenin in a dose-dependent manner. After 24 h, the IC50 cytotoxic dose of diosgenin in HCT-116 was 35°M, while concentrations of 32°M or higher reduced the percentage viable cells by 50%. The viability and growth of HCT-116 cells were reduced by diosgenin in a dose-dependent manner. After 24 h, the IC50 cytotoxic dose of diosgenin in HCT-116 was ∼35°μM, while concentrations of ∼32°μM or higher reduced the percentage viable cells by 50%. Increasing diosgenin concentrations reduced HMG-CoA reductase expression at both the mRNA and protein levels. Food saponin, diosgenin, is a powerful inhibitor of HCT-116 human colon carcinoma cells, inhibiting growth and inducing apoptosis (97). In rats, a dose of diosgenin (40 mg/kg) protected against changes in liver markers and the antioxidant system of red blood cells without causing any side effects (98). Diabetic rats were given diosgenin (40 mg/kg bw) orally, which significantly reduced plasma glucose while increasing insulin levels. Diosgenin may play a protective role against aortic damage caused by oxidative stress in diabetics by modulating antioxidant defence and reducing lipid peroxidation in the aorta (99). These studies demonstrated that diosgenin and its derivatives are non-toxic, highlighting their utility in the treatment of chronic diseases.

Conclusion and future prospective

Recent years have seen an increase in interest in using herbal medicine to supplement conventional therapies or treat a variety of conditions. The growing diabetic population, the demand for new medications from patients, and the potential market for new medications will all continue to drive fresh and creative research into all parts of diabetes therapy. The foundation for the subsequent generation of targeted drug development programmes has been laid by the mainstay medicines, which have been intensively researched throughout the years. Diosgenin, the primary active component of fenugreek, has been the subject of numerous tests by researchers looking at its role in the treatment of diabetes. Additionally, it is well recognized that the majority of diabetic problems are intimately tied to inflammation and oxidative stress in addition to impaired glucose and lipid metabolism. In addition to having an effective anti-inflammatory and antioxidant action, diosgenin also has a positive therapeutic effect on diabetes complications. Numerous studies have demonstrated the pharmacological advantages of diosgenin and its derivatives in the treatment of cancer, diabetes, osteoporosis, Alzheimer's disease, and stroke. It has been demonstrated that diosgenin interacts with a number of molecular targets that are crucial actors in the occurrence and incidence of many major illnesses. Additionally, a multitarget medication strategy targeting various risk factors is a crucial paradigm and a cutting-edge technique for treating neurological diseases with complicated pathophysiology. To treat the various pathogenic characteristics of these disorders, combination therapies of diosgenin with substances exhibiting numerous modes of action are anticipated to be more effective than single medications. Therefore, it is strongly advised that future research use a systematic experimental design to evaluate the long-term effects of DG and/or its derivatives for the treatment of neurodegenerative illnesses and the management of associated symptoms. In-depth research must also be done on the risk assessment and safety evaluation of the pharmaceutical use of diosgenin or its derivatives in the treatment of neurodegenerative diseases.

Author contributions

YT: Conceptualization, Writing – original draft. MK: Conceptualization, Writing – review & editing. AC: Writing – review & editing. MS: Supervision, Writing – review & editing. PV: Writing – review & editing. MB: Conceptualization, Writing – review & editing. CK: Writing – review & editing.

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Conflict of interest

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References

1. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of *Aegle marmelos*. *Biomed Res Int.* (2014) 2014:497606. doi: 10.1155/2014/497606

2. Wani S, Kumar P. Fenugreek: A review on its nutraceutical properties and utilization in various food products. *J Saudi Soc Agric Sci.* (2018) 17:97–106. doi: 10.1016/j.jssas.2016.01.007

3. Rana P, Kumar A, Choudhary A, Kaur H, Singh R. The wisdom of prevention: Holistic, preventive herb approach for healing of the globe. *J Pharm Innov.* (2021) 10:29–46.

4. Ranade M, Mudgalkar N. A simple dietary addition of fenugreek seed leads to the reduction in blood glucose levels: A parallel group, randomized single-blind trial. *Ayu.* (2017) 38(1–2):24–7. doi: 10.4103/ayu.AYU_209_15

5. Syed Q, Rashid Z, Ahmad M, Shukat R, Ishaq A, Muhammad N, et al. Nutritional and therapeutic properties of fenugreek (*Trigonella foenum-graecum*): a review. *Int J Food Prop.* (2020) 23:1777–91. doi: 10.1080/10942912.2020.1825482

6. Paramesha M, Priyanka N, Crassina K. Evaluation of diosgenin content from eleven different Indian varieties of fenugreek and fenugreek leaf powder fortified bread. *J Food Sci Technol.* (2021) 58:4746–54. doi: 10.1007/s13197-021-04967-z

7. Baset M, Ali T, Elshamy H, El Sadek A, Sami D, Badawy M, et al. Anti-diabetic effects of fenugreek (*Trigonella foenum-graecum*): A comparison between oral and intraperitoneal administration - an animal study. *Int J Fun Nut.* (2020) 1:2. doi: 10.3892/ijfn.2020.2

8. Karve A, Hayward R. Prevalence, diagnosis, and treatment of impaired fasting glucose and impaired glucose tolerance in nondiabetic U.S. adults. *Diabetes Care.* (2010) 33:2355–9. doi: 10.2337/dc09-1957

9. Gaddam A, Galla C, Thummisetti S, Marikanty R, Palanisamy U, Rao B. Role of Fenugreek in the prevention of type 2 diabetes mellitus in prediabetes. *J Diabetes Metab Disord.* (2015) 14:74. doi: 10.1186/s40200-015-0208-4

10. Deshpande A, Harris-Hayes M, Schootman M. Epidemiology of diabetes and diabetes-related complications. *Physical therapy* (2008) 88:1254–64. doi: 10.2522/ptj.20080020

11. Fuller S, Stephens J. Diosgenin, 4-hydroxyisoleucine, and fiber from fenugreek: mechanisms of actions and potential effects on metabolic syndrome. *Adv Nutr.* (2015) 6:189–97. doi: 10.3945/an.114.007807

12. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* (2009) 32:S62–7. doi: 10.2337/dc09-S062

13. American Diabetes Association. Lifestyle management: standards of medical care in diabetes-2019. *Diabetes Care.* (2019) 1:S46–60. doi: 10.2337/dc19-S005

14. Atkinson M. The pathogenesis and natural history of type 1 diabetes. *Cold Spring Harb Perspect Med.* (2012) 2:a007641. doi: 10.1101/cshperspect.a007641

15. Haque M, Das J, Xiong X, Song J. Targeting stem cell-derived tissue-associated regulatory t cells for type 1 diabetes immunotherapy. *Curr Diab Rep.* (2019) 19:89. doi: 10.1007/s11892-019-1213-7

16. Pradeepa R, Mohan V. Epidemiology of type 2 diabetes in India. *Indian J Ophthalmol.* (2021) 69:2932–8. doi: 10.4103/ijo.IJO_1627_21

17. Wondmkun Y. Obesity, Insulin Resistance, and Type 2 Diabetes: Associations and Therapeutic Implications. *Diabetes Metab Syndr Obes.* (2020) 13:3611–6. doi: 10.2147/DMSO.S275898

18. Deedwania P. Hypertension, dyslipidemia, and insulin resistance in patients with diabetes mellitus or the cardiometabolic syndrome: benefits of vasodilating β-blockers. *J Clin Hypertens.* (2011) 13:52–9. doi: 10.1111/j.1751-7176.2010.00386.x

19. Martín-Timón I, Sevillano-Collantes C, Segura-Galindo A, Del Ca?izo-Gómez F. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World J Diabetes.* (2014) 5:444–70. doi: 10.4239/wjd.v5.i4.444

20. Quintanilla Rodriguez B, Mahdy H. *Gestational Diabetes. [Updated 2022 Sep 6]. In: StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing (2023).

21. Buchanan T, Xiang A. Gestational diabetes mellitus. *J Clin Invest*. (2005) 115:485–91. doi: 10.1172/JCI24531

22. Spanakis E, Golden S. Race/ethnic difference in diabetes and diabetic complications. *Curr Diab Rep.* (2013) 13:814–23. doi: 10.1007/s11892-013-0421-9

23. Kampmann U, Madsen L, Skajaa G, Iversen D, Moeller N, Ovesen P. Gestational diabetes: A clinical update. *World J Diabetes.* (2015) 6:1065–72. doi: 10.4239/wjd.v6.i8.1065

24. Saeedi M, Cao Y, Fadl H, Gustafson H, Simmons D. Increasing prevalence of gestational diabetes mellitus when implementing the IADPSG criteria: A systematic review and meta-analysis. *Diabetes Res Clin Pract.* (2021) 172:108642. doi: 10.1016/j.diabres.2020.108642

25. Ahmad A, Alghamdi S, Mahmood K, Afzal M. Fenugreek a multipurpose crop: Potentialities and improvements. *Saudi J Biol Sci.* (2016) 23:300–10. doi: 10.1016/j.sjbs.2015.09.015

26. Cortez-Navarrete M, Pérez-Rubio K, Escobedo-Gutiérrez M. Role of Fenugreek, Cinnamon, *Curcuma longa*, Berberine and *Momordica charantia* in Type 2 Diabetes Mellitus Treatment: A Review. *Pharmaceuticals (Basel).* (2023) 16:515. doi: 10.3390/ph16040515

27. Hassani S, Fallahi Arezodar F, Esmaeili S, Gholami-Fesharaki M. Effect of Fenugreek Use on Fasting Blood Glucose, Glycosylated Hemoglobin, Body Mass Index, Waist Circumference, Blood Pressure and Quality of Life in Patients with Type 2 Diabetes Mellitus: A Randomized, Double-Blinded, Placebo-Controlled Clinical Trials. *Galen Med J.* (2019) 8:e1432. doi: 10.31661/gmj.v8i0.1432

28. National Institute of Child Health and Human Development. *Drugs and Lactation Database (LactMed$) [Internet]*. Bethesda (MD): National Institute of Child Health and Human Development (2006).

29. National Institute of Child Health and Human Development, NIH, DHHS. *The NICHD Study of Early Child Care and Youth Development (SECCYD): Findings for Children up to Age 4 1/2 Years (Reference Only) (05-4318).* Washington, DC: U.S. Government Printing Office (2006).

30. Wankhede S, Mohan V, Thakurdesai P. Beneficial effects of fenugreek glycoside supplementation in male subjects during resistance training: A randomized controlled pilot study. *J Sport Health Sci.* (2016) 5:176–82. doi: 10.1016/j.jshs.2014.09.005

31. Singh P, Bajpai V, Gond V, Kumar A, Tadigoppula N, Kumar B. Determination of Bioactive Compounds of Fenugreek (*Trigonella foenum-graecum*) Seeds Using LC-MS Techniques. *Methods Mol Biol.* (2020) 2107:377–93. doi: 10.1007/978-1-0716-0235-5_21

32. Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G, Jiang Y. Saponins from edible legumes: chemistry, processing, and health benefits. *J Med Food.* (2004) 7:67–78. doi: 10.1089/109662004322984734

33. Visuvanathan T, Than L, Stanslas J, Chew S, Vellasamy S. Revisiting *Trigonella foenum-graecum* L.: Pharmacology and Therapeutic Potentialities. *Plants.* (2022) 11:1450. doi: 10.3390/plants11111450

34. Salam S, Rashed M, Ibrahim N, Rahim E, Aly T, Al-Farga A. Phytochemical screening and in-vitro biological properties of unprocessed and household processed fenugreek (*Trigonella foenum-graecum* Linn.) seeds and leaves. *Sci Rep.* (2023) 13:7032. doi: 10.1038/s41598-023-31888-y

35. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* (2004) 74:2157–84. doi: 10.1016/j.lfs.2003.09.047

36. Goyal S, Gupta N, Chatterjee S. Investigating Therapeutic Potential of *Trigonella foenum-graecum* L. as Our Defense Mechanism against Several Human Diseases. *J Toxicol.* (2016) 2016:1250387. doi: 10.1155/2016/1250387

37. Sauvaire Y, Ribes G, Baccou J, Loubatieeres-Mariani M. Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. *Lipids.* (1991) 26:191–7.

38. Pang X, Huang H, Zhao Y, Xiong C. Conversion of furostanol saponins into spirostanol saponins improves the yield of diosgenin from *Dioscorea zingiberensis* by acid hydrolysis. *RSC Adv.* (2015) 5:4831–7. doi: 10.1039/C4RA12709A

39. Navarro del Hierro J, Reglero G, Martin D. Chemical Characterization and Bioaccessibility of Bioactive Compounds from Saponin-Rich Extracts and Their Acid-Hydrolysates Obtained from Fenugreek and Quinoa. *Foods.* (2020) 9:1159. doi: 10.3390/foods9091159

40. Wagner H, Iyengar M, Horhammer L. Vicenin-1 and -2 in the seeds of *Trigonella foenum-graecum*. *Phytochem.* (1973) 12:2548.

41. Rayyan S, Fossen T, Andersen Q. Flavone Cglycosides from seeds of fenugreek, *Trigonella foenumgraecum L. J Agric Food Chem.* (2010) 58:7211–7. doi: 10.1021/jf100848c

42. Sethi G, Shanmugam M, Warrier S, Merarchi M, Arfuso F, Kumar A, et al. Pro-Apoptotic and Anti-Cancer Properties of Diosgenin: A Comprehensive and Critical Review. *Nutrients*. (2018) 10:645. doi: 10.3390/nu10050645

43. Joshi J, Handler P. Biosynthesis of trigonelline. *J Biol Chem.* (1960) 235:2981–3.

44. Spyropoulos C. *Fenugreek—The Genus Trigonella*. London: Taylor and Francis (2002). p. 18–25.

45. Zhang F, Shen B, Jiang W. Hydrolysis extraction of diosgenin from *Dioscorea nipponica* Makino by sulfonated magnetic solid composites. *J Nanopart Res.* (2019) 21:269. doi: 10.1007/s11051-019-4702-3

46. Arya P, Kumar P. Comparison of ultrasound and microwave assisted extraction of diosgenin from *Trigonella foenum-graceum* seed. *Ultrason Sonochem.* (2021) 74:105572. doi: 10.1016/j.ultsonch.2021.105572

47. Wani S, Bishnoi S, Kumar P. Ultrasound and microwave assisted extraction of diosgenin from fenugreek seed and fenugreek-supplemented cookies. *J Food Meas Charact.* (2016) 10:527–32. doi: 10.1007/s11694-016-9331-2

48. Dsouza M, Rufina K, Hana D. Extraction of Diosgenin from Fenugreek and evaluation of its pharmacological role in alleviating Metabolic Syndrome *in vitro*. *Res J Biotechnol.* (2018) 13:10–7.

49. Jesus M, Martins A, Gallardo E, Silvestre S. Diosgenin: Recent Highlights on Pharmacology and Analytical Methodology. *J Anal Methods Chem.* (2016) 2016:4156293. doi: 10.1155/2016/4156293

50. National Center for Biotechnology Information. *PubChem Compound Summary for CID 99474, Diosgenin*. Bethesda, MD: National Center for Biotechnology Information (2023).

51. D?browska-Balcerzak K, Nartowska J, Wawer I, Siudem P, Paradowska K. Spirostanol Sapogenins and Saponins from *Convallaria majalis* L. Structural Characterization by 2D NMR, Theoretical GIAO DFT Calculations and Molecular Modeling. *Molecules.* (2021) 26:2999. doi: 10.3390/molecules26102999

52. Zhou C, Yang Y, Tian J, Wu Y, An F, Li C, et al. 22R- but not 22S-hydroxycholesterol is recruited for diosgenin biosynthesis. *Plant J.* (2022) 109:940–51. doi: 10.1111/tpj.15604

53. Ghayur M, Abdalla M, Khalid A, Ahmad S, Gilani A. *Trigonella foenum-graecum* methanolic extract on isolated smooth muscles and acetylcholinesterase enzyme: an *in vitro* and mechanistic *in silico* investigation. *Biomed Res Int.* (2022) 5:4849464. doi: 10.1155/2022/4849464

54. Abdulai I, Kwofie S, Gbewonyo W, Boison D, Puplampu J, Adinortey M. Multitargeted effects of vitexin and isovitexin on diabetes mellitus and its complications. *Sci World J.* (2021) 2021:6641128. doi: 10.1155/2021/6641128

55. Semwal P, Painuli S, Abu-Izneid T, Rauf A, Sharma A, Da?tan S, et al. Diosgenin: an updated pharmacological review and therapeutic perspectives. *Oxid Med Cell Longev*. (2022) 29:1035441. doi: 10.1155/2022/1035441

56. Sirotkin A, Alexa R, Alwasel S, Harrath A. The phytoestrogen, diosgenin, directly stimulates ovarian cell functions in two farm animal species. *Domest Anim Endocrinol.* (2019) 69:35–41. doi: 10.1016/j.domaniend.2019.04.002

57. Zhao H, Zhang X, Zhang B, Qu X. Gastroprotective effects of diosgenin against HCl/ethanol-induced gastric mucosal injury through suppression of NF-κβ and myeloperoxidase activities. *Open Life Sci.* (2021) 16:719–27. doi: 10.1515/biol-2021-0075

58. Kotani K, Peroni O, Minokoshi Y, Boss O, Kahn B. GLUT4 glucose transporter deficiency increases hepatic lipid production and peripheral lipid utilization. *J Clin Invest.* (2004) 114:1666–75. doi: 10.1172/JCI21341

59. Gromova L, Fetissov S, Gruzdkov A. Mechanisms of Glucose Absorption in the Small Intestine in Health and Metabolic Diseases and Their Role in Appetite Regulation. *Nutrients.* (2021) 13:2474. doi: 10.3390/nu13072474

60. Poulsen S, Fenton R, Rieg T. Sodium-glucose cotransport. *Curr Opin Nephrol Hypertens.* (2015) 24:463–9. doi: 10.1097/MNH.0000000000000152

61. Ghosh S, More P, Derle A, Patil A, Markad P, Asok A, et al. Diosgenin from *Dioscorea bulbifera*: novel hit for treatment of type II diabetes mellitus with inhibitory activity against α-amylase and α-glucosidase. *PLoS One.* (2014) 9:e106039. doi: 10.1371/journal.pone.0106039

62. Zhang H, Xu J, Wang M, Xia X, Dai R, Zhao Y. Steroidal saponins and sapogenins from fenugreek and their inhibitory activity against α-glucosidase. *Steroids.* (2020) 161:108690. doi: 10.1016/j.steroids.2020.108690

63. McAnuff M, Harding W, Omoruyi F, Jacobs H, Morrison E, Asemota H. Hypoglycemic effects of steroidal sapogenins isolated from Jamaican bitter yam, Dioscorea polygonoides. *Food Chem Toxicol.* (2005) 43:1667–72. doi: 10.1016/j.fct.2005.05.008

64. Al-Habori M, Raman A, Lawrence M, Skett P. In vitro effect of fenugreek extracts on intestinal sodium-dependent glucose uptake and hepatic glycogen phosphorylase A. *Int J Exp Diabetes Res.* (2001) 2:91–9. doi: 10.1155/edr.2001.91

65. Sato K, Fujita S, Iemitsu M. Acute administration of diosgenin or dioscorea improves hyperglycemia with increases muscular steroidogenesis in STZ-induced type 1 diabetic rats. *J Steroid Biochem Mol Biol.* (2014) 143:152–9. doi: 10.1016/j.jsbmb.2014.02.020

66. Nakrani M, Wineland R, Anjum F. *Physiology, Glucose Metabolism. [Updated 2022 Jul 25]. In: StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing (2023).

67. Cooper G. *The Cell: A Molecular Approach*. 2nd ed. Sunderland (MA): Sinauer Associates (2000).

68. Upadhyay R. Transendothelial transport and its role in therapeutics. *Int Sch Res Notices.* (2014) 2014:309404. doi: 10.1155/2014/309404

69. Navale A, Paranjape A. Glucose transporters: physiological and pathological roles. *Biophys Rev.* (2016) 8:5–9. doi: 10.1007/s12551-015-0186-2

70. Fu Z, Gilbert E, Liu D. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev.* (2013) 9:25–53.

71. Fang K, Dong H, Jiang S, Li F, Wang D, Yang D, et al. Diosgenin and 5-Methoxypsoralen Ameliorate Insulin Resistance through ER-α/PI3K/Akt-Signaling Pathways in HepG2 Cells. *Evid Based Complement Alternat Med.* (2016) 2016:7493694. doi: 10.1155/2016/7493694

72. Leng J, Li X, Tian H, Liu C, Guo Y, Zhang S, et al. Neuroprotective effect of diosgenin in a mouse model of diabetic peripheral neuropathy involves the Nrf2/HO-1 pathway. *BMC Complement Med Ther.* (2020) 20:126. doi: 10.1186/s12906-020-02930-7

73. Cai B, Zhang Y, Wang Z, Xu D, Jia Y, Guan Y, et al. Therapeutic Potential of Diosgenin and Its Major Derivatives against Neurological Diseases: Recent Advances. *Oxid Med Cell Longev.* (2020) 2020:3153082. doi: 10.1155/2020/3153082

74. Sharabi K, Tavares C, Rines A, Puigserver P. Molecular pathophysiology of hepatic glucose production. *Mol Aspects Med.* (2015) 46:21–33. doi: 10.1016/j.mam.2015.09.003

75. Mahmoud A, Nor El-Din AK. Glucose-6-Phosphate Dehydrogenase Activity and Protein Oxidative Modification in Patients with Type 2 Diabetes Mellitus. *J Biomark.* (2013) 2013:430813. doi: 10.1155/2013/430813

76. Rajas F, Gautier-Stein A, Mithieux G. Glucose-6 Phosphate. A Central Hub for Liver Carbohydrate Metabolism. *Metabolites.* (2019) 9:282. doi: 10.3390/metabo9120282

77. Rajput D, Basha S, Xin Q, Gadekallu T, Kaluri R, Lakshmanna K, et al. Providing diagnosis on diabetes using cloud computing environment to the people living in rural areas of India. *J Ambient Intell Human Comput.* (2022) 13:2829–40. doi: 10.1007/s12652-021-03154-4

78. Valla V. Therapeutics of diabetes mellitus: focus on insulin analogues and insulin pumps. *Exp Diabetes Res.* (2010) 2010:178372. doi: 10.1155/2010/178372

79. Sharma A, Taneja G, Kumar A, Sahu M, Sharma G, Kumar A, et al. Insulin analogs: Glimpse on contemporary facts and future prospective. *Life Sci.* (2019) 219:90–9. doi: 10.1016/j.lfs.2019.01.011

80. Brunetti V, Yu O, Platt R, Filion K. The association of long-acting insulin analogue use versus neutral protamine Hagedorn insulin use and the risk of major adverse cardiovascular events among individuals with type 2 diabetes: A population-based cohort study. *Diabetes Obes Metab.* (2022) 24:2169–81. doi: 10.1111/dom.14802

81. Calimag A, Chlebek S, Lerma E, Chaiban J. Diabetic ketoacidosis. *Dis Mon.* (2023) 69:101418. doi: 10.1016/j.disamonth.2022.101418

82. Thompson A, Lathan P, Fleeman L. Update on insulin treatment for dogs and cats: insulin dosing pens and more. *Vet Med (Auckl).* (2015) 6:129–42. doi: 10.2147/VMRR.S39984

83. Khafagy E, Morishita M, Onuki Y, Takayama K. Current challenges in non-invasive insulin delivery systems: a comparative review. *Adv Drug Deliv Rev*. (2007) 59:1521–46. doi: 10.1016/j.addr.2007.08.019

84. Lee W. MicroRNA, Insulin Resistance, and Metabolic Disorders. *Int J Mol Sci.* (2022) 23:16215. doi: 10.3390/ijms232416215

85. Saravanan G, Ponmurugan P, Deepa M, Senthilkumar B. Modulatory effects of diosgenin on attenuating the key enzymes activities of carbohydrate metabolism and glycogen content in streptozotocin-induced diabetic rats. *Can J Diabetes.* (2014) 38:409–14. doi: 10.1016/j.jcjd.2014.02.004

86. Londzin P, Kisiel-Nawrot E, Kocik S, Janas A, Trawczy?ski M, Cegieła U, et al. Effects of diosgenin on the skeletal system in rats with experimental type 1 diabetes. *Biomed Pharmacother.* (2020) 129:110342. doi: 10.1016/j.biopha.2020.110342

87. Nie K, Gao Y, Chen S, Wang Z, Wang H, Tang Y, et al. Diosgenin attenuates non-alcoholic fatty liver disease in type 2 diabetes through regulating SIRT6-related fatty acid uptake. *Phytomedicine.* (2023) 111:154661. doi: 10.1016/j.phymed.2023.154661

88. Eltamalawy M, Abdel-Aziz A, Mohamed T, Khedr N. The prophylactic treatment of Egyptian, *Trigonella foenum-graecum* L., Extract in comparison to pure diosgenin on experimentally induced non-alcoholic steatohepatitis: New targets via AMPK, RAR, and FXR pathways. *Phytomed Plus*. (2023) 3:100421. doi: 10.1016/j.phyplu.2023.100421

89. Tong Y, Xu S, Huang L, Chen C. Obesity and insulin resistance: Pathophysiology and treatment. *Drug Discov Today.* (2022) 27:822–30. doi: 10.1016/j.drudis.2021.11.001

90. Muscogiuri G, Sorice G, Mezza T, Prioletta A, Lassandro A, Pirronti T, et al. High-normal TSH values in obesity: is it insulin resistance or adipose tissue's guilt? *Obesity (Silver Spring).* (2013) 21:101–6. doi: 10.1002/oby.20240

91. Uemura T, Hirai S, Mizoguchi N, Goto T, Lee J, Taketani K, et al. Diosgenin present in fenugreek improves glucose metabolism by promoting adipocyte differentiation and inhibiting inflammation in adipose tissues. *Mol Nutr Food Res.* (2010) 11:1596–608. doi: 10.1002/mnfr.200900609

92. Wang X, Liu J, Long Z, Sun Q, Liu Y, Wang L, et al. Effect of diosgenin on metabolic dysfunction: Role of ERβ in the regulation of PPARγ. *Toxicol Appl Pharmacol.* (2015) 289:286–96. doi: 10.1016/j.taap.2015.09.015

93. Hirai S, Uemura T, Mizoguchi N, Lee J, Taketani K, Nakano Y. Diosgenin attenuates inflammatory changes in the interaction between adipocytes and macrophages. *Mol Nut Food Res.* (2010) 54:797–804. doi: 10.1002/mnfr.200900208

94. Sangeetha M, ShriShri Mal N, Atmaja K, Sali V, Vasanthi H. PPAR's and Diosgenin a chemico biological insight in NIDDM. *Chem Biol Interact.* (2013) 206:403–10. doi: 10.1016/j.cbi.2013.08.014

95. Arya P, Kumar P. Diosgenin: An ingress towards solving puzzle for diabetes treatment. *J Food Biochem.* (2022) 46:e14390. doi: 10.1111/jfbc.14390

96. Kiss R, Pesti-Asbóth G, Szarvas M, Stündl L, Cziáky Z, Hegedûs C, et al. Diosgenin and Its Fenugreek Based Biological Matrix Affect Insulin Resistance and Anabolic Hormones in a Rat Based Insulin Resistance Model. *Biomed Res Int.* (2019) 4:7213913. doi: 10.1155/2019/7213913

97. Raju J, Patlolla J, Swamy M, Rao C. Diosgenin, a steroid saponin of *Trigonella foenum graecum* (Fenugreek), inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. *Cancer Epidemiol Biomarkers Prev.* (2004) 13:1392–8.

98. Raju J, Bird R. Diosgenin, a naturally occurring steroid [corrected] saponin suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells. *Cancer Letters.* (2007) 255:194–204. doi: 10.1016/j.canlet.2007.04.011

99. Manivannan J, Barathkumar TR, Sivasubramanian J, Arunagiri P, Raja B, Balamurugan E. Diosgenin attenuates vascular calcification in chronic renal failure rats. *Mol Cell Biochem.* (2013) 378:9–18. doi: 10.1007/s11010-013-1588-8

100. Pari L, Monisha P, Mohamed Jalaludeen A. Beneficial role of diosgenin on oxidative stress in aorta of streptozotocin induced diabetic rats. *Eur J Pharmacol.* (2012) 691:143–50. doi: 10.1016/j.ejphar.2012.06.038

101. Suresh P, Singh P, Padwad Y, Sharma U. Steroidal saponins from Trillium govanianum as α-amylase, α-glucosidase, and dipeptidyl peptidase IV inhibitory agents. *J Pharm Pharmacol.* (2021) 73:487–95. doi: 10.1093/jpp/rgaa038

102. Ghosh S, Ahire M, Patil S, Jabgunde A, Bhat Dusane M, Joshi B, et al. Antidiabetic Activity of *Gnidia glauca* and *Dioscorea bulbifera*: Potent Amylase and Glucosidase Inhibitors. *Evid Based Complement Alternat Med.* (2012) 2012:929051. doi: 10.1155/2012/929051

103. Gad M, El-Sawalhi M, Ismail M, El-Tanbouly N. Biochemical study of the anti-diabetic action of the Egyptian plants fenugreek and balanites. *Mol Cell Biochem.* (2006) 281:173–83. doi: 10.1007/s11010-006-0996-4

104. Uemura T, Goto T, Kang M, Mizoguchi N, Hirai S, Lee J, et al. Diosgenin, the main aglycon of fenugreek, inhibits LXRα activity in HepG2 cells and decreases plasma and hepatic triglycerides in obese diabetic mice. *J Nutr.* (2011) 141:17–23. doi: 10.3945/jn.110.125591

105. Mayakrishnan T, Nakkala J, Jeepipalli S. Fenugreek seed extract and its phytocompounds- trigonelline and diosgenin arbitrate their hepatoprotective effects through attenuation of endoplasmic reticulum stress and oxidative stress in type 2 diabetic rats. *Eur Food Res Technol.* (2015) 240:223–32. doi: 10.1007/s00217-014-2322-9

106. Naidu P, Ponmurugan P, Begum M, Mohan K, Meriga B, Ravindar N, et al. Diosgenin reorganises hyperglycaemia and distorted tissue lipid profile in high-fat diet-streptozotocin-induced diabetic rats. *J Sci Food Agric.* (2015) 95:3177–82. doi: 10.1002/jsfa.7057

107. Hua S, Li Y, Su L, Liu X. Diosgenin ameliorates gestational diabetes through inhibition of sterol regulatory element-binding protein-1. *Biomed Pharmaco.* (2016) 84:1460–5. doi: 10.1016/j.biopha.2016.10.049

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Enhancing immune regulation *in vitro*: the synergistic impact of 3′-sialyllactose and osteopontin in a nutrient blend following influenza virus infection

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Natural components of breast milk, human milk oligosaccharides (HMOs) and osteopontin (OPN) have been shown to have a variety of functional activities and are widely used in infant formulas. However, the preventive and therapeutic effects of both on influenza viruses are not known. In this study, antiviral assays using a human laryngeal carcinoma cell line (HEP-2) showed that 3′-sialyllactose (3′-SL) and OPN had the best antiviral ability with IC_{50} values of 33.46 μM and 1.65 μM, respectively. 3′-SL (10 μM) and OPN (4 μM) were used in combination to achieve 75% inhibition. Further studies found that the combination of 200 μg/mL of 3′-SL with 500 μg/mL of OPN exerted the best antiviral ability. The reason for this was related to reduced levels of the cytokines TNF-α, IL-6, and iNOS in relation to mRNA expression. Plaque assay and $TCID_{50}$ assay found the same results and verified synergistic effects. Our research indicates that a combination of 3′-SL and OPN can effectively reduce inflammatory storms and exhibit anti-influenza virus effects through synergistic action.

KEYWORDS

3′-sialyllactose, osteopontin, influenza virus, immune cytokines, human milk oligosaccharide

1 Introduction

Influenza is a respiratory disease that spreads easily and causes symptoms such as a runny nose, sore throat, fever, and in severe cases, pneumonia and complications in other organs (1). Scientific data reveals that influenza viruses are responsible for hundreds of millions of infections worldwide annually, with infants and children particularly vulnerable due to their weakened immune system. In 2018, there were nearly 109.5 million reported

cases of viral infections among children under the age of 5 and approximately 34,800 deaths attributed to influenza or its complications globally (2). These figures demonstrate the significant threat that influenza viruses pose to human health, particularly in young children. While antiviral drugs currently play a crucial role in treating influenza. However, ethical concerns and strict regulations surrounding clinical trials in infants and children have restricted access to appropriate antiviral medications for this demographic (3, 4), Moreover, the high mutagenicity of influenza viruses has led to reduced efficacy of vaccines, further limiting treatment options (5). Indeed, there is an urgent need for new approaches to improve infants and childrens resistance to viruses.

Breast milk is widely recognized as the optimal food for infants, offering adequate nutrition as well as numerous active ingredients that promote anti-inflammatory (6), anti-infective (7), and immune development in early infancy (8). Human milk oligosaccharides (HMOs), the third most abundant component in human milk, confer important benefits for early colonization of the infants gut microbiota, intestinal barrier function, and immune modulation (9). HMOs have been shown to have unique antiviral properties and some studies have demonstrated their potential in reducing the risk of infection from a wide range of viruses. Clinical evidence supports the idea that HMOs have the ability to withstand the harsh conditions in the stomach and anterior small intestine. This enables them to reach the distal small intestine where they can stabilize G10P rotaviruses that infect cells (10). In a controlled dietary model of rotavirus-induced diarrhea in piglets, the experimental group received milk powder with HMOs showed a significant reduction in the duration of diarrhea compared to the control group (11). The 2-fucosyllactose (2-FL) mimics the human norovirus receptor, the histo-blood group antigen (HBGA), and acts as a decoy to prevent norovirus binding to HBGA, ultimately reducing the symptoms of infection (12). Osteopontin (OPN) is a highly abundant multifunctional non-collagenous matrix phosphoprotein in breast milk (13, 14). Research has demonstrated that OPN exhibits antiviral properties, and that mice with impaired OPN gene expression showed reduced immunity to viral and bacterial infections (15). Furthermore, a recent study discovered that both 2-FL and OPN were highly effective in reversing DNCB-induced dermatitis in mouse models, with an even more pronounced restorative effect observed when the two compounds were used together (16). In summary, the active components found in breast milk, particularly HMOs and OPN, have shown remarkable antiviral properties and may potentially exhibit synergistic effects. These findings provide strong motivation for further research into their effectiveness against influenza viruses and for studying the unique impact they can have when used together.

We utilized 2′-fucosyllactose (2′-FL), 3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), and OPN to intervene in an *in vitro* experiment of human laryngeal cancer cells infected with H1N1 influenza virus. Our aim was to investigate the potential therapeutic effects of these compounds on infected cells. The most effective HMO was then selected for combination with OPN to observe their synergistic effects and explore possible mechanisms of influence. Our objective is to propose a new personalized approach for infants and children with influenza virus, distinct from conventional drug treatment.

2 Materials and methods

2.1 Cells and strains

The HEP-2 human laryngeal carcinoma cell line was obtained from the American Type Culture Collection (ATCC) and cultured in modified DMEM medium (GibcoTM Life Technologies Inc., Grand Island, USA), supplemented with 10% fetal bovine serum (FBS, GibcoTM Life Technologies Inc., Grand Island, USA) and 1% dual antibodies (10,000 U/mL penicillin-10,000 g/mL streptomycin, Solarbio life sciences, Beijing, China). Influenza A (H1N1) virus (A/Zhejiang-Kecheng/SWL1219/2023) was provided by the Chinese Centre for Disease Control and Prevention and stored at -80°C. The virus was passaged twice through chicken embryos before the experiment to determine its potency for use, the first titer is 2^{-6}, the second is 2^{-7}, and the virus with the smaller titer is taken for follow up experiments. TNF-α, iNOS, and IL-6 ELISA kits were obtained from MULTISCIENCES (Shenzhen, China).

2.2 HMOs and OPN

This study utilized five different breast milk oligosaccharides (HMOs), with purities exceeding 95%, purchased from Royal DSM Group in the Netherlands. These HMOs included one fucose-based neutral oligosaccharide, 2′-fucosyllactose (2′-FL), two non-fucose-based neutral oligosaccharides, lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT), and two acidic oligosaccharides, 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL). Additionally, Lacprodan OPN-10 bone bridging protein with a purity level of over 95% was provided by Arla Foods Ingredients. The HMOs were dissolved in a 20 mM master batch using dimethyl sulfoxide (DMSO, Sigma, USA), while the OPN was dissolved in a 2 mg/mL master batch using sterile PBS for use. The OPN and HMOs used in the study were extracted and purified from bovine milk or synthesized using methods such as enzymatic reaction and microbial fermentation to obtain humanized osteopontin. Both production and use have been certified by the European Food Safety Authority (EFSA).

2.3 H1N1 virus $TCID_{50}$ analysis

A frozen solution of the H1N1 influenza virus was diluted in a 10-fold gradient with a serum-free DMEM medium. The diluted virus solution (100 μL/well) was added to 96-well plates lined with monolayers of cells, with six replicate wells used for each concentration, and a control group of normal cells. The plates were incubated in a 5% CO_2 incubator at 37°C for two hours, then the virus dilution solution was replaced with cell maintenance solution, and the plates were allowed to incubate for a further 48 hours, with

the degree of cellular lesions and number of wells recorded by observing cell morphology. When cell controls were close to normal morphology, wells with ≥50% virus-infected cytopathic lesions were considered diseased wells. The amount of virus infection in half of the cell cultures ($TCID_{50,}$ 50% tissue culture infective dose) was calculated using the Reed-Muench method.

2.4 Cytotoxicity analysis

HEP-2 cells were recovered and passaged 3-4 times until they grew well. The cells were then trypsin digested, diluted to a concentration of 1.5 x 10^5 cells/mL with complete medium, mixed, and blown before being inoculated into 96-well plates at 100 μL/well. The plates were incubated at 37°C in a 5% CO2 incubator for 24 h. The substances being tested were serially diluted in pairs with culture medium, and each concentration was added to the 96-well plates at 100 μL/well, with three replicate wells for every concentration, and a blank control group was included. The cells were incubated at 37°C in a 5% CO_2 incubator for 48 h. Afterward, cell morphology was observed and the supernatant was removed. CCK-8 was prepared as a 10% solution in PBS and 100 μL was added to each well. After one hour of action, the absorbance of the cells was measured using an enzyme marker at a wavelength of 540 nm.

2.5 Cytopathic inhibition assay

The culture medium was aspirated, and 100 $TCID_{50}$ of virus solution (control and treatment groups) or culture medium (normal group) was added at 100 μL/well. After adsorbing in a 37°C, 5% CO_2 incubator for 4 h, the influenza virus liquid was aspirated and 100 μL of compounds at appropriate doses were added to each well, with three replicate wells per concentration repeated three times. The cells were observed for 48 h to check for any morphological changes, and the cell supernatant was discarded. Next, CCK-8 was prepared as a 10% solution in PBS, and 100 μL was added to each well. After being given one hour to interact with the cells, enzymatic markers measured cell absorbance at a wavelength of 540 nm. Using the formula: Inhibition rate (%) = (Drug average A value - Virus control group average A value)/(Cell control average A value - Virus control group average A value) ×100%, calculate the degree of suppression of viral-induced cellular deterioration by the drug.

2.6 ELISA analysis

After a 4-hour adsorption period at 37°C in a 5% CO_2 incubator, the influenza virus solution was aspirated, and 100 μL of compounds at varying doses were added to 3 replicate wells per concentration, which was repeated 3 times. Following a 48-hour incubation period, the supernatant was collected and analyzed using an ELISA kit to measure the levels of inflammatory factors, including TNF-α, iNOS, and IL-6.

2.7 RT-qPCR analysis

After adsorbing the influenza virus solution for 4 hours at 37° C in a 5% CO_2 incubator, the solution was removed and replaced with 100 μL L of each compound at the appropriate dose across 3 wells per concentration, repeated 3 times. After 48 hours, the supernatant was removed, and cells were washed twice with pre-chilled PBS. Total RNA was extracted from the cells using Trizol according to the manufacturers instructions. Subsequently, mRNA expression levels of TNF-α, iNOS, and IL-6 inflammatory factors were measured by real-time fluorescence PCR after reverse transcription. GAPDH was used as an endogenous control, and the primers used in this study were: GADPH-Forward-(5-GACCCCTTCATTGACCTCAAC-3), GADPH-Reverse-(5-CATACCAGGAAATGAGCTTG-3), TNF-α-Forward-(5-CTGCTGCACTTTGGAGTGAT-3), TNF-α-Reverse-(5-AGATGATCTGACTGCCTGGG-3), IL-6-Forward-(5-AGCCACTCACCTCTTCAGAAC-3), IL-6-Reverse-(5-GCCTCTTTGCTGCTTTCACAC-3), iNOS-Forward-(5-CATCCTCTTTGCGACAGAGAC-3), iNOS-Reverse-(5-GCAGCTCAGCCTGTACTTATC-3). 2-ΔΔCTwas used to quantitatively analyze the mRNA of genes.

2.8 Inhibition of influenza H1N1 virus by the compositions

Plaque Assay: Inoculate HEP-2 cells into 6-well plates ($1.5x10^5$/well) and incubate overnight at 37°C, 5% CO_2. The culture medium was aspirated, and 100 $TCID_{50}$ of virus solution (H1N1 and treatment groups) or culture medium (control group) was added at 1 mL/well. After adsorbing in a 37°C, 5% CO_2 incubator for 4 h. Control group, 3′-SL group (200 μg/mL), OPN group (500 μg/mL), 3′-SL group (200 μg/mL), and 3′-SL+OPN group (200 μg/mL+500 μg/mL) were set up with the H1N1-infected group, adding 1mL of the compound to the corresponding wells. After that, each well was covered with a nutrient mixture containing 0.2% Bovine Serum Albumin (BSA, Beyotime, Shanghai, China), 0.6% Agar (Beyotime, Shanghai, China), and 0.3% DEAE (Beyotime, Beijing, China). The plates were then incubated at 37°C under 5% CO_2 conditions for 2-3 days. Subsequently, the wells were fixed and stained using a 0.5% crystal violet solution with formalin, and the plaque was observed.

$TCID_{50}$ Assay: A frozen solution of the H1N1 influenza virus was diluted in a 10-fold gradient with a serum-free DMEM medium. The diluted virus solution (100 μL/well) was added to 96-well plates lined with monolayers of cells, with six replicate wells used for each concentration, and a control group of normal cells. The plates were incubated in a 5% CO_2 incubator at 37°C for two hours, then the virus dilution solution was replaced with cell maintenance solution, then 100 μL of each of the compounds at the appropriate dose was added. Incubate for 48 h to observe the lesions (CPE) after viral infection of the cells, and the $TCID_{50}$ of the different drug concentration groups was determined by the Reed-Muench method.

2.9 Statistical analysis

The significance statistics were performed by One-way ANOVA analysis, Duncans multiple comparison test, and paired-samples T test of variance with SPSS 22 Version. A P value <0.05 was considered indicative of statistical significance. All experiments were repeated at least 3 times, and the data were expressed as mean ± standard deviation (Mean ± SD).

3 Result

3.1 Determination of $TCID_{50}$ of H1N1 influenza virus

The influenza virus was passaged twice in SPF-grade chicken embryos, and the number of wells in which the H1N1 virus appeared cytopathic for each virus dilution at 42h was observed. The $TCID_{50}$ value for the infection of cells by the H1N1 strain of influenza virus was calculated to be $10^{-3.48}$/0.1mL using the Reed-Muench method.

3.2 Toxic effects of HMOs and OPN on HEP-2 cells

We first assessed the toxic effects of these six nutrients in HEP-2 cells before *in vitro* antiviral assays as a way to avoid using concentrations that are toxic to cells for antiviral assays. The six nutrients were found to have close to 100 percent cell survival whether administered at high or low concentrations (Figures 1A, B), suggesting that they have no significant effect on cell survival and are sufficiently safe.

3.3 Inhibitory effect of HMOs and OPN on HEP-2 cytopathy caused by H1N1 virus

To investigate whether these six nutrients could inhibit H1N1 influenza virus infection, antiviral assays were performed using HEP-2 cells and only OPN and 3′-SL were found to have a significant inhibitory effect on H1N1-infected cells., we calculated the IC_{50} values of six nutrients against the H1N1 virus using a non-linear fit (Table 1). The IC_{50} values for 3′-SL and OPN were 33.46 μM and 1.65 μM, respectively, while several others including fucosyl-neutral and non-fucosyl-neutral and other acidic oligosaccharides showed less than 50% inhibition at all concentrations, indicating that the anti-H1N1 influenza virus was not effective. Based on these results, we determined the excellent antiviral properties of OPN with 3′-SL.

3.4 Inhibition of viral HEP-2 cytopathic lesions by 3′-SL complex OPN

Preliminary antiviral experiments were conducted to assess the combined antiviral properties of 3′-SL+OPN. Based on the data, we observed inhibition rates of 40% and 70% for H1N1 at OPN concentrations of 1 μM and 4 μM, respectively, and inhibition rates of 45% and 60% at 3′-SL concentrations of 10 μM and 40 μM, respectively. A combination of OPN (4 μM) and 3′-SL (10 μM) demonstrated higher inhibition rates (75%) than the monomers at lower concentrations (Table 2), suggesting the need to investigate optimal concentration for inhibiting the pathogenic effect of 3′-SL in combination with OPN against H1N1 virus.

3.5 H1N1 influenza virus inhibition assay

The impact of nutrients and their combinations on inflammatory factors was evaluated following infection of cells with the H1N1 virus, as illustrated in Figures 2A–C. The control group displayed levels of TNF-a, IL-6, and iNOS at 59.67 ± 6.67 pg/mL, 246.39 ± 8.24 pg/mL, and 2.87 ± 0.09 pg/mL, respectively. On the other hand, the model group showed a significant increase ($p \leq 0.001$) in all three inflammatory factors after virus infection, with TNF-a, IL-6, and iNOS levels at 144.11 ± 1.92 pg/mL, 417.62 ± 6.46 pg/mL, and 5.14 ± 0.25 pg/mL, respectively. The results of this

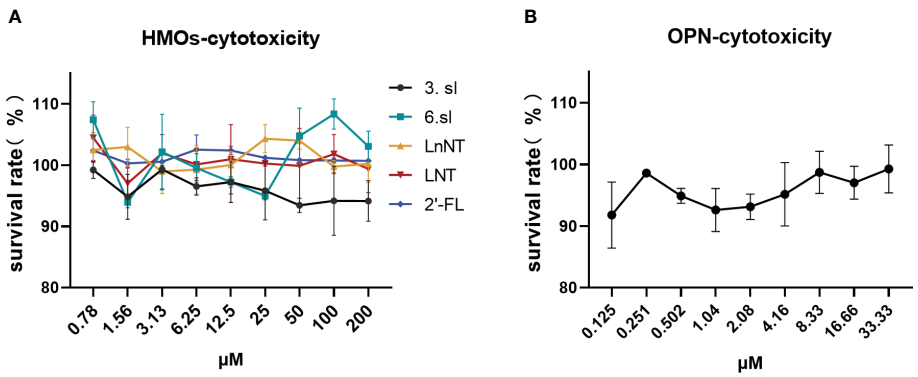

FIGURE 1
Toxic effects of HMOs and OPN on HEP-2 cells. **(A)** Toxic effects of five breast milk oligosaccharides on HEP-2 cells. **(B)** Toxic effects of bone bridge protein (OPN) on HEP-2 cells. Data are represented as mean ± SD (n= 3). Cell viability was calculated according to the formula: % cell viability = absorbance value of the administered group A/absorbance value of the cell control group A x 100%.

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TABLE 1 Results of nutrient inhibition of viral cytopathogenesis.

Nutrients	IC_{50}
2′-FL	>200 μM
3′-SL	33.46 μM
6′-SL	>200 μM
LNT	>200 μM
LNNT	>200 μM
OPN	1.65 μM

study suggest that the H1N1 virus upregulates pro-inflammatory factors, which can trigger an inflammatory storm and lead to tissue damage. After treatment with different doses of OPN and 3′-SL, a decrease in pro-inflammatory factors was observed. However, analysis of variance indicated that six different concentrations of the nutrients exhibited high sensitivity ($p \leq 0.001$) in inhibiting IL-6 production compared to the model group. In contrast, for TNF-a, significant differences were only found at medium and high concentrations of 3′-SL in combination with three concentrations of OPN, while no significant differences were observed at low concentrations ($p>0.05$). As for iNOS production, significant differences were found at medium and high concentrations of both OPN and 3′-SL, but no significant differences were observed at low concentrations when compared to the model group ($p>0.05$). These findings indicate that OPN and 3′-SL have a potential therapeutic effect on inflammation induced by H1N1 influenza virus.

Combination of two nutrients, OPN and 3′-SL, demonstrates superior inhibition of inflammatory factors. Analysis of variance showed highly significant differences ($p < 0.01$) in IL-6 and TNF-a compared to the model group in all nine groups with different dose combinations of high, medium and low. Additionally, iNOS production was highly significantly different from the model group ($p<0.001$) in all combinations except for 3′-SL+OPN (ML). Based on these results, it can be concluded that the synergistic effect of combining the two nutrients is more potent in suppressing H1N1 virus-induced inflammation as compared to using either nutrient alone.

Further, a comparative analysis of the inhibitory effect of nine combinations on the cytopathogenic effect of the H1N1 virus was

TABLE 2 Inhibition rate of viral cytopathogenic lesions by nutrients and their combinations.

OPN	concentration (μM)	1	4
	Inhibition rate (%)	40	70
3′-SL	concentration (μM)	10	40
	Inhibition rate (%)	45	60
OPN+3′-SL	concentration	4 (μM)+10 (μM)	
	Inhibition rate (%)	75.21 ± 6.15	

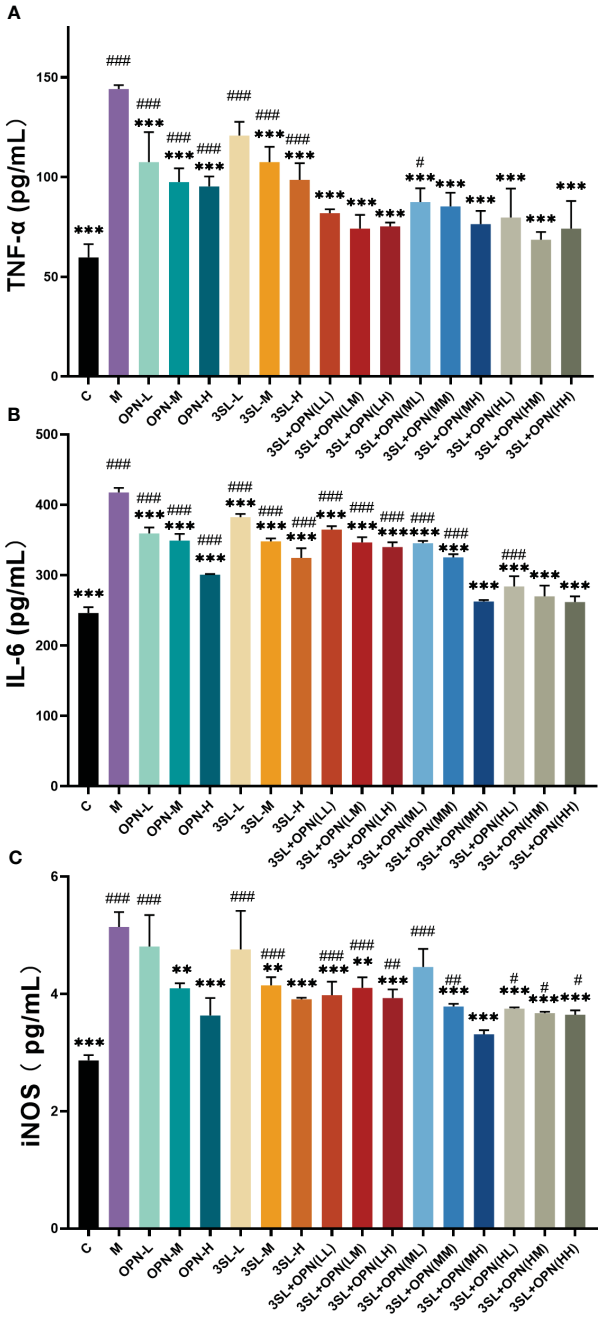

FIGURE 2
Effects of nutrient concentrations and combinations on suppression of inflammation following H1N1 infection. The study included the following groups: control group (C), a model group (M), and various groups treated with different doses of 3′-SL and OPN alone, including low dose (20 μg/mL, 3SL-L), medium dose (200 μg/mL, 3SL-M), and high dose (600 μg/mL, 3SL-H) of 3′-SL, and low dose (60 μg/mL, OPN-L), medium dose (200 μg/mL, OPN-M), and high dose (500 μg/mL, OPN-H) of OPN. 3′-SL+OPN group: (20 μg/mL + 60 μg/mL, LL), (20 μg/mL + 200 μg/mL, LM), (20 μg/mL + 500 μg/mL, LH), (200 μg/mL + 60 μg/mL, ML), (200 μg/mL + 200 μg/mL, MM), (200 μg/mL + 600 μg/mL, MH), (500 μg/mL + 60 μg/mL, HL), (500 μg/mL + 200 μg/mL, HM), and (500 μg/mL + 600 μg/mL, HH). **(A)** TNF-a, **(B)** IL-6, **(C)** iNOS. Data are represented as mean ± SD (n= 3). *P< 0.05; **P <0.01; ***P <0.001, compared with the Model group M. $^{\#}$P <0.05; $^{\#\#}$P< 0.01; $^{\#\#\#}$P <0.001, compared with the control group C.

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carried out. Optimal inhibition of H1N1 virus pathogenicity by the combination 3′-SL+OPN (MH). Its treatment resulted in no significant difference in the production of inflammatory factors and iNOS production in cells compared to non-viral treated controls ($p>0.05$), indicating recovery of the inflammatory phenomenon caused by H1N1 virus infection.

3.6 RT-qPCR

To further confirm the inhibitory effect of nutrients on the virus, this study also examined the expression of mRNA for inflammatory factors at the gene level using RT-qPCR. The expression of mRNA for inflammatory factors in the control group was very low, while the mRNA expression of the inflammatory factors TNF-a and IL-6 was found to be 100 and 150 times higher, respectively, after viral H1N1 infection (Figures 3A, B), and the mRNA expression of iNOS was elevated by about 9 times (Figure 3C). A significant reduction in the expression of mRNA for the three inflammatory factors was also found after treatment with both nutrients, particularly in the 3′-SL +OPN (MH) group where the mRNA expression of all three inflammatory factors reached the lowest levels of all groups: TNF-a 48.52 ± 1.53, IL-6 53.27 ± 6.88 and iNOS 3.87 ± 0.28, which is similar to the results for inflammatory factor production.

3.7 Inhibition of influenza H1N1 virus by the compositions

To confirm the synergistic effect of the combination of 3′-SL +OPN (200 μg/mL+500 μg/mL) in inhibiting viral cytopathogenic lesions, a plaque assay was conducted, and the results are presented in Figure 4. The number of plaques was significantly increased in the H1N1 group compared to the non-virus-infected group C. 3′-SL (600 μg/mL) and OPN (500 μg/mL) exhibited an inhibitory effect on viral plaques, but this effect was more significant when the two compounds were combined together. The results suggest that 3′-SL and OPN, especially when combined, had a direct effect on the virus, resulting in a reduction of viral infectivity.

The $TCID_{50}$ assay was used to investigate the reduction of H1N1 virus in HEP-2 cells under nutrient combination intervention. Table 3 presents the $TCID_{50}$ values for H1N1 virus in different conditions. Without nutrient intervention, the $TCID_{50}$ of H1N1 was $10^{-3.71}$/0.1 mL. However, when OPN (500 μg/mL) or 3′-SL (600 μg/mL) was administered alone, the H1N1 virus was significantly suppressed, resulting in $TCID_{50}$ values of $10^{-3.23}$/0.1 mL and $10^{-2.9}$/0.1 mL, respectively. Remarkably, the combination of OPN and 3′-SL exhibited an even more pronounced suppression of the H1N1 virus, with a $TCID_{50}$ value of $10^{-2.65}$/0.1 mL.

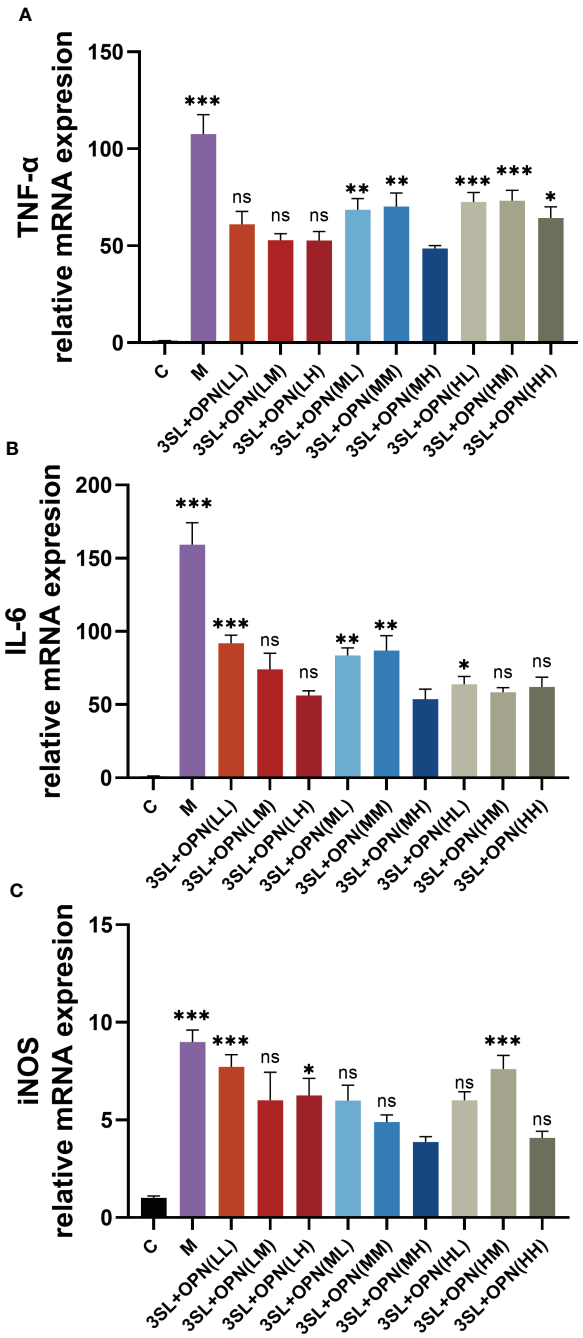

FIGURE 3
Expression of mRNA for inflammatory factors and iNOS during anti-H1N1 virus by nutrients and their different combinations. The study included the following groups: control group (C), a model group (M), 3′-SL+OPN group (20 μg/mL + 60 μg/mL, LL), (20 μg/mL + 200 μg/mL, LM), (20 μg/mL + 500 μg/mL, LH), (200 μg/mL + 60 μg/mL, ML), (200 μg/mL + 200 μg/mL, MM), (200 μg/mL + 600 μg/mL, MH), (500 μg/mL + 60 μg/mL, HL), (500 μg/mL + 200 μg/mL, HM), and (500 μg/mL + 600 μg/mL, HH). Data are represented as mean ± SD (n= 3). *P< 0.05; **P <0.01; ***P <0.001, ns, not significant, compared with the 3SL+OPN (MH) group.

4 Discussion

The H1N1 virus is a common influenza virus that primarily affects the upper respiratory tract in humans and causes a range of symptoms such as fever, body fatigue, sore throat, dry cough and flu (17). The respiratory tract is an important structure connecting the larynx to the lungs and includes the nasal cavity, pharynx, trachea, bronchi and alveolar tissues (18), Therefore, it is crucial to maintain

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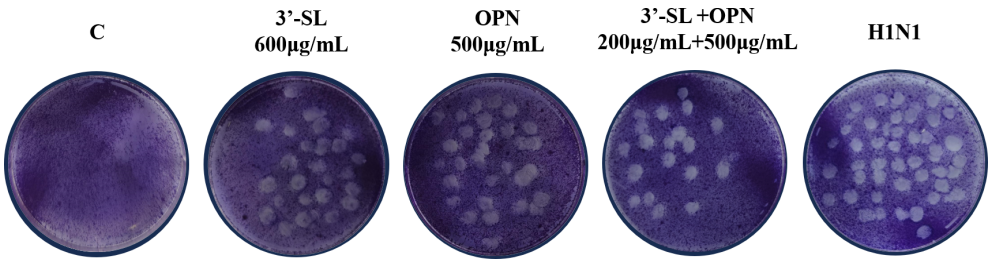

FIGURE 4
Inhibitory effect of 3′-SL, OPN and 3′-SL+OPN on H1N1 detected by plaque assay.

a healthy respiratory tract. Human laryngeal cancer cells are extensively utilized in viral and antiviral drug research due to their relatively high susceptibility to viral infection. The selection of the HEP-2 human laryngeal cancer cell line for this study was based on the fact that it represents the organ site where HMOs and OPNs come into direct contact with the host.

The nutrients used in this study are derived from natural breast milk ingredients and their safety has been scientifically proven in numerous studies. In our experiments, cells came into direct contact with nutrients after infection with the H1N1 virus, and a potential precursor cause could be that the nutrients inhibited the attachment of the virus to the cells, altering the binding efficiency of H1N1 to HEP-2 cells, and thus inhibiting the virus from being effective in causing the cells to become diseased. A recent study has shown that glycosaminoglycans (HM-GAGs) in breast milk prevent cells from binding to cytomegalovirus (19), highlighting the active function of breast milk. In addition, some HMOs can also reduce the binding ability of most pathogens (e.g. Escherichia coli, Vibrio cholerae, Philis Salmonella and Helicobacter pylori) to intestinal epithelial cells *in vitro* (20, 21), thus reducing gastrointestinal diseases. This ability to enhance the antiviral effect by mitigating attachment may be related to the unique structure of nutrients and viruses. For example, the ability of breast milk oligosaccharide 2′-FL to mimic the norovirus receptor, blood group antigen (HBGA), and act as a decoy to prevent norovirus from binding to HBGA (12). HMOs and OPNs may have similar decoy mechanisms to influenza viruses (22). It is worth noting that while the present study only identified 3′-SL as being resistant to H1N1, other studies have indicated that 6′-SL may also possess broad-spectrum antiviral activity. This is attributed to the ability of salivary acidified molecules to bind to crucial influenza virus proteins, specifically the hemagglutinin proteins, and compete with host cells (23). Our data show variability in the inhibitory potency of different structures of HMOs against H1N1 viruses and find that salivary acidified 3-SL exerts the best inhibitory effect, consistent with the previously reported ability of salivary acidification to reduce selectin-mediated leukocyte adhesion (24). This result provides new evidence and insight into the link between the structure of HMOs and their ability to resist viruses. However, the detailed structural mechanisms targeting the attenuation of influenza virus attachment deserve further exploration.

TABLE 3 Inhibition of Influenza H1N1 Virus by the Compositions.

Nutrients	concentration	$TICD_{50}$(/0.1mL)
3′-SL	600 mg/mL	$10^{-3.23}$
OPN	500 μg/mL	$10^{-2.9}$
3′-SL+ OPN	200 μg/mL+500 μg/mL	$10^{-2.65}$
No Nutrients		$10^{-3.71}$

Numerous *in vitro* and *in vivo* experiments have shown that the hyperinflammatory response to influenza virus infection is a key factor in organismal damage (25–27). The TOLL receptor pathway, which triggers inflammatory damage during viral cytopathogenesis, is often thought to be highly associated with increased influenza morbidity and significant changes in TNF-α, IL-6 and iNOS during Toll receptor pathway activation (28, 29). Pro-inflammatory cytokines such as TNF-α and IL- 6 play a key regulatory role in the inflammation-induced immune response (30, 31). Tumor necrosis factor-a (TNF-α) is a pleiotropic cytokine produced by a variety of cells in response to inflammatory and immunomodulatory stimuli and can induce cytopathic regulation (32). IL-6 is a strong activator of the acute phase response, contributing to the systemic and local inflammatory response, and excess IL-6 can induce a variety of chronic inflammatory diseases (33). NO radicals are also critical in inflammatory and immune responses and are synthesized by enzymes such as NOS (eNOS) and iNOS via the l-arginine pathway (34). Under normal physiological conditions, iNOS is dormant in dormant cells; however, under pathological conditions, it produces large amounts of NO and plays a dual role in chronic infections, inflammation (35). Reducing NO production may be an effective strategy for treating a wide range of inflammatory diseases (36). In addition, influenza virus induces proliferation of TNF-α, IL-6, and iNOS, and overexpression of IL-6 and TNF-α promotes influenza virus replication (37), which, together with iNOS, are involved in the hyperinflammatory response to influenza virus infection (38). Breast milk-derived active substances have been reported to exhibit significant inflammatory and immunomodulatory effects both *in vitro* and *in vivo*. Powdered infant formula supplemented with HMOs protects the colon from infection and reduces the inflammatory response by preventing necrotizing small intestinal colitis (NEC) in mice or piglets and by inhibiting activation of the TLR4

signaling NF-κb signaling pathway (39). Moreover, *In vitro*, 2′-FL directly inhibits lipopolysaccharide (LPS)-induced inflammatory responses in intestinal epithelial cells (IECs), decreases IL-8 release, and suppresses transcription and translation of CD14, whose overexpression increased inflammatory responses (40). Recent studies have demonstrated that 2′-FL can enhance immunomodulation and reduce inflammatory responses following influenza vaccination (41). This activity may also have an direct impact on respiratory inflammation caused by influenza viruses. Our data show that TNF-α, IL- 6 and iNOS are increased to varying degrees in HEP-2 cells after H1N1 virus infection. In terms of mRNA expression, the expression of TNF-α and IL- 6 increases up to an alarming 100-fold and 150-fold after H1N1 virus infection, and the excessive inflammatory factors cause inflammatory damage to human respiratory tissues, resulting in discomfort such as sore throat and dry cough. However, after 3′-SL and OPN treatment, the levels and expression of TNF-α, IL- 6 and iNOS were significantly reduced, alleviating cellular inflammation. The plaque assay and $TCID_{50}$ also indicated that lower concentrations of the combined nutrients were more effective in inhibiting the H1N1 virus compared to higher concentrations of single nutrient interventions.

5 Conclusion

Overall, our work demonstrated the ability of 3′-SL and OPN to inhibit H1N1 influenza virus cytopathogenesis *in vitro* and found that the most effective combination of antiviral doses was 200 μg/mL of 3′-SL combined with 500 μg/mL of OPN. HMOs and OPN have almost no toxic effect on HEP-2 cells. Acidic breast milk oligosaccharide 3′-SL had a more effective inhibitory effect on the cellular attack of H1N1 virus. When used in combination with OPN, its combined antiviral capacity exceeded the effect of a single substance. This antiviral capacity was associated with a reduction in the inflammatory response in HEP-2 cells, and 3′-SL with OPN was able to significantly reduce the levels and mRNA expression of the cytokines TNF-α, IL-6 and iNOS as a result of H1N1 virus infection, enhancing the innate immunity of cells to H1N1 virus *in vitro*. The synergistic effect of the same phenomenon was observed using both plaque assay and $TCID_{50}$ assay, confirming its effectiveness. However, further exploration is required to understand the mechanism of synergy, along with conducting human clinical studies to validate the findings of the *in vitro* studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval and written informed consent were not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

ZG: Data curation, Formal analysis, Investigation, Writing – original draft. QX: Data curation, Methodology, Writing – review & editing. QR: Data curation, Methodology, Writing – review & editing. YL: Data curation, Methodology, Writing – review & editing. KL: Data curation, Methodology, Writing – review & editing. BL: Project administration, Writing – review & editing. JL: Project administration, Writing – review & editing.

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Conflict of interest

Authors QX, QR, YL, KL and JL are employed by Heilongjiang Feihe Dairy Co.

The remaining 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/fimmu.2024.1271926/full#supplementary-material

References

1. Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, Doherty PC, et al. Influenza. *Nat Rev Dis Primers* (2018) 4:3. doi: 10.1038/s41572-018-0002-y

2. Wang X, Li Y, OBrien KL, Madhi SA, Widdowson MA, Byass P, et al. Global burden of respiratory infections associated with seasonal influenza in children under 5 years in 2018: a systematic review and modelling study. *Lancet Glob Health* (2020) 8: e497–510. doi: 10.1016/s2214-109x(19)30545-5

3. Needham AC, Kapadia MZ, Offringa M. Ethics review of pediatric multi-center drug trials. *Paediatr Drugs* (2015) 17:23–30. doi: 10.1007/s40272-014-0098-9

4. Gonzalez D, Sinha J. Pediatric drug-drug interaction evaluation: drug, patient population, and methodological considerations. *J Clin Pharmacol* (2021) 61 Suppl 1: S175–s187. doi: 10.1002/jcph.1881

5. Hsu AC. Influenza virus: A master tactician in innate immune evasion and novel therapeutic interventions. *Front Immunol* (2018) 9:743. doi: 10.3389/fimmu.2018.00743

6. Masi AC, Embleton ND, Lamb CA, Young G, Granger CL, Najera J, et al. Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut* (2021) 70:2273–82. doi: 10.1136/gutjnl-2020-322771

7. Slomski A. Human milk protein prevents infections in preterm infants. *Jama* (2016) 316:2078. doi: 10.1001/jama.2016.17037

8. Lyons KE, Ryan CA, Dempsey EM, Ross RP, Stanton C. Breast milk, a source of beneficial microbes and associated benefits for infant health. *Nutrients* (2020) 12 (4):1039–69. doi: 10.3390/nu12041039

9. Rousseaux A, Brosseau C, Le Gall S, Piloquet H, Barbarot S, Bodinier M. Human milk oligosaccharides: their effects on the host and their potential as therapeutic agents. *Front Immunol* (2021) 12:680911. doi: 10.3389/fimmu.2021.680911

10. Ramani S, Stewart CJ, Laucirica DR, Ajami NJ, Robertson B, Autran CA, et al. Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. *Nat Commun* (2018) 9:5010. doi: 10.1038/s41467-018-07476-4

11. Li M, Monaco MH, Wang M, Comstock SS, Kuhlenschmidt TB, Fahey GC Jr., et al. Human milk oligosaccharides shorten rotavirus-induced diarrhea and modulate piglet mucosal immunity and colonic microbiota. *ISME J* (2014) 8:1609–20. doi: 10.1038/ismej.2014.10

12. Koromyslova A, Tripathi S, Morozov V, Schroten H, Hansman GS. Human norovirus inhibition by a human milk oligosaccharide. *Virology* (2017) 508:81–9. doi: 10.1016/j.virol.2017.04.032

13. Du Y, Mao L, Wang Z, Yan K, Zhang L, Zou J. Osteopontin - The stirring multifunctional regulatory factor in multisystem aging. *Front Endocrinol (Lausanne)* (2022) 13:1014853. doi: 10.3389/fendo.2022.1014853

14. Zhu J, Yu X, Wang Y, Bai S, Lai J, Tong X, et al. Longitudinal changes of lactopontin (milk osteopontin) in term and preterm human milk. *Front Nutr* (2022) 9:962802. doi: 10.3389/fnut.2022.962802

15. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al. Eta-1 (Osteopontin): an early component of type-1 (Cell-mediated) immunity. *Science* (2000) 287:860–4. doi: 10.1126/science.287.5454.860

16. Chen X, Yang C, Zeng J, Zhu Z, Zhang L, Lane JA, et al. The protective effects of human milk components, 2′-fucosyllactose and osteopontin, against 2,4-dinitrochlorobenzene-induced atopic dermatitis in mice. *J Funct Foods* (2021) 87:104806. doi: 10.1016/j.jff.2021.104806

17. Sullivan SJ, Jacobson RM, Dowdle WR, Poland GA. H1N1 influenza. *Mayo Clin Proc* (2009) 85:64–76. doi: 10.4065/mcp.2009.0588

18. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* (2017) 15:259–70. doi: 10.1038/nrmicro.2017.14

19. Francese R, Donalisio M, Ritta M, Capitani F, Mantovani V, Maccari F, et al. Human milk glycosaminoglycans inhibit cytomegalovirus and respiratory syncytial virus infectivity by impairing cell binding. *Pediatr Res* (2022) 1–8. doi: 10.1038/s41390-022-02091-y

20. Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. Human Milk Oligosaccharides Inhibit the Adhesion to Caco-2 Cells of Diarrheal Pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. *Pediatr Res* (2006) 59:377–82. doi: 10.1203/01.pdr.0000200805.45593.17

21. Simon PM, Goode PL, Mobasseri A, Zopf D. Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect Immun* (1997) 65:750–7. doi: 10.1128/iai.65.2.750-757.1997

22. Etzold S, Bode L. Glycan-dependent viral infection in infants and the role of human milk oligosaccharides. *Curr Opin Virol* (2014) 7:101–7. doi: 10.1016/j.coviro.2014.06.005

23. Pandey RP, Kim DH, Woo J, Song J, Jang SH, Kim JB, et al. Broad-spectrum neutralization of avian influenza viruses by sialylated human milk oligosaccharides: *in vivo* assessment of 3′-sialyllactose against H9N2 in chickens. *Sci Rep* (2018) 8:2563. doi: 10.1038/s41598-018-20955-4

24. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk oligosaccharides. *Adv Nutr* (2012) 3:383S–91S. doi: 10.3945/an.111.001404

25. Snelgrove RJ, Godlee A, Hussell T. Airway immune homeostasis and implications for influenza-induced inflammation. *Trends Immunol* (2011) 32:328–34. doi: 10.1016/j.it.2011.04.006

26. Jha A, Dunning J, Tunstall T, Thwaites RS, Hoang LT, Kon OM, et al. Patterns of systemic and local inflammation in patients with asthma hospitalised with influenza. *Eur Respir J* (2019) 54(4):949–61. doi: 10.1183/13993003.00949-2019

27. Liang X, Huang Y, Pan X, Hao Y, Chen X, Jiang H, et al. Erucic acid from Isatis indigotica Fort. suppresses influenza A virus replication and inflammation *in vitro* and *in vivo* through modulation of NF-κB and p38 MAPK pathway. *J Pharm Anal* (2020) 10:130–46. doi: 10.1016/j.jpha.2019.09.005

28. Duan T, Du Y, Xing C, Wang HY, Wang RF. Toll-like receptor signaling and its role in cell-mediated immunity. *Front Immunol* (2022) 13:812774. doi: 10.3389/fimmu.2022.812774

29. Khanmohammadi S, Rezaei N. Role of Toll-like receptors in the pathogenesis of COVID-19. *J Med Virol* (2021) 93:2735–9. doi: 10.1002/jmv.26826

30. Bradley JR. TNF-mediated inflammatory disease. *J Pathol* (2008) 214:149–60. doi: 10.1002/path.2287

31. Jones SA, Jenkins BJ. Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. *Nat Rev Immunol* (2018) 18:773–89. doi: 10.1038/s41577-018-0066-7

32. Ruddle NH. Tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta). *Curr Opin Immunol* (1992) 4:327–32. doi: 10.1016/0952-7915(92)90084-r

33. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol* (2014) 6:a016295. doi: 10.1101/cshperspect.a016295

34. Rao KM. Molecular mechanisms regulating iNOS expression in various cell types. *J Toxicol Environ Health B Crit Rev* (2000) 3:27–58. doi: 10.1080/109374000281131

35. Connelly ST, Macabeo-Ong M, Dekker N, Jordan RC, Schmidt BL. Increased nitric oxide levels and iNOS over-expression in oral squamous cell carcinoma. *Oral Oncol* (2005) 41:261–7. doi: 10.1016/j.oraloncology.2004.09.007

36. Bahadoran Z, Mirmiran P, Ghasemi A. Role of nitric oxide in insulin secretion and glucose metabolism. *Trends Endocrinol Metab* (2020) 31:118–30. doi: 10.1016/j.tem.2019.10.001

37. Julkunen I, Sareneva T, Pirhonen J, Ronni T, Melen K, Matikainen S. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev* (2001) 12:171–80. doi: 10.1016/s1359-6101(00)00026-5

38. Mgbemena V, Segovia JA, Chang TH, Tsai SY, Cole GT, Hung CY, et al. Transactivation of inducible nitric oxide synthase gene by Kruppel-like factor 6 regulates apoptosis during influenza A virus infection. *J Immunol* (2012) 189:606–15. doi: 10.4049/jimmunol.1102742

39. Sodhi CP, Wipf P, Yamaguchi Y, Fulton WB, Kovler M, Nino DF, et al. The human milk oligosaccharides 2-fucosyllactose and 6-sialyllactose protect against the development of necrotizing enterocolitis by inhibiting toll-like receptor 4 signaling. *Pediatr Res* (2021) 89:91–101. doi: 10.1038/s41390-020-0852-3

40. He Y, Liu S, Kling DE, Leone S, Lawlor NT, Huang Y, et al. The human milk oligosaccharide 2-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. *Gut* (2016) 65:33–46. doi: 10.1136/gutjnl-2014-307544

41. Xiao L, Leusink-Muis T, Kettelarij N, van Ark I, Blijenberg B, Hesen NA, et al. Human milk oligosaccharide 2-fucosyllactose improves innate and adaptive immunity in an influenza-specific murine vaccination model. *Front Immunol* (2018) 9:452. doi: 10.3389/fimmu.2018.00452

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Efficacy and safety of Wuhu oral liquid in treating acute soft tissue injuries: a multicenter, randomized, double-blind, double-dummy, parallel-controlled trial

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Background: Wuhu Oral Liquid (WHOL) is a modified preparation derived from the famous Wuhu Powder, which has a long history of use in treating traumatic injuries. This preparation has anti-inflammatory and analgesic properties and accelerates recovery following acute soft tissue injuries.

Aims: To evaluate the efficacy and safety of WHOL in treating acute soft tissue injury associated with qi stagnation and blood stasis syndrome and to provide a basis for applying for the protection of varieties of Chinese medicine for WHOL.

Methods: This study was a randomized, controlled, double-blind, multicenter clinical trial in which Fufang Shang Tong Capsule (FFSTC) was selected as the control drug. A total of 480 subjects with acute soft tissue injury associated with qi stagnation and blood stasis syndrome were randomly divided into a test and control group in a 3:1 ratio. The duration of drug treatment was 10 days. The primary outcome was Visual Analogue Scale (VAS) score for pain (including pain at rest and pain on activity). Secondary outcomes included the disappearance time of the pain at rest and on activity; the curative effect of TCM syndrome and improvement in the individual symptoms of TCM (swelling, ecchymosis, and dysfunction); and changes in C-reactive protein (CRP) and interleukin-6 (IL-6) levels. Safety was assessed using vital signs, laboratory examinations, electrocardiograms, and physical examinations.

Results: Patient compliance was satisfactory in both groups (all between 80% and 120%). After 4 days of treatment, the WHOL group was superior to the FFSTC group in decreasing the VAS scores for pain at rest (–1.88 ± 1.13 vs. –1.60 ± 0.93, $p < 0.05$) and on activity (–2.16 ± 1.18 vs. –1.80 ± 1.07, $p < 0.05$). After 7 days of treatment, the WHOL group was superior to the FFSTC group in decreasing the VAS scores for pain on activity (–3.87 ± 1.60 vs. –3.35 ± 1.30, $p < 0.01$) and

improving swelling (cure rate: 60.4% vs. 46.2%, $p < 0.05$; obvious effective rate: 60.7% vs. 47.0%, $p < 0.05$). After 10 days of treatment, the WHOL group was superior to the FFSTC group in decreasing the levels of CRP (−0.13 ± 2.85 vs. 0.25 ± 2.09, $p < 0.05$) and improving the TCM syndrome (cure rate: 44.1% vs. 30.8%, $p < 0.05$) and swelling (cure rate: 75.6% vs. 67.5%, $p < 0.01$; obvious effective rate: 75.6% vs. 68.4%, $p < 0.05$; effective rate: 77.0% vs. 71.8%, $p < 0.05$). The disappearance time of pain at rest was 8 days in both groups and 9 days on activity in both groups. In addition, there was no statistical difference between the incidence of adverse events (4.5% vs. 2.6%, $p > 0.05$) and adverse reactions (0.3% vs. 0%, $p > 0.05$) between the WHOL group and the FFSTC group. No serious adverse events occurred in either group, and no subjects were withdrawn because of adverse events.

Conclusion: WHOL relieves the symptoms caused by acute soft tissue injury associated with qi stagnation and blood stasis syndrome more rapidly than FFSTC, and it is effective and safe in the treatment of acute soft tissue injury. Future studies still need a larger sample size to verify its efficacy and safety.

Clinical Trial Registration: https://www.chictr.org.cn/showproj.html?proj=149531, Identifier ChiCTR2200056411.

KEYWORDS

Chinese medicine, Wuhu oral liquid, acute soft tissue injury, multicenter, double-blind, parallel-controlled

1 Introduction

Acute soft tissue injuries refer to a range of trauma syndromes caused by direct or indirect damage to soft tissues or skeletal muscles. These include acute injury to tissues such as muscles, ligaments, fascia, tendons, synovium, fat, and joint capsules, as well as peripheral nerves and blood vessels (Sloan, 2008). Acute soft tissue injuries represent a large proportion of cases seen in emergency medicine departments, constituting approximately 5%–10% of emergency department attendances in the United Kingdom (Handoll et al., 2007; Jones et al., 2020). In addition, the costs associated with acute soft tissue injuries, which may seem "minor," are substantial, and approach $2 billion annually in Australia and over $100 million in New Zealand (Jones et al., 2020).

Acute soft tissue injuries are characterized by aseptic inflammation. The common clinical symptoms and signs of acute soft tissue injuries include pain, swelling, stasis, and dysfunction of the affected tissue (Jiang et al., 2020). The P.R.I.C.E principle or the P.O.L.I.C.E principle is commonly applied in modern medicine to treat acute soft tissue injuries. However, these principles cannot be used as standards for treatment because they do not apply to all types of acute soft tissue injuries (Bleakley, 2013). In addition, non-steroidal anti-inflammatory drugs (NSAIDs) are usually recommended for the pharmacological treatment of acute tissue injuries (Sloan, 2008; Jones et al., 2020). However, the use of NSAIDs is associated with several adverse reactions, among which gastrointestinal reactions are the most common. Other NSAID-associated adverse reactions include acute renal failure, cardiovascular adverse reactions, hemorrhagia, and bronchospasm (Jones et al., 2020). Therefore, the use of NSAIDs is restricted in patients with preexisting gastrointestinal disease, cardiovascular disease, and renal insufficiency, as well as the elderly (Wang, 2021). Traditional Chinese medicine (TCM) has shown great value in treating acute soft tissue injuries due to its efficacy, simplicity of operation, and few side effects (Chen et al., 2011; Wang et al., 2016; Huang et al., 2020).

TCM classifies acute soft tissue injury as "tendon injury." The underlying pathogenesis involves qi stagnation and blood stasis. TCM theory suggests that the normal flow of blood in the vessels is propelled by Qi. When the movement of Qi is obstructed (Qi stagnation), blood flow loses its momentum and stagnates, forming blood stasis. Meanwhile, blood stasis can hinder the circulation of Qi and exacerbate Qi stagnation. Qi stagnation and blood stasis are mutually causal and generally coexist. The Qi stagnation and blood stasis syndrome is the pathological state of Qi stagnation and blood stasis coexisting (Huang et al., 2020). Following trauma, vessels in the injured area are damaged, obstructing the circulation of Qi and blood, resulting in stagnation of blood stasis in the vessels and poor circulation of Qi, leading to Qi stagnation and blood stasis syndrome.

WHOL is a liquid product prepared using the Wuhu Powder formula outlined in the Chinese Pharmacopoeia. The original prescription of Wuhu Powder dates back to the Qing Dynasty and has been used to treat traumatic injuries for more than 130 years. It has been recorded in all editions of the Chinese Pharmacopoeia. An experimental study demonstrated the remarkable analgesic and anti-inflammatory effects of the ethanolic extract of Wuhu Powder 30 years ago (Wang et al., 1990). There have also been reports of favorable outcomes in patients with acute soft tissue injuries who received treatment with Wuhu Powder (Lv and Rong, 2005). Notably, the use of Wuhu Powder in its powdered form has several drawbacks. These drawbacks include inconvenience in administration, poor bioavailability, and challenges in quality control (Deng et al., 2000). Jiangsu Jiuxu Pharmaceutical Group Co., Ltd. addressed the limitations of Wuhu Powder by developing a new dosage form (WHOL). A study (Deng et al., 2000) demonstrated that WHOL has

superior and rapid analgesic and anti-inflammatory effects compared to Wuhu Powder.

This study aimed to evaluate the efficacy and adverse effects of WHOL in treating acute soft tissue injury with Qi stagnation and blood stasis using FFSTC as control. FFSTC has also been clinically proven effective in the treatment of acute soft tissue injury (Zheng et al., 2012; Du, 2016; Ge and Fan, 2017; Chang et al., 2021). Furthermore, the study provides a basis for applying for the protection of WHOL as an innovative TCM.

2 Materials and methods

2.1 Investigational medications

As a "Type A extract" (Heinrich et al., 2022), WHOL is composed of five botanical drugs. Through modern pharmaceutical technology, these five botanical drugs were mixed in different proportions, extracted, concentrated, and filtered to obtain WHOL. The quality control analysis of WHOL used high-performance liquid chromatography (HPLC) (see Supplementary Material for details). The proportion of botanical drugs contained in each standard dose (10 mL) of WHOL is as follows: *Angelica sinensis* (Oliv.) Diels [Apiaceae; Angelica sinensis radix]: 1.28 g; *Carthamus tinctorius* L. [Asteraceae; Carthami flos]: 1.28 g; *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. [Apiaceae; Saposhnikoviae radix]: 1.28 g; *Arisaema erubescens* (Wall.) Schott [Araceae; Arisaematis rhizoma]: 1.28 g; and *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. [Apiaceae; Angelicae dahuricae radix]:0.88 g. WHOL has been approved by the National Medical Products Administration (NMPA) (approval number: Z20184009). The oral liquid was manufactured by Jiangsu Jiuxu Pharmaceutical Group Co., Ltd. (Batch number: 20210701/20210601).

FFSTC, the control drug, is composed of eight botanical drugs, including *Rheum palmatum* L. [Polygonaceae; Rhei radix et rhizoma], *Bupleurum chinense* DC. [Apiaceae; Bupleuri radix], *Angelica sinensis* (Oliv.) Diels [Apiaceae; Angelica sinensis radix], *Prunus persica* (L.) Batsch [Rosaceae; Persicae semen], *Carthamus tinctorius* L. [Asteraceae; Carthami flos], *Corydalis yanhusuo* (Y.H.Chou & Chun C.Hsu) W.T.Wang ex Z.Y.Su & C.Y.Wu [Papaveraceae; Corydalis rhizoma], *Trichosanthes kirilowii* Maxim.[Cucurbitaceae; Trichosanthis radix], *Glycyrrhiza uralensis* Fisch. ex DC. [Fabaceae; Glycyrrhizae radix et rhizoma]. FFSTC has been approved by NMPA (approval number: Z20073054). The capsule was manufactured by Gansu Province Xifeng Pharmaceutical Co. Ltd. (Batch number: 105009/13210902).

The placebo in both test and control groups was composed of maltodextrin. Jiangsu Jiuxu Pharmaceutical Group Co., Ltd. provided all of the investigational medications.

2.2 Study design

This study was a multicenter, randomized, double-blind, parallel-controlled clinical trial. A total of 480 patients with acute soft tissue injury syndrome associated with qi stagnation and blood stasis were enrolled across 14 clinical trial centers from November 2021 to August 2022. This study was approved by the Clinical Research Ethics Committee of Longhua Hospital, Shanghai University of TCM (approval number: 2021LCSY114) and the 13 other sites. The protocol was also registered in the Chinese Clinical Trial Registry (registration number ChiCTR2200056411). All participants signed an informed consent form. Plant use in this study complied with the relevant laws and regulations of the national and local governments to protect biodiversity. The study was conducted per the requirements of the CONSORT Extension for Chinese Herbal Medicine Formulas (Cheng et al., 2017), the Code for Quality Management of Drug Clinical Trials (National Medical Products Administration, 2020), the Guidelines for the Protection of Varieties of Chinese Medicine (National Medical Products Administration, 2009), the Guiding Principles for Clinical Research of New Chinese Medicines (Zheng, 2002), and the Declaration of Helsinki. Each hospital had a designated investigator responsible for the quality of the clinical trial, and standardized training was provided to the designated investigator and participating investigators at each hospital before the start of the trial.

2.3 Diagnostic, inclusion and exclusion criteria

Diagnostic criteria of Western medicine: 1) Obvious history of trauma; 2) Obvious pressure and pain at the injured site, skin bruising, petechiae, localized swelling, and, in severe cases, subcutaneous hematoma and limb dysfunction; 3) X-ray examination: mainly used for checking whether there are fracture, dislocation, and osteopathy at the injured site, which has certain reference value for assessing tendon, ligament rupture, and cartilage injuries. Formulated with reference to the Guiding Principles for Clinical Research of New Chinese Medicines (Zheng, 2002) and the Expert Consensus on Diagnosis, Treatment, and Pain Management of Acute Closed Soft Tissue Injuries (Wang, 2021).

Diagnostic criteria of TCM (Li et al., 2022; Zhu et al., 2022; Li et al., 2023): Qi stagnation and blood stasis syndrome: 1) Primary symptoms: localized, stabbing pain in a definite place or with impaired mobility; 2) Secondary symptoms: localized swelling, bruises, and petechiae or hematoma; 3) Tongue: purplish and dark tongue or petechiae; 4) Pulse: stringy and astringent pulse. These primary symptoms are essential, with or without other symptoms. Formulated with reference to the Guiding Principles for Clinical Research of New Chinese Medicines (Zheng, 2002) and the Chinese Medicine Industry Standard of the People's Republic of China-Diagnostic Efficacy Criteria for TCM Evidence (ZY/T001.9).

Inclusion criteria: 1) Met the diagnostic criteria for acute closed soft tissue injuries in "*The Expert Consensus on Diagnosis, Treatment, and Pain Management of Acute Closed Soft Tissue Injuries*" (Wang, 2021), including injuries to subcutaneous tissues or muscles, tendons, fascia, ligaments, and joint capsules attached to the skeletal structure. The skin and mucous membranes at the injury site should remain intact without an open wound; 2) Met the diagnostic criteria of acute soft tissue injury in Western medicine; 3) Met the diagnostic criteria of qi stagnation and blood stasis syndrome in TCM; 4) Duration of soft tissue

injury ≤48 h; 5) Visual analogue scale (VAS) score at rest >3 points, VAS score on activity <9 points; 6) Aged 18–65, regardless of gender; 7) Participants who gave written informed consent.

Exclusion criteria: 1) Soft tissue injury with fracture, bone fissure, open wound, or complete rupture of muscle, tendon, and ligament; 2) Soft tissue injury sites ≥2; 3) Presence of comorbid inflammatory pain diseases such as rheumatoid arthritis, psoriatic arthritis, and gout; 4) Participants who had taken NSAIDs, similar medicines, corticosteroids, or antibiotics within a week, or had received alternative therapies such as acupuncture, physiotherapy, or manipulation before the first dose; 5) Severe heart disease, renal failure, hematological disease, or abnormal liver and kidney function; 6) Pregnant, suspected pregnant, or lactating women; 7) Participants with physical or mental diseases that affect cooperation, or serious diseases such as tumors that affect survival; 8) Suspected or proven history of alcohol or drug abuse; 9) Known allergy to trial drug, alcohol, or protocol-prescribed emergency medication; 10) Participants who engaged in high-altitude, high-risk work, or professional driver.

2.4 Randomization and control

According to the requirements of the Guiding Principles for the Protection of Varieties of TCM issued by the National Medical Products Administration (NMPA), which states that the selection of parallel control drugs should follow the principles of "widely recognized, of the same category, and based on excellence." FFSTC is recommended by the expert consensus of the Chinese Academy of TCM for the treatment of stasis syndrome in acute chest contusion injuries or blood stasis and Qi stagnation syndrome in acute soft tissue injuries. Therefore, the use of FFSTC in the treatment of Qi stagnation and blood stasis syndrome in acute soft tissue injuries is in line with the principle of "widely recognized." Both FFSTC and WHOL are purely Chinese medicine preparations that are used to promote blood circulation and remove blood stasis, and have similar therapeutic effects, thus meeting the principle of "of the same category." FFSTC has been on the market for more than 10 years and has been widely used in departments such as orthopedics, thoracic surgery, emergency medicine, and pain management. FFSTC's clinical efficacy in treating soft tissue injuries has been confirmed, and it is considered a high-quality product among similar drugs, conforming to the principle of "based on excellence." Therefore, FFSTC was selected as the parallel control drug.

Block randomization was applied. Random allocation codes (code range 001–480) were generated using the SAS software. The recruitment capacity for each trial center was determined before the start of the trial. Furthermore, a statistician evaluated and designed the appropriate number of blocks and block lengths based on the number of subjects expected to be recruited at each trial center (finalized number of blocks = 120 and block length = 4). Code segments were assigned to each center in multiples of 4 based on the number of subjects expected to be recruited at each trial center. The different code segments were sent to each trial center after the allocation, with corresponding treatment kits. During the trial, cases were deployed based on the progress of enrollment in each center to ensure that all centers completed the trial at the same time. The randomization sequence was designed by a statistician who did not participate in the trial. After obtaining the consent of the subjects, the research physicians overseeing recruitment at each site contacted the appointed drug administrator at the respective site. This drug administrator, kept independent of the recruitment process, was contacted by phone.

2.5 Blinding

The trial was a double-blind study and ensured that neither the subjects, investigators, supervisors, nor data analysts were aware of the distribution of the treatment drugs. The sponsor provided the test drug, with uniform internal and external packaging. Because the dosage forms of both test and control drugs were different, the oral liquid placebo and capsule placebo were made separately to ensure the reliability of the blinding method. Patients in the test group received WHOL and FFSTC placebo, while those in the control group received FFSTC and WHOL placebo. The appearance, daily frequency of administration, and dose of the two groups of drugs were consistent. Furthermore, the drugs were packaged in sealed, opaque boxes with the same label. The label outside each box indicated the clinical study drug (WHOL), the same usage and dosage, the storage conditions, and the expiry date. Appointed clinical trial drug administrators at each site were responsible for receiving, storing, distributing, and retrieving surplus drugs and used drug cartridges.

The blinding envelope contained the specific treatment group (test group, control group) corresponding to each subject. The blinding envelopes were sealed separately, in duplicate, and one copy was kept at the team leader's office and the other in the applicant's office. Each test drug was assigned a unique code, and corresponding emergency letters were provided for each group to break the blind in the event of an emergency. The emergency letters were sealed and sent, along with the corresponding coded test drugs, to each clinical trial center. The centers were responsible for the preservation of the letters and did not open the letters unless it was necessary. In the event of an emergency, such as a serious adverse event or when a subject needed to be rescued, it was necessary to know what treatment the subject was receiving. Once the blind was broken for any particular subject, that subject was withdrawn from the trial and treated as a dropped case, and the investigator recorded the reason for withdrawal in a case report form. After all the case report forms were entered into a database and challenged, verified, and blinded for review, the data were locked. The unblinding was carried out by the staff member who kept the blinded envelopes.

2.6 Intervention and efficacy evaluation

The study period consisted of visits V1 to V5. V1 was at day 0 for screening and baseline laboratory examinations, V2 was at 0.5 h of drug administration, V3 was at day 4, V4 was at day 7, and V5 was at day 10. Eligible subjects were assigned to test or control groups. The test and control drugs were administered orally, and the doses administered were: Oral Liquid, 10 mL twice daily; Capsule, 3 capsules thrice daily. The treatment duration was 10 days. NSAIDs were administered when a subject continued to

TABLE 1 TCM syndrome Scoring Scale*.

Primary symptom	Normal (0 score)	Mild (2 scores)	Moderate (4 scores)	Severe (6 scores)
Pain at rest (VAS score)	0	≤3	3–7	≥7
Pain on activity (VAS score)	0	≤3	3–7	≥7
Dysfunction	None	Mildly restricted, able to perform normal activities	Significantly limited, but still able to care for themselves	Loss of activity function
Secondary symptom	**Normal (0 score)**	**Mild (1 score)**	**Moderate (2 scores)**	**Severe (3 scores)**
Swelling	None	Light swelling, no obvious indentation when pressing the swollen area with the finger	Swelling is obvious, but skin texture is still smooth, press the swollen part with a finger, and there can be obvious depression	Significant swelling, elongated skin texture, tense shiny skin, and even tense blisters
Ecchymosis	None	Ecchymosis pale purple, area <2 × 2 cm	Ecchymosis purple, area ≥2 × 2 cm but <4 × 4 cm	Ecchymosis dark purple, area ≥4 × 4 cm
Tongue and Pulse	**Normal (0 score)**	**Mild (1 score)**	**Other (no score)**	
Tongue color	Light red	Dark purple or ecchymosis	Other ( )	
Pulse condition	Normal pulse	Wiry and unsmooth pulse	Other ( )	

*The TCM, syndrome score is the sum of the 3 primary symptom scores, the 2 secondary symptom scores, and the tongue and pulse scores. The score for the primary symptom is twice as high as the score for the secondary symptom and the tongue and pulse.

experience pain that they could not tolerate after 24 h of receiving the assigned treatment. Subjects were immediately withdrawn in instances where the pain persisted after at least two doses of NSAIDs were administered, and medical attention was promptly sought. All relevant drug information, including drug name, dosage, indication, frequency, and duration of use, were documented on the case report form to allow for comprehensive analysis and reporting. Compliance was calculated as the actual dosage/prescribed dosage × 100%, and compliance between 80% and 120% was considered satisfactory; compliance <80% or >120% was considered poor.

The primary efficacy indicator was VAS scores, including reductions in VAS scores for pain at rest and pain on activity at 0.5 h, 4 days, 7 days, and 10 days after treatment. Evaluation method: Change values = post-treatment (rest/activity) pain VAS score—pre-treatment (rest/activity) pain VAS score. The secondary efficacy indicators were: 1) Disappearance time of the pain at rest and on activity, Evaluation method: time (days) when (rest/activity) pain VAS score = 0; 2) Curative effect of TCM syndrome at 4, 7, and 10 days after treatment, Evaluation method: Curative effect of TCM syndrome = (pre-treatment TCM syndrome score - post-treatment TCM syndrome score)/pre-treatment TCM syndrome score × 100%. Results ≥95%, ≥70%, ≥30%, and <30% corresponded to cured, significantly effective, effective, and ineffective, respectively; 3) Individual symptoms of TCM syndrome, including improved swelling, ecchymosis, and dysfunction at 4, 7, and 10 days after treatment, Evaluation method: Disappearance of symptoms or signs = cured; Significant improvement in symptoms or signs (from severe to mild) = obvious effective; Improvement in symptoms or signs (from severe to moderate, or moderate to mild) = effective; No change or insignificant reduction in symptoms or signs, or aggravation = ineffective; 4) Levels of C-reactive protein (CRP) and interleukin (IL)-6 before and 10 days after treatment, Evaluation method: Change values = post-treatment (CRP/IL-6) measured value—pre-treatment (CRP/IL-6) measured value. The details of the TCM syndrome score and the assessment of the severity of pain, swelling, and dysfunction are presented in Table 1.

2.7 Safety assessment

Safety was assessed using vital signs, laboratory examinations, electrocardiograms, and physical examinations. The vital signs included body temperature, respiratory rate, pulse, and blood pressure. The laboratory examinations included complete blood count (white blood cells, red blood cells, hemoglobin, and platelets); urine tests (erythrocytes, leukocytes, glucose, and protein); liver function (alanine transaminase [ALT], aspartate transaminase [AST], alkaline phosphatase [ALP], total bilirubin, and gamma-glutamyl transpeptidase); and renal function (SCr and blood urea nitrogen). A 12-lead electrocardiogram was performed to assess cardiac activity and function. A thorough physical examination was conducted, including an assessment of the head, neck, chest, abdomen, and skin of the extremities for any signs of allergic reactions.

When an adverse event occurred, the investigators carefully observed and evaluated the association between the adverse event and the test drug, the severity of the adverse event, and the outcome of the adverse event. At the same time, necessary interventions such as dose adjustment and temporary dosing interruption were made, as well as a decision to withdraw the subject from the trial. The severity of adverse events was graded. Mild adverse events were tolerated by the subject, did not affect treatment, did not require special treatment, and had no effect on recovery; moderate adverse events were intolerable to the subject, required withdrawal or special

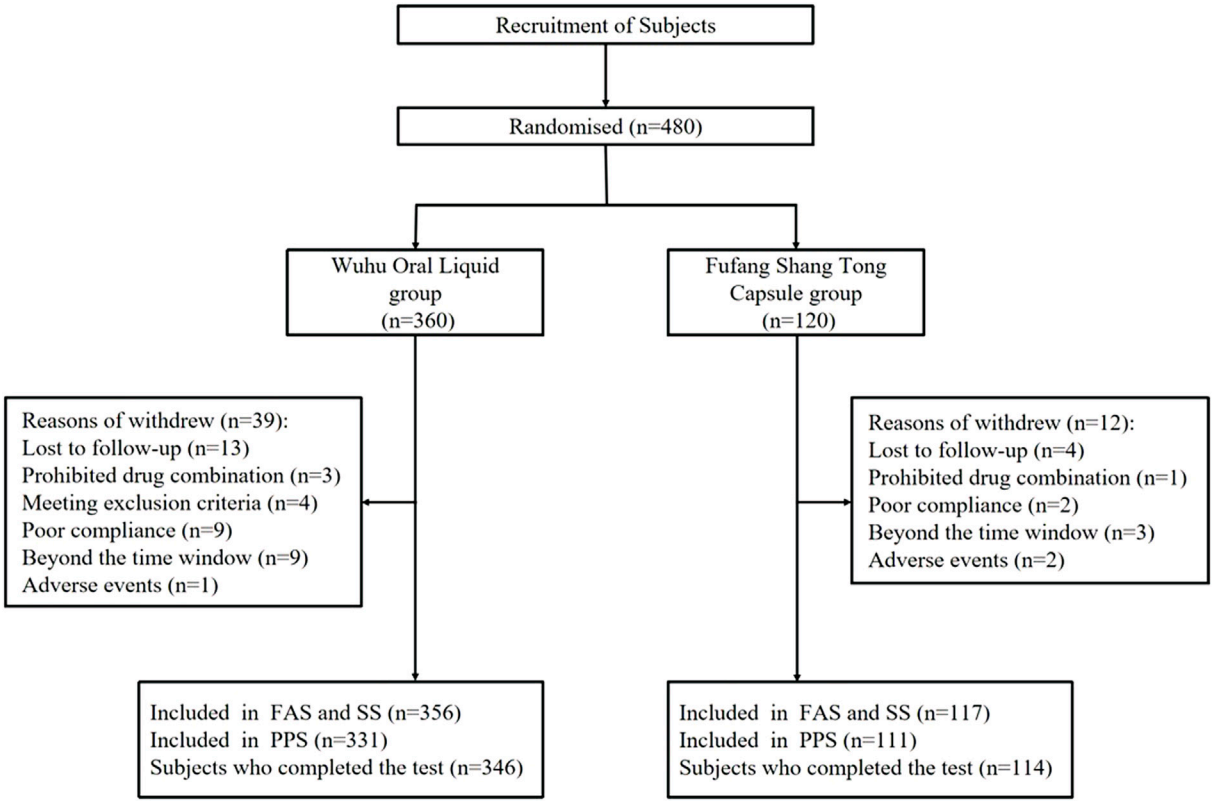

FIGURE 1
Study flowchart.

treatment, and had a direct impact on recovery; and severe adverse events were life-threatening, fatal, or disabling to the subject, required immediate withdrawal or emergency treatment.

2.8 Sample size and statistical analysis

According to the Guiding Principles for the Protection of Varieties of TCM (National Medical Products Administration, 2009), the sample size needed to meet statistical requirements, with the test group typically having not less than 300 subjects. Under the premise of meeting the statistical requirements, the control group was generally designed to contain 1/3 of the number of subjects in the test group. Thus, a minimum of 400 subjects were needed. Given a 20% drop-off rate, a total of 480 subjects were included, of which 360 were in the test group and 120 were in the control group. In addition, the guideline requires that when drugs are used to treat multiple conditions, the number of cases for the primary condition in the test group must generally not be less than 60 cases. Therefore, acute soft tissue injury of the ankle joint was considered the primary condition in this trial, and the test group comprised not less than 60 cases.

According to the intention-to-treat (ITT) principle in the statistical principles for clinical trials (ICH E9) (JA, 1999), the full analysis set (FAS) (defined as subjects who received at least one dose of the therapeutic drug and had the corresponding efficacy evaluation) was used to evaluate the primary and secondary efficacy indicators in superiority trials. Adverse event assessment was based on the safety analysis set (SS) (defined as subjects who received at least one dose of the drug). Quantitative data are presented as mean ± standard deviation (SD) or median, and two-tailed Student's t-test or Wilcoxon-Mann-Whitney test were used to test for differences between groups before and after treatment. For normally distributed data, the two-tailed Student's t-test was used. Otherwise, the Wilcoxon-Mann-Whitney test was adopted. Qualitative data were described using frequency or composition ratio, and chi-square and Fisher's exact tests were used to analyze the differences between the two groups of qualitative data results. Missing observations were imputed using the last-observation-carried-forward (LOCF) method, where the last value observed before dropout was used as the outcome for participants who withdrew from the trial prematurely. All tests were two-sided, and $p < 0.05$ was considered significant. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, United States).

3 Results

3.1 Baseline characteristics

This clinical trial was conducted at 14 sites, and a total of 480 subjects were enrolled: 360 in the test group and 120 in the control group. However, 460 subjects completed the trial: 114

TABLE 2 Comparison of subject characteristics at baseline.

Characteristics	Test group (n = 356)	Control group (n = 117)	p-values
Age (years, $\bar{\chi} \pm s$)			
Mean (SD)	37.14 ± 13.44	37.20 ± 11.98	0.529
Min, Max	16.00–65.00	15.00–64.00	
Median (Q3-Q1)	34 (25.00–49.00)	34 (27.00–48.00)	
Sex (%)			
Men	169 (47.5)	52 (44.4)	0.569
Women	187 (52.5)	65 (55.6)	
Nationality (%)			
Han ethnic	339 (95.2)	109 (93.2)	0.387
Non-Han ethnic	17 (4.8)	8 (6.8)	
Marital status (%)			
Married	216 (60.7)	77 (65.8)	0.321
Others	140 (39.3)	40 (34.2)	
Occupation (%)			
Mental labor	246 (69.1)	79 (67.5)	0.643
Physical labor	92 (25.8)	34 (29.1)	
Others	18 (5.1)	4 (3.4)	
Height (cm, $\bar{\chi} \pm s$)	166.60 ± 8.18	166.52 ± 8.19	0.934
Weight (kg, $\bar{\chi} \pm s$)	63.72 ± 10.27	64.96 ± 11.52	0.595
BMI (kg/m^2 $\bar{\chi} \pm s$)	22.87 ± 2.65	23.32 ± 2.98	0.143
Past medical History (%)	45 (12.6)	18 (15.4)	0.449
Medication for other diseases (%)	15 (4.2)	7 (6.0)	0.430
Allergy history (%)	4 (1.1)	3 (2.6)	0.372
Injured part (%)			0.532
Ankle	102 (28.7)	36 (30.8)	
Knee	71 (19.9)	32 (27.4)	
Low back	58 (16.3)	16 (13.7)	
Neck	28 (7.9)	6 (5.1)	
Chest	27 (7.6)	7 (6.0)	
Wrist	23 (6.5)	5 (4.3)	
Shoulder	15 (4.2)	5 (4.3)	
Elbow	8 (2.2)	3 (2.6)	
Calf	7 (2.0)	0 (0)	
Hand	6 (1.7)	1 (0.9)	
Foot	5 (1.4)	4 (3.4)	
Hip	3 (0.8)	0 (0)	
Sacrococcygeal	1 (0.3)	0 (0)	
Abdomen	1 (0.3)	0 (0)	
Buttock	1 (0.3)	2 (1.7)	

Statistics: p-values were calculated for continuous outcomes with the Wilcoxon-Mann-Whitney test; The chi-square test and Fisher's exact test were performed for categorical outcomes; Injury parts were calculated using a Monte Carlo Estimate of the exact test.

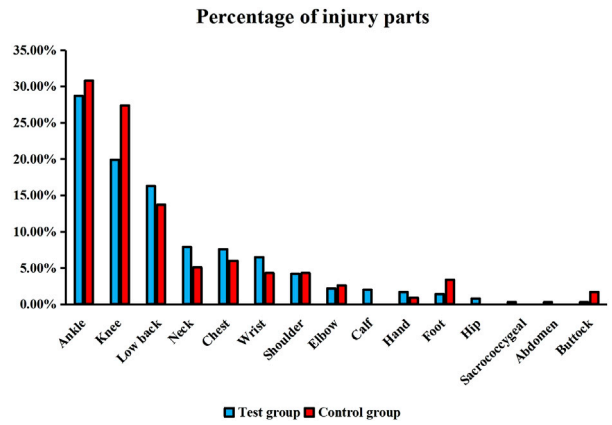

FIGURE 2
Percentage of injury sites.

(95.00%) in the control group and 346 (96.10%) in the test group (Figure 1). Subject characteristics are presented in Table 2. There were no statistical differences between the two groups in terms of demographic characteristics, past medical history, medication for other diseases, allergy history, and injured part ($p > 0.05$). The percentages of injury sites are presented in Figure 2. Notably, the medication adherence was 92.17% ± 6.97% in the test group and 92.73% ± 6.77% in the control group, and the difference in medication adherence between these two groups was not statistically significant. Meanwhile, the overall medication adherence in both groups was satisfactory and ranged from 80% to 120% (Table 3).

3.2 Primary indicators

3.2.1 Pain at rest

The VAS scores for pain at rest at the injury site were 5.05 ± 1.33 in the test group and 5.07 ± 1.38 in the control group before treatment, with no statistically significant difference ($p > 0.05$). Figure 3A presents the VAS score changes for pain at rest in the test and control groups at each follow-up visit. Compared to baseline, the VAS scores for pain at rest at 0.5 h, 4 days, 7 days, and 10 days after treatment decreased by 0.55 ± 0.65, 1.88 ± 1.13, 3.34 ± 1.58, and 4.49 ± 1.54, respectively, in the test group and by 0.56 ± 0.65, 1.60 ± 0.93, 2.96 ± 1.29, and 4.33 ± 1.40, respectively, in the control group (Table 4).

The test group exhibited significantly greater reductions in scores compared to the control group after 4 days of treatment ($p < 0.05$), indicating that WHOL was more effective in relieving pain at rest at the injury site than FFSTC. The difference between the two groups at 0.5 h, 7 days, and 10 days of treatment was not statistically significant ($p > 0.05$), suggesting that the efficacy of the two groups in relieving pain at rest at the injury site was not significantly different at 0.5 h, 7 days, and 10 days of treatment.

3.2.2 Pain on activity

Before treatment the VAS scores for pain on activity at the injury site were 6.77 ± 1.18 in the test group and 6.77 ± 1.26 in the control group, with no statistically significant difference ($p > 0.05$). Figure 3B presents the VAS score changes for pain on activity in the test and control groups at each follow-up visit. Compared to baseline, the VAS scores for pain on activity at 0.5 h, 4 days, 7 days, and 10 days after treatment decreased by 0.57 ± 0.69, 2.16 ± 1.18,

TABLE 3 Medication adherence.

Category	Test group (%)	Control group (%)	P- values
Overall medication adherence			
Mean ± SD	92.17 ± 6.97	92.7 3 ± 6.77	0.703
Min—Max Q1–Q3	45.00–120.00 88.34–95.84	70.89–120.00 88.34–97.50	
Median	91.67	92.50	

Statistics: *p*-values were calculated with Wilcoxon–Mann–Whitney test.

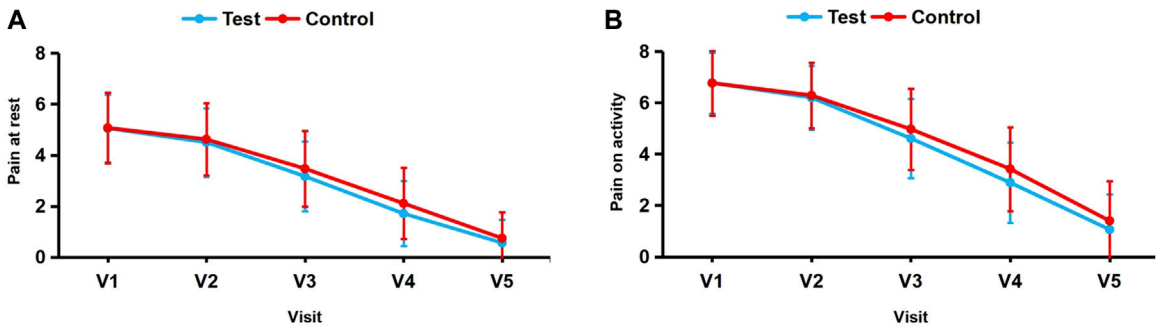

FIGURE 3
(A, B) Changes in mean VAS scores for pain at rest and on activity from baseline (visit 1) to visit 5.

TABLE 4 Measured values (MV) and change values (CV) relative to baseline in VAS scores for pain at rest and on activity at different follow-up times[a].

Category	Test group (N = 356)		Control group (N = 117)		*p*-values
Pain at rest	MV	CV	MV	CV	
V1 (Baseline)	5.05 ± 1.33		5.07 ± 1.38		0.859
V2 (0.5 h)	4.50 ± 1.35	−0.55 ± 0.65	4.62 ± 1.41	−0.44 ± 0.58	0.380
V3 (Day 4)	3.17 ± 1.36	−1.88 ± 1.13	3.47 ± 1.49	−1.60 ± 0.93	0.031
V4 (Day 7)	1.71 ± 1.28	−3.34 ± 1.58	2.11 ± 1.39	−2.96 ± 1.29	0.082
V5 (Day 10)	0.56 ± 0.91	−4.49 ± 1.54	0.74 ± 1.03	−4.33 ± 1.40	0.365
Pain on activity	MV	CV	MV	CV	
V1 (Baseline)	6.77 ± 1.18		6.77 ± 1.26		0.929
V2 (0.5 h)	6.20 ± 1.25	−0.57 ± 0.69	6.29 ± 1.28	−0.47 ± 0.68	0.865
V3 (Day 4)	4.61 ± 1.54	−2.16 ± 1.18	4.97 ± 1.59	−1.80 ± 1.07	0.011
V4 (Day 7)	2.89 ± 1.57	−3.87 ± 1.60	3.42 ± 1.63	−3.35 ± 1.30	0.082
V5 (Day 10)	1.06 ± 1.37	−5.71 ± 1.63	1.40 ± 1.54	−5.37 ± 1.60	0.101

[a]Data are presented as Mean ± SD, Change values (CV), Measured values (MV)—Baseline values at different follow-up times.
Statistics: *p*-values were calculated with Wilcoxon–Mann–Whitney test. Differences at baseline were compared based on measured values (MV) in both groups, while differences between both groups at different follow-up time points were compared based on change values (CV).

TABLE 5 Disappearance time of the pain at rest and on activity.

	Test group (day)	Control group (day)	*p*-values
Pain at rest	8.00	8.00	0.179
Pain on activity	9.00	9.00	0.514

Statistics: The disappearance time of pain was skewness distribution and presented by a median. *p*-values were calculated with Wilcoxon–Mann–Whitney test.

3.87 ± 1.60, and 5.71 ± 1.63, respectively, in the test group and by 0.47 ± 0.68, 1.80 ± 1.07, 3.35 ± 1.30, and 5.37 ± 1.60, respectively, in the control group (Table 4).

The test group exhibited significantly greater reductions in VAS scores compared to the control group on days 4 and 7 ($p > 0.05$), suggesting that the effectiveness of WHOL in relieving the pain on activity at the injury site was superior to FFSTC at 4 and 7 days of treatment. However, there was no significant difference between the two groups in relieving the pain on activity at the injury site at 0.5 h and 10 days of treatment ($p > 0.05$).

3.3 Secondary indicators

3.3.1 Disappearance time of the pain at rest and pain on activity

The median disappearance time of pain at rest was 8 days in both groups, and the median disappearance time of pain on activity was 9 days in both groups ($p > 0.05$) (Table 5).

3.3.2 Curative effect of TCM syndrome

The TCM syndrome scores of the test group and control group were 14.26 ± 2.55 and 14.65 ± 2.68 ($p > 0.05$), respectively. The curative effect of TCM syndrome at days 4, 7, and 10 was analyzed, and the cure rate was 44.1% in the test group and 30.8% in the control group at day 10 ($p < 0.05$). Resultantly, it was suggested that the curative effect of WHOL in improving qi stagnation and blood stasis syndrome was superior to FFSTC after 10 days of treatment. However, the difference in total effective rate between the two groups was not statistically significant ($p > 0.05$) (Table 6).

3.3.3 Improvement in individual symptoms

There were no significant differences in the severity of dysfunction (2.79 ± 0.99 vs. 2.90 ± 1.03, $p > 0.05$), swelling (1.16 ± 0.38 vs. 1.25 ± 0.46, $p > 0.05$), and ecchymosis (1.28 ± 0.45 vs. 1.26 ± 0.48, $p > 0.05$) between the two groups before treatment. After 7 and 10 days of treatment, the test group exhibited a higher cure rate and obvious effective rate compared to the control group. The effective rate of the test group was also superior to that of the control group after 10 days of treatment. The differences were statistically significant ($p < 0.05$), suggesting that the effectiveness of WHOL in relieving swelling in the test group was superior to that of FFSTC after 7 and 10 days of treatment. In addition, there was no significant difference between the two groups in the improvement of dysfunction and ecchymosis (Table 7).

3.3.4 Levels of CRP and IL-6

Before treatment, there was no statistically significant difference in CRP levels between the test group (4.19 ± 4.16 mg/L) and the control group (4.00 ± 4.10 mg/L) ($p > 0.05$). However, after 10 days of treatment, the test group exhibited a greater decrease in CRP levels compared to the control group (−0.13 ± 2.85 mg/L vs. 0.25 ±

TABLE 6 Curative effect of TCM syndrome.

Category		Test group	Control group	*p*-values
TCM syndrome	Efficacy			
N (Missing)		356 (0)	117 (0)	
V3 (Day 4)	Cured	5 (1.4%)	0 (0.0%)	0.339
	Obvious effective	7 (2.0%)	0 (0.0%)	0.201
	Effective	144 (40.4%)	49 (41.9%)	0.785
V4 (Day 7)	Cured	18 (5.1%)	1 (0.9%)	0.055
	Obvious effective	93 (26.1%)	20 (17.1%)	0.047
	Effective	317 (89.0%)	104 (88.9%)	0.964
V5 (Day 10)	Cured	157 (44.1%)	36 (30.8%)	0.010
	Obvious effective	249 (69.9%)	76 (65.0%)	0.301
	Effective	345 (96.9%)	112 (95.7%)	0.257

Statistics: Chi-square test or Fisher's exact test was performed for curative effect analysis.

2.09 mg/L, $p < 0.05$). The IL-6 levels before treatment were 2.21 ± 3.77 pg/mL and 1.90 ± 3.43 pg/mL in the test and control groups, respectively, with no statistically significant difference ($p > 0.05$). After 10 days of treatment, there was no statistically significant difference in the IL-6 level-decreasing effect of both groups ($p > 0.05$) (Table 8).

3.4 Safety assessment

Out of the total number of subjects in the test group, 16 (4.5%) experienced adverse events. Among these, 15 cases were classified as mild, and 1 was considered moderate. One of the mild adverse events, specifically abnormal liver function, was determined to have a potential relationship with the test drug and was classified as an adverse drug reaction. The remaining adverse events were deemed possibly unrelated to the test drug. At the end of the trial, 3 cases of mild adverse events were of unknown cause; 1 case of moderate adverse event was relieved; and the remaining 12 cases of mild adverse events were cured. A total of 3 cases (2.6%) of adverse events occurred in the control group, encompassing 2 mild adverse events and 1 moderate adverse event, all of which were determined to be possibly unrelated to the control drug. At the end of the trial, all adverse events were cured except for one mild adverse event of unknown cause (Table 9).

In summary, the incidence of adverse events and adverse reactions (abnormal liver function) was 4.5% and 0.3%, respectively, in the test group after 10 days of treatment; in the control group, the incidence of adverse events was 2.6% and no adverse reactions occurred. The two groups had no statistical difference in the incidence of adverse events and adverse reactions ($p > 0.05$). The investigators concluded that all adverse events were unrelated to the trial drug except for one case of abnormal liver function. In addition, there were no serious adverse events in either group, and adverse events did not cause the withdrawal of any subject from the trial.

4 Discussion

The frequency of outdoor activities or physical exercise has increased with the growing emphasis on healthy lifestyles. However, this has led to an increased risk of injury, with acute soft tissue injuries being the most common (Sloan, 2008; Bleakley, 2013). Acute soft tissue injuries include sprains, contusions, falls, or impact injuries. An injury may result when the mechanical load on a particular tissue exceeds the tensile strength of the tissue. Local tissue trauma caused by such injury can lead to microcirculatory disturbance and an aseptic inflammatory response, resulting in local tissue pain, swelling, skin petechiae and ecchymosis, and even local functional limitations (Bleakley and Davison, 2010; Jones et al., 2020; Wang, 2021). Acute soft tissue injuries can adversely affect the work and life of a patient and often reduce productivity. Rapid and appropriate medical intervention for acute soft tissue injuries is essential for a favorable prognosis (Bleakley et al., 2010). Given that the P.R.I.C.E. or P.O.L.I.C.E. principles are not universal for all acute soft tissue injuries and the implementation is cumbersome and time-consuming, individuals prefer simple and effective treatment when the injury is mild. In addition, pain is the most common clinical manifestation of acute soft tissue injuries and often necessitates the use of oral analgesics. However, treatment with NSAIDs is limited because of the potential for adverse effects such as gastrointestinal irritation (Jones et al., 2020; Wang, 2021). Therefore, exploring new treatment options for acute soft tissue injuries is necessary.

TCM believes that acute soft tissue injuries can lead to the development of Qi stagnation and blood stasis syndrome, in which blood stasis predominates (Liu et al., 2012). The Golden Mirror of Medicine (edited by Wu, Q. in 1742) states: "The syndrome of traumatic injuries are treated based on the theory of blood; The symptoms of injury, which appear as swelling and pain, are caused by the clotting of blood stasis." TCM theory holds that "obstruction causes pain." After acute soft tissue injury, since blood stasis blocks the injured area and the movement of Qi is obstructed, typical localized stabbing pain occurs. This classical pain is key in diagnosing Qi stagnation and blood stasis syndrome.

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TABLE 7 Improvement in individual symptoms.

Category		Test group	Control group	*p*-values
Dysfunction	Efficacy			
N (Missing)		348 (8)	115 (2)	
V3 (Day 4)	Cured	49 (13.8%)	17 (14.5%)	0.852
	Obvious effective	49 (13.8%)	17 (14.5%)	0.852
	Effective	158 (44.4%)	57 (48.7%)	0.438
V4 (Day 7)	Cured	172 (48.3%)	53 (45.3%)	0.533
	Obvious effective	173 (48.6%)	54 (46.2%)	0.607
	Effective	263 (73.9%)	93 (79.5%)	0.235
V5 (Day 10)	Cured	306 (86.0%)	96 (82.1%)	0.243
	Obvious effective	306 (86.0%)	96 (82.1%)	0.243
	Effective	327 (91.9%)	104 (88.9%)	0.194
Swelling				
N (Missing)		292 (64)	96 (21)	
V3 (Day 4)	Cured	89 (25.0%)	21 (17.9%)	0.105
	Obvious effective	89 (25.0%)	21 (17.9%)	0.105
	Effective	120 (33.7%)	35 (29.9%)	0.421
V4 (Day 7)	Cured	215 (60.4%)	54 (46.2%)	0.001
	Obvious effective	216 (60.7%)	55 (47.0%)	0.002
	Effective	239 (67.1%)	71 (60.7%)	0.101
V5 (Day 10)	Cured	269 (75.6%)	79 (67.5%)	0.005
	Obvious effective	269 (75.6%)	80 (68.4%)	0.013
	Effective	274 (77.0%)	84 (71.8%)	0.045
Ecchymosis				
N (Missing)		163 (193)	57 (60)	
V3 (Day 4)	Cured	32 (9.0%)	11 (9.4%)	0.956
	Obvious effective	32 (9.0%)	11 (9.4%)	0.956
	Effective	55 (15.4%)	18 (15.4%)	0.765
V4 (Day 7)	Cured	105 (29.5%)	35 (29.9%)	0.684
	Obvious effective	105 (29.5%)	36 (30.8%)	0.865
	Effective	132 (37.1%)	45 (38.5%)	0.739
V5 (Day 10)	Cured	138 (38.8%)	47 (40.2%)	0.488
	Obvious effective	138 (38.8%)	47 (40.2%)	0.488
	Effective	152 (42.7%)	53 (45.3%)	0.519

Statistics: Chi-square test or Fisher's exact test was performed for curative effect analysis.

Additionally, if blood stasis does not disperse and overflows from the vessel to the muscle surface, swelling or petechiae may be observed, and these are also symptoms of Qi stagnation and blood stasis syndrome. Moreover, TCM considers a person as a whole. The pathological status of local Qi and blood may impact the Qi and blood status of various parts of the body, such as changes in tongue color (turning from normal light red into purple or dark) and pulse condition (diminished sense of pulse beat), and indirectly reflect Qi stagnation and blood stasis syndrome. Therefore, the main TCM principle for acute soft tissue injuries is to promote blood circulation and remove stasis (Liu et al., 2012; Wang et al., 2022).

TABLE 8 Changes in CRP and IL-6 levels*.

Category	Test group		Control group		*p*-values
N (Missing)	354 (2)		116 (1)		
CRP	MV	CV	MV	CV	
V1 (Baseline)	4.19 ± 4.16		4.00 ± 4.10		0.594
V5 (Day 10)	4.06 ± 4.29	−0.13 ± 2.85	4.25 ± 4.11	0.25 ± 2.09	0.033
N (Missing)	354 (2)		116 (0)△		
IL-6	MV	CV	MV	CV	
V1 (Baseline)	2.21 ± 3.77		1.90 ± 3.43		0.923
V5 (Day 10)	2.05 ± 3.59	−0.18 ± 3.68	1.74 ± 2.00	−0.15 ± 2.89	0.095

Statistics: *p*-values were calculated with Wilcoxon–Mann–Whitney test. Differences at baseline were compared based on measured values (MV) in both groups, while differences between both groups at different follow-up time points were compared based on change values (CV).
*CRP, values are in milligrams per liter (mg/L); IL-6, values are in picograms per milliliter (pg/mL).
△ The statistical analysis was performed after excluding one case of apparently abnormal extreme IL-6, values in the control group.

TABLE 9 Occurrence of adverse events (AE), adverse drug reaction (ADR), and serious adverse events (SAE).

Category	Test group (N = 356)	Control group (N = 117)	Total (N = 473)	*p*-values
AE	16 (4.5)	3 (2.6)	19 (4.0)	0.356
SAE	0 (0.0)	0 (0.0)	0 (0.0)	-
ADR	1 (0.3)	0 (0.0)	1 (0.2)	1.000
AE unrelated to drug	15 (4.2)	3 (2.6)	18 (3.8)	1.000
AE severity				0.298
mild	15 (4.2)	2 (1.7)	17 (3.6)	
moderate	1 (0.3)	1 (0.9)	2 (0.4)	
serious	0 (0.0)	0 (0.0)	0 (0.0)	
AE results				1.000
cured	12 (3.4)	2 (1.7)	14 (3.0)	
relieved	1 (0.3)	0 (0.0)	1 (0.2)	
unknown	3 (0.8)	1 (0.9)	4 (0.8)	
Withdrew due to AE	0 (0.0)	0 (0.0)	0 (0.0)	-
Withdrew due to ADR	0 (0.0)	0 (0.0)	0 (0.0)	-

Statistics: *p*-values were calculated with chi-square test or Fisher's exact test.

Oral Chinese patent medicines (CPMs) are commonly used in TCM as a treatment method for acute soft tissue injuries because of their convenience of administration and lasting therapeutic effect, but solid CPMs are limited by their slow absorption rate (Wang, 2021). WHOL is a liquid CPM improved from a solid preparation. Liquid preparations have high bioavailability and are not associated with dysphagia (Bende et al., 2016; Liu et al., 2023). The development of WHOL provides a safe, efficient, convenient, and universal treatment for acute soft tissue injuries. This study was conducted to verify the clinical application of WHOL in the treatment of acute soft tissue injuries, obtain scientific and objective clinical data, and provide a clinical basis for the protection of Chinese medicine varieties.

WHOL is made up of five botanical drugs and functions to promote blood circulation, alleviate blood stasis, reduce swelling, and relieve pain. This preparation is primarily used for the treatment of acute soft tissue injury associated with qi stagnation and blood stasis syndrome caused by traumatic injuries and sprains. Acute and long-term toxicity tests showed no abnormal changes in various physiological indices of animals, indicating that the preparation was safe (see Supplementary Material for details). Modern research has found that the microcirculatory disturbance experienced by patients with blood stasis syndrome is associated with abnormal hemorheology, while the pain and swelling are caused by the release of various inflammatory mediators triggered by an aseptic inflammatory response following injury (Liu et al., 2012; Moshiri et al., 2017). Moreover, Pharmacodynamic studies have demonstrated that the metabolites in Angelica sinensis radix and Carthami flos significantly improve hemorheology in rats with blood stasis. Furthermore, combining these two botanical drugs enhances the hemorheological effects (Li et al., 2009; Liu et al., 2011; Yuan et al., 2019). The metabolites found in Saposhnikoviae radix, Arisaematis rhizoma, and Angelicae dahuricae radix have demonstrated notable anti-inflammatory and analgesic effects (Yang et al., 2020; Jin et al., 2021; Qi et al., 2021). In addition,

WHOL has been proven to possess anti-inflammatory, analgesic, and hemorheological improvement properties (Deng et al., 2000). These findings provide a pharmacological basis for the use of WHOL in treating acute soft tissue injuries.

According to the relevant provisions of the Guiding Principles for the Protection of Varieties of Chinese Medicine (National Medical Products Administration, 2009), an application for the protection of varieties of TCM can be submitted if the effectiveness of the main treatment option is superior to that of similar varieties. The basic condition is to conduct clinical studies to prove that it has significant clinical advantages or superior efficacy compared to similar varieties. The study must have a sufficient sample size (not less than 300 subjects in the test group) and follow standardized adverse event evaluation procedures.

FFSTC is effective in treating acute soft tissue injuries (Zheng et al., 2012; Du, 2016; Ge and Fan, 2017; Chang et al., 2021). Notably, FFSTC demonstrated superior efficacy compared to similar Chinese medicines that relieve pain and swelling during a large-scale clinical trial (Zheng et al., 2012). Additionally, FFSTC demonstrated an analgesic effect equivalent to NSAIDs but better swelling reduction within 48 h (Ge and Fan, 2017). This study compared the efficacy of WHOL to that of FFSTC. Meanwhile, the sample size of the WHOL group exceeded 300 subjects (356 subjects). FAS was used in the efficacy analysis to better reflect the actual efficacy of the drug in clinical use, and SS was used in the adverse event assessment to ensure its reliability and comprehensiveness (JA, 1999).

WHOL outperformed FFSTC in various aspects. After 4 days of treatment, WHOL demonstrated superior efficacy in relieving pain at rest and on activity at the injury site. After 7 days, it demonstrated better effectiveness in alleviating pain on activity and reducing swelling at the injury site. Furthermore, after 10 days of treatment, WHOL was more effective in reducing swelling, improving the syndrome of qi stagnation and blood stasis, and lowering CRP levels compared to FFSTC. These findings highlight the potential clinical advantages of WHOL in managing acute soft tissue injuries. The disappearance time of pain at rest was 8 days in both groups and 9 days on activity in both groups. Meanwhile, medication compliance was satisfactory in both groups. In addition, there was no statistical difference in the incidence of adverse events and adverse reactions between the two groups during drug treatment. No serious adverse events occurred in either group, and no subjects were withdrawn because of adverse events. WHOL exhibited superior efficacy in relieving pain and swelling of acute soft tissue injury compared to the FFSTC. It also improved qi stagnation and blood stasis syndrome with fewer adverse effects and satisfactory compliance. These findings support the potential eligibility of WHOL for protection as a variety of TCM.

This study has some limitations. Quality of life was not assessed, and this limited the evaluation of the medication's impact on improving quality of life. Additionally, a larger sample size is recommended for future clinical studies to further validate the efficacy and adverse effects of WHOL.

5 Conclusion

This study confirmed WHOL's efficacy and safety in treating acute soft tissue injury associated with qi stagnation and blood stasis syndrome. It is particularly effective in relieving pain and swelling, with satisfactory patient compliance. Thus, WHOL is a safe and effective new alternative for patients with acute soft tissue injury associated with qi stagnation and blood stasis syndrome in the future.

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.

Ethics statement

The studies involving humans were approved by the Clinical Research Ethics Committee of Longhua Hospital, Shanghai University of TCM (approval number: 2021LCSY114). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. 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

W-HZ: Writing–original draft, Writing–review and editing. YS: Data curation, Methodology, Project administration, Writing–review and editing. YX: Formal Analysis, Visualization, Writing–original draft. QS: Supervision, Writing–review and editing. Z-XF: Data curation, Investigation, Writing–review and editing. Y-QF: Data curation, Investigation, Writing–review and editing. H-BW: Data curation, Investigation, Writing–review and editing. BQ: Data curation, Investigation, Writing–review and editing. JZ: Data curation, Investigation, Writing–review and editing. W-QZ: Data curation, Investigation, Writing–review and editing. G-HX: Conceptualization, Project administration, Writing–review and editing. X-QW: Project administration, Supervision, Writing–review and editing. D-ZT: Project administration, Resources, Supervision, Writing–review and editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1335182/full#supplementary-material

References

Bende, G., Biswal, S., Bhad, P., Chen, Y., Salunke, A., Winter, S., et al. (2016). Relative bioavailability of diclofenac potassium from softgel capsule versus powder for oral solution and immediate-release tablet formulation. *Clin. Pharmacol. Drug Dev.* 5 (1), 76–82. doi:10.1002/cpdd.215

Bleakley, C. M. (2013). Acute soft tissue injury management: past, present and future. *Phys. Ther. Sport* 14 (2), 73–74. doi:10.1016/j.ptsp.2013.01.002

Bleakley, C. M., and Davison, G. W. (2010). Management of acute soft tissue injury using protection rest ice compression and elevation: recommendations from the association of chartered physiotherapists in sports and exercise medicine (ACPSM). *Antimicrob. Agents Chemother.* 1, 1–24.

Bleakley, C. M., O'Connor, S. R., Tully, M. A., Rocke, L. G., Macauley, D. C., Bradbury, I., et al. (2010). Effect of accelerated rehabilitation on function after ankle sprain: randomised controlled trial. *Bmj* 340, c1964. doi:10.1136/bmj.c1964

Chang, R., Pan, Y., and Zeng, X. C. (2021). The clinical efficacy analysis of Fufang Shang Tong Capsule in treating acute ankle sprain. *J. Prac. Tradit. Chin. Med.* 37 (09), 1481–1482.

Chen, P. D., Chen, G. Z., and Xu, Y. X. (2011). Study strategies for bloodletting therapy in treatment of acute soft tissue injuries. *Chin. J. Integr. Med.* 9 (3), 237–241. doi:10.3736/jcim20110302

Cheng, C. W., Wu, T. X., Shang, H. C., Li, Y. P., Altman, D. G., Moher, D., et al. (2017). CONSORT extension for Chinese herbal medicine formulas 2017: recommendations, explanation, and elaboration. *Ann. Intern. Med.* 167 (2), 112–121. doi:10.7326/m16-2977

Deng, H. Z., Chen, Y. Y., Liu, H. H., Chen, F. L., and Huang, X. L. (2000). Pharmacodynamic studies of Wuhu koufuye. *Chin. J. First Mil. Med. Univ.* (3), 269–271. doi:10.3321/j.issn:1673-4254.2000.03.030

Du, W. P. (2016). Exploring the clinical efficacy of Fufang Shang Tong Capsule in treating acute soft tissue injuries. *World Latest Med. infor.* 16 (8), 88–93. doi:10.3766/j.issn:1671-3141.2016.08.022

Ge, R., and Fan, J. F. (2017). Efficacy of Fufang Shang Tong capsule on acute sprain of ankle. *Chin. Pharm.* 26 (04), 50–52. doi:10.3969/j.issn.1006-4931.2017.04.014

Handoll, H. H., Gillespie, W. J., Gillespie, L. D., and Madhok, R. (2007). Moving towards evidence-based healthcare for musculoskeletal injuries: featuring the work of the Cochrane Bone, joint and Muscle Trauma Group. *J. R. Soc. Promot. Health* 127 (4), 168–173. doi:10.1177/1466424007079491

Heinrich, M., Jalil, B., Abdel-Tawab, M., Echeverria, J., Kulić, Ž., McGaw, L. J., et al. (2022). Best Practice in the chemical characterisation of extracts used in pharmacological and toxicological research-The ConPhyMP-Guidelines. *Front. Pharmacol.* 13, 953205. doi:10.3389/fphar.2022.953205

Huang, Y. X., Xu, D. Q., Yue, S. J., Chen, Y. Y., Tao, H. J., Fu, R. J., et al. (2020). Deciphering the active compounds and mechanisms of qixuehe capsule on qi stagnation and blood stasis syndrome: a network Pharmacology study. *Evid. Based Complement. Altern. Med.* 2020, 5053914. doi:10.1155/2020/5053914

Ja, L. (1999). Statistical principles for clinical trials (ICH E9): an introductory note on an international guideline. *Statistics Med.* 18 (15), 1903–1942. doi:10.1002/(sici)1097-0258(19990815)18:15<1903::aid-sim188>3.0.co;2-f

Jiang, S., Chen, Z., Lai, W., Mai, Q., Chen, D., Sun, S., et al. (2020). Decoction of heat-clearing, detoxifying and blood stasis removing relieves acute soft tissue injury via modulating MiR-26b-5p/COX2 Axis to inhibit inflammation. *Biosci. Rep.* 40 (12). doi:10.1042/bsr20201981

Jin, Y. H., Chen, T., Liu, Q., Huang, S. H., Xu, Y. Y., Wen, C. W., et al. (2021). Virtual screening of anti-inflammatory and analgesic coumarins in Angelica dahurica. *Infor. Tradit. Chin. Med.* 38 (10), 9–13. doi:10.19656/j.cnki.1002-2406.20211002

Jones, P., Lamdin, R., and Dalziel, S. R. (2020). Oral non-steroidal anti-inflammatory drugs versus other oral analgesic agents for acute soft tissue injury. *Cochrane Database Syst. Rev.* 8 (8), Cd007789. doi:10.1002/14651858.CD007789.pub3

Li, H. X., Han, S. Y., Wang, X. W., Ma, X., Zhang, K., Wang, L., et al. (2009). Effect of the carthamins yellow from Carthamus tinctorius L. on hemorheological disorders of blood stasis in rats. *Food Chem. Toxicol.* 47 (8), 1797–1802. doi:10.1016/j.fct.2009.04.026

Li, J., Yu, X. Q., Xie, Y., Yang, S. G., Zhao, L., Zhou, M., et al. (2022). Efficacy and safety of traditional Chinese medicine treatment for idiopathic pulmonary fibrosis: an exploratory, randomized, double-blinded and placebo controlled trial. *Front. Pharmacol.* 13, 1053356. doi:10.3389/fphar.2022.1053356

Li, W., Xie, L., Zhu, X., Yang, Y., Wang, L., Yang, M., et al. (2023). Effectiveness and safety of Qingfei Dayuan granules for treating influenza and upper respiratory tract infections manifested by the pulmonary heat-toxin syndrome: a multicenter, randomized, double-blind, placebo-controlled trial. *Front. Pharmacol.* 14, 1133560. doi:10.3389/fphar.2023.1133560

Liu, H., Li, W., Zhang, W., Sun, S., and Chen, C. (2023). Levothyroxine: conventional and novel drug delivery formulations. *Endocr. Rev.* 44 (3), 393–416. doi:10.1210/endrev/bnac030

Liu, L., Duan, J. A., Tang, Y., Guo, J., Yang, N., Ma, H., et al. (2012). Taoren-Honghua herb pair and its main components promoting blood circulation through influencing on hemorheology, plasma coagulation and platelet aggregation. *J. Ethnopharmacol.* 139 (2), 381–387. doi:10.1016/j.jep.2011.11.016

Liu, L., Duan, J. A., Zhu, Z. H., Shi, X. Q., Su, S. L., Tang, Y. P., et al. (2011). Correlation of combinations between Angelicae sinensis radix and Carthami flos. *Chin. Tradit. Herb. Drugs* 42 (05), 929–934.

Lv, Z., and Rong, X. H. (2005). Wuhu powder is effective in the treatment of trauma. *J. Tradit. Chin. Med.* 10, 797. doi:10.13288/j.11-2166/r.2005.10.040

Moshiri, M., Moallem, S. A., Attaranzadeh, A., Saberi, Z., and Etemad, L. (2017). Injury to skeletal muscle of mice following acute and sub-acute pregabalin exposure. *Iran. J. Basic Med. Sci.* 20 (3), 256–259. doi:10.22038/ijbms.2017.8352

National Medical Products Administration (2009). The issuance of guiding principles for the protection of varieties of Chinese medicine. Available at: https://www.nmpa.gov.cn/xxgk/fgwj/gzwj/gzwjyp/20090203120001961.html (Accessed February 3, 2009).

National Medical Products Administration (2020). Code for quality management of drug clinical trials. Available at: https://www.nmpa.gov.cn/yaopin/ypggtg/20200426162401243.html (Accessed April 23, 2020).

Qi, C. Y., Wang, J., Wu, X., He, S. R., Zhang, Q., Wu, J. H., et al. (2021). Botanical, traditional use, phytochemical, and toxicological of Arisaematis rhizoma. *Evid. Based Complement. Altern. Med.* 2021, 9055574. doi:10.1155/2021/9055574

Sloan, J. (2008). Soft tissue injuries: introduction and basic principles. *Emerg. Med. J.* 25 (1), 33–37. doi:10.1136/emj.2007.050971

Wang, C. L. (2021). The expert consensus on Diagnosis, treatment, and pain management of acute closed soft tissue injuries. *Natl. Med. J. Chin.* 101 (21), 1553–1559. doi:10.3760/cma.j.cn112137-20210123-00225

Wang, S., Li, T., Qu, W., Li, X., Ma, S., Wang, Z., et al. (2016). The effects of xiangqing anodyne spray on treating acute soft-tissue injury mainly depend on suppressing activations of AKT and p38 pathways. *Evid. Based Complement. Altern. Med.* 2016, 9213489. doi:10.1155/2016/9213489

Wang, W. J., Yang, A. F., Wang, W., and Hu, X. Y. (2022). Protective effect and mechanism of taohong siwu decoction on rats with acute soft tissue injury. *Chin. J. Integr. Med.* 42 (10), 1207–1212. doi:10.7661/j.cjim.20220509.127

Wang, X. L., Cui, H. S., and Li, M. (1990). Anti-inflammatory and analgesic effects of alcohol extract of Wuhu powder. *Chin. Tradit. Med.* 44 (09).

Yang, M., Wang, C. C., Wang, W. L., Xu, J. P., Wang, J., Zhang, C. H., et al. (2020). Saposhnikovia divaricata-an ethnopharmacological, phytochemical and pharmacological review. *Chin. J. Integr. Med.* 26 (11), 873–880. doi:10.1007/s11655-020-3091-x

Yuan, Z., Zhong, L., Hua, Y., Ji, P., Yao, W., Ma, Q., et al. (2019). Metabolomics study on promoting blood circulation and ameliorating blood stasis: investigating the mechanism of Angelica sinensis and its processed products. *Biomed. Chromatogr.* 33 (4), e4457. doi:10.1002/bmc.4457

Zheng, C. H., Wang, X. L., Zhao, J., and Cao, L. J. (2012). Short-term effect of Fufang Shang Tong Capsule on emergency injury of soft tissue. *Hebei. J. Tradit. Chin. Med.* 34 (06), 821–822. doi:10.3969/j.issn.1002-2619.2012.06.008

Zheng, X. Y. (2002). *Guiding principles for clinical research of new Chinese medicines*. Beijing: China Medicine Science and Technology Press.

Zhu, M., Wei, J., Li, Y., Wang, Y., Ren, J., Li, B., et al. (2022). Efficacy and mechanism of buyang huanwu decoction in patients with ischemic heart failure: a randomized, double-blind, placebo-controlled trial combined with proteomic analysis. *Front. Pharmacol.* 13, 831208. doi:10.3389/fphar.2022.831208

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Dietary supplementation with plant extracts for amelioration of persistent myofascial discomfort in the cervical and back regions: a randomized double-blind controlled study

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Background: Back pain is a common health problem that affects both workers and older people, reducing their quality of life. The primary objective was to assess the effect of dietary supplementation with plant extracts of rosemary, ashwagandha, and sesame consumed for 12 weeks on the intensity of back pain.

Methods: A single-center randomized double-blind study with three parallel arms depending on the product consumed. The duration of treatment was 12 weeks. The investigational product, Berelief®, contained a blend of three polyphenolic standardized extracts: rosemary (*Rosmarinus officinalis* L.), ashwagandha (*Withania somnifera* L.), and sesame (*Sesamum indicum* L.) seed. Two doses were tested: low dose (400 mg) and high dose (800 mg). There were 42 subjects in the placebo group, 39 in the low dose and 42 in the high dose groups. Study variables included back pain intensity [VAS score, Patient-Reported Outcomes Measurement Information System (PROMIS-29), and Cornell Musculoskeletal Discomfort Questionnaire; functionality Roland-Morris Disability (RMD) questionnaire]; quality of life (QoL) [36-item Short Form Survey (SF-36), the Beck Depression Inventory-II (BDI-II), the State–Trait Anxiety Inventory (STAI), and the Perceived Stress Scale (PSS)]; sleep quality [accelerometer and Pittsburgh Sleep Quality Index (PSQI)].

Results: The improvement in back pain recorded by the visual analogue scale (VAS) at the study visits after the beginning of treatment, as well as on a weekly basis recorded in the diary card was significantly higher in the intervention group than in the placebo group ($p < 0.044$ dose-low; $p < 0.005$ dose-high). Significant differences in pain intensity of the PROMIS-29 ($p = 0.002$) and upper back pain in the Cornell questionnaire ($p = 0.011$) in favour of the investigational product were found. Furthermore, benefits in improving health-related quality of life, mood and sleep quality were also detected.

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Conclusion: Dietary supplementation for 12 weeks of a blend of polyphenolic standardized extracts of rosemary, ashwagandha, and sesame was effective in reducing the intensity of pain in subjects with chronic myofascial cervical and back pain.

KEYWORDS

back pain, rosemary, ashwagandha, sesame, quality of life, herbal extracts, randomized clinical trial, food supplement

1 Introduction

In today's hectic lifestyle, many people experience some form of discomfort that negatively affects their ability to carry out daily activities efficiently. The escalating prevalence of discomfort experienced by individuals is primarily attributed to the on-going demographic change and concomitant population aging, as well as unhealthy lifestyles. If these episodic aches are not managed properly, they can turn into chronic conditions and led to more severe problems.

Back pain, whether in the upper back or lumbar area, is one of the most frequent sources of pain due to musculoskeletal disorders and is an important public health problem that can affect quality of life (1). Also, it is one of the most common reasons for adult patients to seek medical care both in the primary and emergency care setting (2) and represents the highest percentage of referrals and workload for physical therapy utilization (3). Back pain is widespread in the adult population leading to great economic and social costs (4). Estimates from the Global Burden of Disease Study 2017 have shown an age-standardized point prevalence for lower back pain of 8.2%, higher in females than males, and increasing with age (5). Systematic reviews have provided evidence of a high prevalence of chronic lower back pain in older adults, with a variable pooled 12-month prevalence of 21 to 68% (6, 7). Since the world population of adults aged 60 years or older is estimated to increase from 1 billion in 2020 to 1.4 billion in 2030 (with 2.1 billion in 2050) (8), it is critically important to identify proper prevention and treatment strategies to be implemented for individuals at risk.

In most people, acute back pain symptoms resolve spontaneously, but in some patients the symptoms continue and become persistent or even chronic (longer than 12 weeks despite treatment). Clinical studies have searched for signs or parameters for predicting pain chronicity, but no consistent neurobiological, behavioural or psychological factors have emerged (9, 10). Chronic back pain management depends on whether the pain is specific (can be explained by an underlying cause) or non-specific/idiopathic, being the latter case the most common (11). Although there are numerous pharmacological and non-pharmacological treatments, including pain-killers, physical therapies, lifestyle changes, education, self-care, and psychological support, clinical trials evaluating the efficacy of a variety of treatment indicate limited efficacy for the majority of the commonly applied interventions and approaches (12). A systematic review from the American College of Physician Practice Guideline of pharmacological therapies for lower back pain concluded that systemic medications [acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), skeletal muscle relaxants, benzodiazepines, systemic corticosteroids, anti-seizure medications, and opioids] were ineffective or associated with small to moderate primarily short-term effects (13). On the other hand, non-pharmacological measures, such as exercise, multi-disciplinary rehabilitation, psychological therapies, spinal manipulation, massage therapy, mindfulness-based stress reduction, or acupuncture have shown to provide small short-term benefits (14).

Due to the limited effectiveness, increased risks, or adverse effects of available medication options, there is a need for safe therapeutic alternatives that can reduce pain and complement other treatment methods. Adopting healthy habits such as regular exercise, balanced nutrition, and maintaining a healthy weight can be effective in preventing chronic pain. However, in some cases, these habits may not be sufficient. In such cases, food supplements can help alleviate pain and enable people to engage in other active treatment modalities as part of a holistic and comprehensive treatment plan.

Although nutritional supplements are growing in popularity, there is limited scientific evidence regarding their efficacy. Rigorous scientific inquiry is needed to determine their true efficacy and safety. Botanical products have recently gained increasing attention for their potential to reduce pain and improve function in chronic non-specific lower back pain. However, evidence of the benefits of herbal medicine in the back pain setting is scarce. A systematic review of 14 randomized controlled studies with 2050 patients with acute, subacute, or chronic non-specific low back pain found some improvements in pain and functional status during 4 to 6 weeks with the use of *Solidago chilensis* (Brazilian arnica) gel, *Capsicum frutescens* cream, and oral doses of *Harpagophytum procumbens* (devil's claw) (standardized 50 or 100 mg harpagoside), *Salix alba* (white willow bark), (standardized to 120 mg or 240 mg salicin), and *Symphytum officinale* L. (comfrey root extract) compared to placebo (15). In another in-depth review of the management of chronic low back pain with herbal, vitamin, mineral, and homeopathic supplements, the use of *Camphora molmol*, *Capsicum frutescens*, *Salix alba*, *Maleluca alternifolia*, *Angelica sinensis*, *Aloe vera*, *Thymus officinalis*, *Menthe peperita*, *Arnica montana*, *Curcuma longa*, *Tancaetum parthenium*, *Harpagophytum procumbens*, and *Zingiber officinalis* showed some short-term beneficial effect versus placebo (16). It should be noted that the efficacy of a combination of herbal extracts has not been evaluated in none of these studies. It is possible that supplementation with a mixture of herbal products may produce a synergistic effect, potentially increasing the effect of each compound and further reducing back pain symptoms.

For this reason, it was considered of interest to design a randomized controlled clinical trial to assess the benefits of a herbal ingredient composed of rosemary, ashwagandha, and sesame seed extracts, administered in two different doses, in subjects with chronic myofascial back pain compared to placebo.

2 Materials and methods

2.1 Study design

A single-center randomized double-blind study with three parallel arms depending on the product consumed (investigational product dose 1 or low dose and dose 2 or high dose, and placebo) was conducted at the Department of Health Sciences of San Antonio Catholic University of Murcia (UCAM), in Murcia, Spain. The study period was from September 21, 2022 to April 26, 2023.

The study was conducted in accordance with the World Medical Association's (WMA) Helsinki Declaration and its amendments. Both the study protocol and the in-formed consent form were approved by the Ethics Committee of San Antonio Catholic University (code CE062205 approval date July 24, 2022) (Murcia, Spain) and was registered in the ClinicalTrials.gov (NCT05597189).

The primary objective was to assess the effect of the study products consumed for 12 weeks on the intensity of back pain. Secondary objectives included reduction in the frequency and/or amount of analgesic medication; assessment of the degree of back pain and functionality using patient-centered outcome measures; health-related quality of life; sleep quality; inflammatory biomarkers; body composition; level of physical activity; and safety.

2.2 Eligibility criteria for participants

Participants were recruited from the database for clinical studies available at UCAM. Inclusion criteria included healthy men and women aged between 20 and 65 years, episodic persistent myofascial back pain (cervical, dorsal, lumbar areas) with a value of at least 3 using a 1–10 cm visual analogue scale (VAS) and for at least 3 months, and body mass index (BMI) between 18.5 and 29.9 kg/m^2. Exclusion criteria were as follows: injury-associated pain; pain caused by chronic conditions, such as rheumatoid arthritis, herniated disks, ankylosing spondylitis, etc.; severe or terminal illnesses; subjects with known allergy to any of the components of the investigational product; subjects undergoing physiotherapy treatment during the course of the study; pregnant or lactating women; and inability to understand the informed consent.

In addition, subjects are requested to respect the following requirements during the whole study (after inclusion): avoid initiating or altering hormonal/medical treatments without justification; abstain from treatments affecting study parameters; refrain from consuming food supplements and avoid modifying regular dietary patterns, particularly in relation to flavonoid-rich foods such as fruits, vegetables, coffee, etc.; and maintain consistent physical activity habits throughout the study. A signed informed consent form was obtained from all the subjects participating in the study before any study-related procedure took place.

2.3 Randomization, masking and study groups

Eligible subjects who provided the written informed consent were randomized to one of the three study groups (1:1:1) using a computer-generated randomization list with the Epidat 4.1 software program. The study groups were as follows: (a) low dose of the investigational product, (b) high dose of the investigational product, and (c) placebo. Both investigators and participants remained blinded to group assignments. Products were coded with unique numbers, detailed on a sheet indicating subject and product codes. The randomization sheet, signed and dated, revealed the product assignments only at the study's conclusion.

The investigational product was a commercially available food supplement ingredient (Berelief®) supplied by Monteloeder, S.L. (Elche, Alicante, Spain), which is a botanical blend of three standardized herbal extract. Specifically, it contains 66% of ashwagandha (*Withania somnifera* L.) root extract standardized in whitanolides by high-performance liquid chromatography (HPLC), 22% sesame (*Sesamum indicum* L.) seed extract standardized in sesamin by HPLC, and *Rosmarinus officinalis* extract standardized in carnosic acid by HPLC. In total, w/w, this blend comprises a minimum content of 2.5% carnosic acid, 2.8% withanolides, and 5.5% sesamin. The ingredients present in the final formula included transient receptor potential vanilloid 1 (TRPV1), the voltage-gated sodium channel isoform 1.7 (NaV1.7), and tropomyosin receptor kinase A (TrkA), which were selected for their individual and complementary effects in inhibiting ion channels involved in pain-related signaling cascades (data not shown).

In the case of low dose, in each jelly capsule 36.4% (200 mg) of the product was the botanical blend ingredient, 45.4% microcrystalline cellulose, and 18.2% encapsulation, whereas in high dose, capsules were composed of 66.7% botanical blend ingredient (400 mg), 16.7% microcrystalline cellulose, and 16.6% encapsulation. The placebo product was composed of 18.2% magnesium stearate, 63.6% microcrystalline cellulose, and 18.2% of the capsule. Both the dietary supplement and placebo products were in opaque colored capsules with identical appearances. They were pre-packed in blisters and consecutively numbered for each subject according to the randomization list.

Subjects were instructed to take two capsules of the assigned product, 200 and 400 mg per capsule in the low and high dose groups, respectively, or placebo capsules, once a day, 30 min before breakfast for 84 consecutive days (12 weeks).

2.4 Study procedures and compliance

The study included a screening visit (visit 0), a baseline visit (visit 1) and two intermediate visits at 4 (visit 2) and 8 (visit 3, end of study) weeks followed by a final visit at week 12 (visit 4). The screening visit took place within ±7 days prior to the baseline visit, in which the inclusion criteria were checked, the written informed con-sent was obtained, and randomization was performed.

At the baseline visit (visit 1), subjects received the study product, a diary, and a sleep accelerometer and the following variables were recorded: concomitant medication; back pain intensity [VAS score, Patient-Reported Outcomes Measurement Information System (PROMIS-29)], and Cornell Musculoskeletal Discomfort Questionnaire; functionality [Roland-Morris Disability (RMD) questionnaire]; quality of life (QoL) [36-item Short Form Survey (SF-36), the Beck Depression Inventory-II (BDI-II), the State–Trait Anxiety Inventory (STAI), and the Perceived Stress Scale (PSS)]; sleep quality [accelerometer and Pittsburgh Sleep Quality Index (PSQI)].

Concomitant medication, study variables recorded at visit 1 (except for subjective sleep quality and physical activity), and adverse

events were evaluated at visits 2 and 3. At the final visit (visit 4), all study variables were assessed, the study product and the diary were collected, adverse events were registered, and a blood sample was drawn for analyses of inflammatory markers and safety testing.

Compliance at the final visit was defined as the number of capsules taken by the participant during the study, divided by the number of capsules expected to be taken ($n = 168$), and multiplied by 100. Subjects were required to consume at least 80% of the total treatment, so they could only leave 34 capsules unconsumed corresponding to 17 out of the 84 days of supplementation.

2.5 Study variables

Clinical variables included age, sex, body mass index (BMI), blood pressure, and percentage of decrease of concomitant analgesic medication.

Pain was the primary efficacy variable and was measured using a 1–10 cm VAS scale (0 = no pain, 10 = worst pain imaginable) and the PROMIS-29 and the Cornell questionnaires. VAS scores were assessed at the study visits and also recorded daily by the study subjects on their diary cards.

PROMIS-29 (v2.0) is a 29-item validated questionnaire (17, 18) that assesses 7 do-mains: pain interference, physical function, anxiety, depression, fatigue, sleep disturbance, satisfaction with participation in social roles, and finally a pain intensity scale. The first seven domains are assessed with 4 questions each. On symptom-oriented (negatively worded) domains of PROMIS-29 (anxiety, depression, fatigue, pain interference, and sleep disturbance), higher scores represent worse symptomatology. On the function-oriented (positively worded) domains (physical function and social role) higher scores represent better functioning.

The Cornell Musculoskeletal Discomfort Questionnaire is a 54-item questionnaire developed for sedentary and standing workers (19) that includes a body chart and questions about occurrence of pain (frequency, intensity, and work interference) in 20 parts of the body over the last work week. Firstly, the level of discomfort recorded by the subject is calculated as "never (0), 1 or 2 times/week (1.5), 3 or 4 times/week (3.5), every day (5), or several times every day (10)." In order to reach the weighted musculoskeletal discomfort level, the result is then multiplied by the severity rate ("slightly uncomfortable = 1, moderately uncomfortable = 2, very uncomfortable = 3") and interference rating ("not at all = 1, slightly interfered = 2, substantially interfered = 3"). Thus, the product of the weighted responses on the three scales gives a weighted score for each body part which ranges between 0 (i.e., "never" on the frequency scale) and 90 (i.e., 10 on the frequency scale x 3 on the severity scale x 3 on the work interference scale). The following areas were independently evaluated: neck, shoulder (right, left), upper back, and lower back.

The Roland Morris Disability (RMD) questionnaire is a 24-item patient-reported outcome measure inquiring about pain-related disability resulting from low back pain. Items are scored 0 if left blank or 1 if endorsed, for a total RMD score ranging from 0 to 24; higher scores represent higher levels of pain-related disability. A change of ≥2 points is clinically relevant (20). A Spanish validated version was used (21).

The SF-36 is a 36-item scale which measures eight domains of health status: physical functioning, physical role limitations, bodily pain, general health perceptions, energy/vitality, social functioning, emotional role limitations, and mental health. A health transition question (HTQ) estimates changes in health status compared to the previous year. Scores of the eight dimensions are transformed to range from 0 where the respondent has the worst possible health to 100 where the respondent is in the best possible health. A Spanish validated version of the instrument was used (22, 23).

The BDI-II is a 21-item rating questionnaire to assess symptoms of depression that occurred during the previous month. Each question has four possible answers, scoring from 0 to 3. The total score ranges from 0 to 63. In non-clinical populations, scores above 20 indicate depression. In those diagnosed with depression the standard cutoff values are 0–13 for minimal depression, 14–19 for mild, 20–28 for moderate, and 29–63 for severe. A Spanish validated version was used (24).

The STAI questionnaire measures state (STAI-state) and trait (STAI-trait) of anxiety based on 20 questions for each domain, scores can vary between 0 and 60 with higher scores indicating greater anxiety levels. A Spanish validated version was used (25).

The PSS scale includes 14 items measuring the frequency or extent of a certain stress-signaling event occurrence of a 5-point scale (from 0: never to 4: very often). Total perceived stress level score ranges between 0 and 56 (scores 0–18 indicate low stress, scores 19–37 indicate moderate stress, and scores 38–56 indicate severe stress). A Spanish validated version was used (26).

The PSQI was administered to assess the quality of sleep. It is a self-reported questionnaire that assesses sleep quality over an interval of 1 month. The overall score ranges between 0 and 21 and is the sum of seven components (sleep latency, subjective sleep quality, duration of sleep and sleep disturbances, habitual sleep efficiency, need of medication to sleep, and daytime dysfunction), with higher scores indicating poorer sleep quality, with an overall score of more than 5 indicating a "poor" sleeper. A Spanish validated version of the PSQI was used (27).

Sleep quality was also evaluated by actigraphy (ActiGraph wGT3X-BT accelerometer, ActiGraph, Pensacola, FL, United States) and the following variables were recorded: sleep latency, sleep efficiency, total time in bed, total sleep time, wakefulness after sleep onset, number of awakenings, and average number in minutes of awakenings.

Variables related to bias control were considered during the study, including body composition and physical activity. Body composition was analyzed by bioelectrical impedance analysis (BIA) on a whole-body BIA analyzer Tanita BC-420MA (Tanita Corporation, Tokyo, Japan). Variables analyzed included weight (kg), BMI (kg/m2), fat mass (kg), percentage of fat mass, and muscle mass (kg). The physical activity level was measured with the same accelerometer used to assess sleep quality. Assessments were conducted both at the study's outset and after 12 weeks of product consumption. The evaluation period for the accelerometer was 3 weekdays and 1 weekend day, aiming to provide a weekly average. METs (metabolic equivalents) were used as the variable to determine physical activity levels, with 1 MET corresponding to the minimum oxygen consumption required to maintain vital functions, serving as a unit to compare the energy cost of daily activities.

2.6 Safety

The occurrence of adverse events (AEs) was monitored throughout the study by the investigators and based on subjects'

diary entries. Investigators rated the reported AEs as being either severe or non-severe based on their potential relationship to study treatment.

A blood analysis was conducted to determine the values of enzymes such as GOT (Glutamic Oxaloacetic Transaminase), GPT (Glutamic Pyruvic Transaminase GGT) (Gamma-Glutamyl Transferase), LDH (Lactate Dehydrogenase), and bilirubin for the assessment of liver function, as well as biomolecules like urea and creatinine to evalu-ate renal function. Additionally, a complete blood count (hemogram) was performed to assess red and white blood cell series, as well as platelets. Blood samples were obtained under fasting conditions at baseline and after 12 weeks of product consumption.

2.7 Statistical analysis

Frequencies and percentages were used for the expression of categorical variables, and mean ± standard deviation (SD) for continuous variables. The chi-square test or the Fisher's exact test was used for the comparison of categorical variables between the study groups, and the Student's t test for the comparison of quantitative variables. The analysis of variance (ANOVA) for repeated measures was used to assess the change of variables corresponding to each group throughout the study period. Within subject factor included data at baseline and at 8 weeks, and between-subject factor for paired data included the product administered that is, medicinal plant extract high dose, medicinal plant extract low dose, and placebo. The Turkey's or Bonferroni's correction was applied for post-hoc analyses. In the evaluation of changes in the Cornell Musculoskeletal Discomfort Questionnaire, patients who determined that they had no pain during the entire study in the indicated area of the body (score 0) were not included in the analysis. In order to assess the effect of the study product in subjects with minimal depression, anxiety, and perceived stress symptoms at the beginning of the study, secondary analyses were also performed using cut-points of ≥4 for BDI-II, ≥ 14 for STAI-state, and ≥ 16 for PSS. A $p < 0.05$ was considered statistically significant. Data analyses were performed with the SPSS version 25.0 (IBM Corp., Armonk, NY, United States) software program.

3 Results

3.1 General characteristics of participants

A total of 302 subjects were initially selected, 135 of which were eligible, but 167 were excluded because the selection criteria were not met ($n = 104$) or refusal to participate ($n = 63$). The 135 eligible subjects were randomized 45 in each of the three study groups. However, 12 subjects were lost to follow-up (3 in the placebo group, 6 in the low dose group, and 3 in the high dose group). The analysis was finally carried out in 123 subjects (42 in the placebo group, 39 in the low dose group, and 42 in the high dose group). The flow chart of the study population is shown in Figure 1.

The study population included 43 men and 80 women, with a mean age of 30.9 ± 12.0 years. The mean VAS score of pain was 5.7 ± 1.4. Differences in demographic and clinical data at baseline between the study groups were not found (Table 1).

3.2 Pain intensity

3.2.1 Monthly VAS scores

In all study groups, VAS scores of pain intensity showed a statistically significant decrease at the end of the study as compared to baseline, but within-group differences were of greater magnitude in subjects assigned to the investigational product groups (Table 2). Also, between-group analyses showed statistically significant differences of VAS scores of the two groups of the investigational product as compared to placebo. The differences were noticeable from the second visit, i.e., from 28 days of product consumption ($p < 0.006$ dose-low; $p < 0.001$ dose-high). At the 56-day visit, the decrease was not as pronounced as in the early days of consumption, but significant differences persisted between the experimental products and the placebo ($p < 0.050$ dose-low; $p < 0.017$ dose-high). These significant differences remained at the end of the study ($p < 0.044$ dose-low; $p < 0.005$ dose-high).

At the end of the study (day 84) both experimental product doses led to a more significant and sustained reduction in pain compared to the placebo, with the low-dose product showing a reduction of approximately 56%, and the high-dose product demonstrating a reduction of about 59% as compared to their corresponding baseline values. The evolution of VAS scores over the study period is shown in Figure 2.

3.2.2 Weekly VAS scores

Results obtained in weekly VAS scores of pain intensity were similar than changes observed at the monthly study visits (Figure 3 and Table 3). In the placebo group, there were statistically significant within-group differences from the seventh week until the end of the study (28% VAS reduction), while in the two groups of the investigational product, statistically significant within-group differences were already observed from the first week reaching a 55.3 and 62.7% reduction for the low and high dose, respectively, from baseline by the end of the 12-week period.

On the other hand, there were between-group statistically significant differences of VAS scores of the two groups of the investigational product as compared to placebo from the first week of treatment until the end. Differences between the low and high dose groups of the investigational product were not found ($p = 0.726$ at the end of the study).

3.3 Analgesic medication

At the beginning of the study, 70 (56.9%) participants [placebo group 22 (31.4%), experimental group-low dose 23 (32.9%), experimental group-high dose 25 (35.7%)] used some analgesic medication for the relief of back pain. Medications initially consumed were ibuprofen (24.4%), paracetamol (17%), dexketoprofen trometamol (12.2%), metamizole magnesium (4.9%), naproxen (3.3%), and diclofenac (1.6%). Local heat pads were used by 12.2% of subjects. Throughout the study, the modification of these treatments was monitored, revealing a decrease in consumption across all study groups after 28 days (Table 4). Apart from identifying notable variations in treatment reduction during each study visit, both experimental groups exhibit considerable statistical significance when compared to placebo group ($p < 0.001$) from the first visit. Regarding the low dose and high dose groups, only significant differences were detected only in the first visit (28 days $p < 0.043$; 56 days $p = 0.077$;

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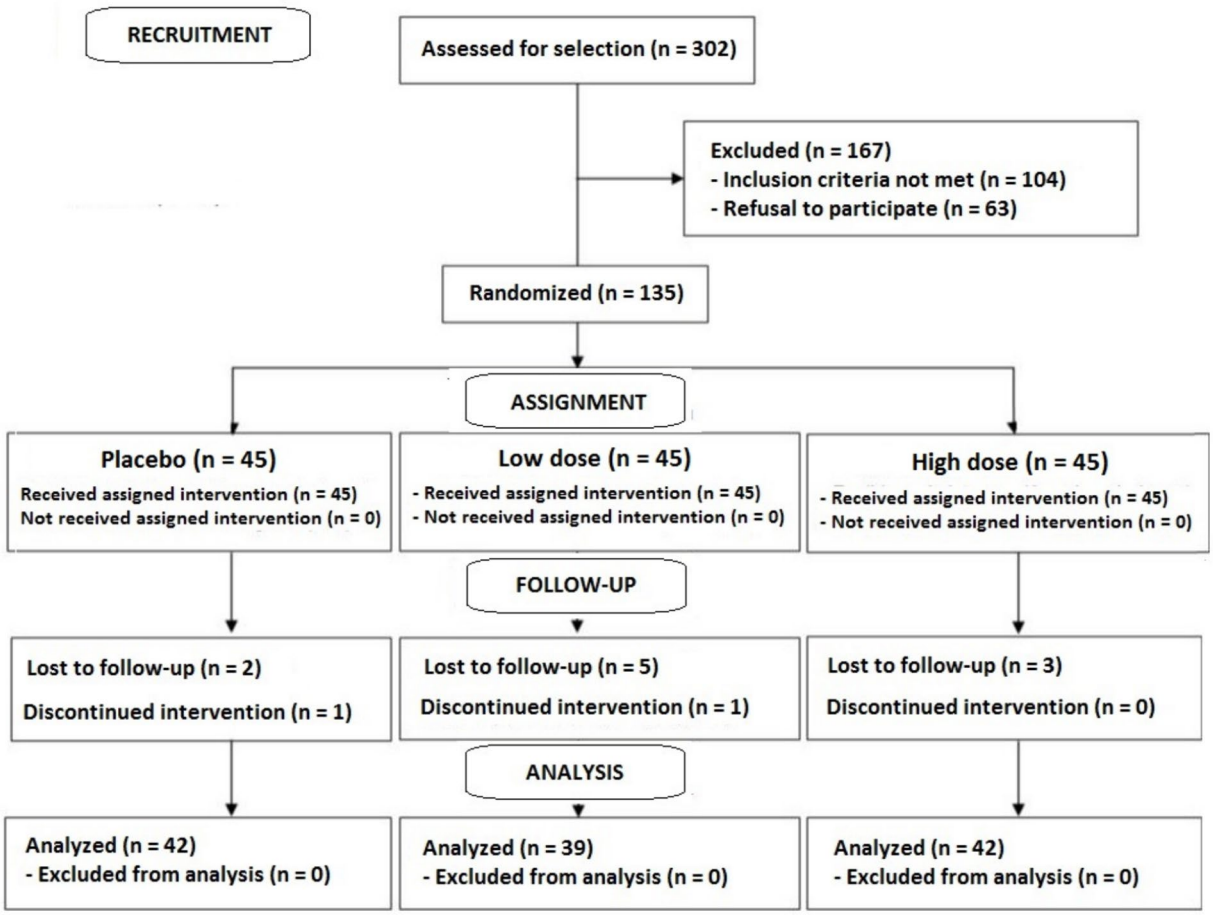

FIGURE 1
Flow chart of the study population.

TABLE 1 Demographic and clinical data at baseline.

Variables	Placebo (*n* = 42)	Investigational product		Total (*n* = 123)
		Low dose (*n* = 39)	High dose (*n* = 42)	
Age, years	33.1 ± 12.2	31.4 ± 13.4	28.2 ± 10.0	30.9 ± 12.0
Weight, kg	70.5 ± 14.7	68.5 ± 13.2	67.6 ± 12.1	68.9 ± 13.3
BMI, kg/m2	24.1 ± 3.6	23.5 ± 3.4	23.6 ± 3.2	23.7 ± 3.4
Systolic BP, mmHg	115.2 ± 14.6	115.0 ± 13.3	113.5 ± 13.5	114.6 ± 13.8
Diastolic BP, mmHg	73.7 ± 9.0	74.0 ± 8.1	74.9 ± 9.3	74.2 ± 8.8
VAS score	5.7 ± 1.4	5.7 ± 1.4	5.9 ± 1.4	5.8 ± 1.4

Data as mean ± standard deviation; BMI, body mass index; BP, blood pressure; VAS, visual analogue scale.

84 days $p = 0.330$). At the end of the study, most of the subject in the experimental groups reduced their medication/heat treatment (87% in the low dose and 96% in the high dose group) (Table 4).

3.4 Cornell musculoskeletal discomfort questionnaire

Results obtained of the Cornell questionnaire in the different body areas are shown in Table 5. The two doses of the investigational product were more effective than placebo in the relief of the level of discomfort in all five body areas, especially in the neck and back pain, which were the variables used for the inclusion of the subjects. However, despite the clear trend of improvement with both doses of the product, statistically significant differences compared to the start of the study were observed in all areas for the high dose and only in the neck and back pain for the low dose. The reason for this could be attributed to the fact that there were very few participants with shoulder pain. Additionally, subjects taking the low dose started with lower pain values, particularly in subjects with left shoulder

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TABLE 2 VAS scores of pain intensity at baseline and throughout the study period.

Study groups	VAS score, mean ± SD				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Placebo ($n = 42$)	5.7 ± 1.4	4.7 ± 1.7	4.1 ± 2.1	3.7 ± 1.9	0.001	< 0.001
Investigational product						
Low dose ($n = 39$)	5.6 ± 1.4	3.4 ± 2.2	3.0 ± 2.5	2.5 ± 2.1	0.001	
High dose ($n = 42$)	5.9 ± 1.5	3.4 ± 2.0	3.0 ± 2.3	2.4 ± 2.4	0.001	

VAS, visual analogue scale; SD, standard deviation.

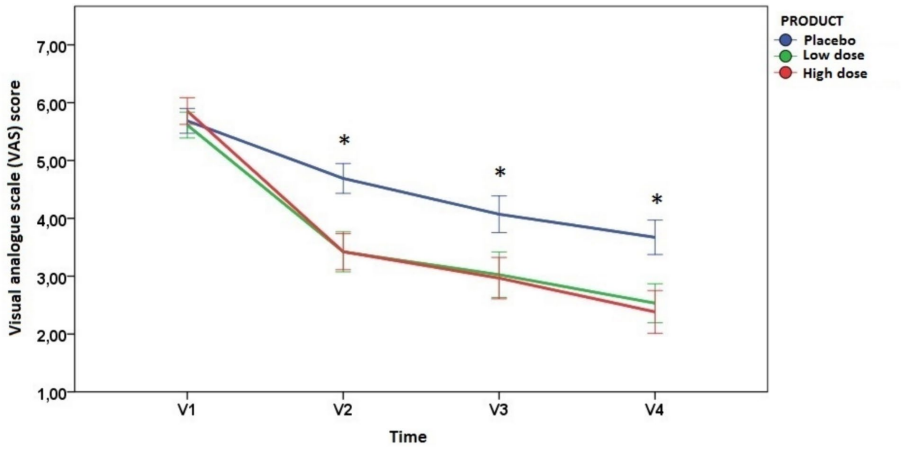

FIGURE 2
Changes of VAS scores during the study period (error bars ±1 standard deviation) (*indicates statistically significant differences between the placebo group and the investigational product groups; see text for *p* values at each time points).

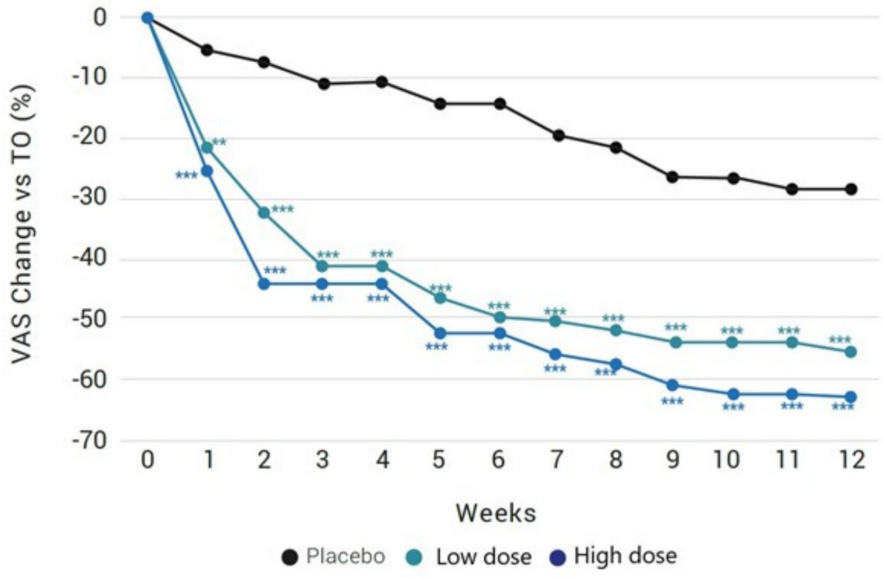

FIGURE 3
Weekly percentage changes of VAS scores in the study groups. *Significant differences in comparison with placebo.

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pain. Between-group significant differences were found in the analysis of neck and upper back areas, with a lower level of discomfort in the investigational product groups as compared to placebo.

3.5 Pain-related disability

Baseline scores of the RMD questionnaire were not homogeneous among the three study groups, with higher scores in the high dose group (4.9 ± 2.8) compared to the low dose (3.9 ± 2.6) ($p = 0.197$) and the placebo group (3.2 ± 2.1) ($p < 0.007$). Scores of the RMD questionnaire decreased significantly in all groups throughout the study, as well as significant within-group differences, with greater improvements in the investigational product groups (Table 6). However, clinically relevant changes (≥ 2 points reduction) were only found in the groups treated with the supplement ingredient (in the low dose −2.3 points and high dose −2.9 points).

TABLE 3 Weekly changes of VAS scores of pain intensity in the three study groups.

Time point	Study groups, VAS score, mean ± SD		
	Placebo (n = 42)	Investigational product	
		Low dose (n = 39)	High dose (n = 42)
Baseline	5.7 ± 1.4	5.6 ± 1.4	5.9 ± 1.5
Week 1	5.4 ± 1.6	4.4 ± 1.8	4.4 ± 1.6
Week 2	5.3 ± 1.6	3.8 ± 1.8	3.3 ± 1.6
Week 3	5.3 ± 1.7	3.3 ± 1.8	3.3 ± 1.6
Week 4	5.1 ± 1.7	3.3 ± 2.0	3.4 ± 1.7
Week 5	4.9 ± 1.8	3.0 ± 2.1	2.8 ± 2.0
Week 6	4.9 ± 1.8	2.8 ± 2.0	2.8 ± 1.9
Week 7	4.6 ± 1.8	2.8 ± 2.1	2.6 ± 1.9
Week 8	4.5 ± 1.8	2.7 ± 2.1	2.5 ± 1.8
Week 9	4.2 ± 1.9	2.6 ± 2.1	2.3 ± 2.0
Week 10	4.2 ± 1.8	2.6 ± 2.0	2.2 ± 2.0
Week 11	4.1 ± 1.8	2.6 ± 2.1	2.2 ± 2.0
Week 12	4.1 ± 1.8	2.5 ± 2.0	2.2 ± 2.2
Within-group p value	0.015 (from week 7)	< 0.001 (from week 1)	< 0.001 (from week 1)
Between-group p value	< 0.001		

SD, standard deviation.

3.6 Quality of life

3.6.1 Quality of life assessed with the SF-36 questionnaire

As shown in Table 7, statistically significant differences favoring the investigational product in both low and high dose doses compared to placebo were found in the domains of bodily pain and the health transition question, which coincidently were the domains with the lowest starting average scores. In the domains of physical functioning, physical role limitations, and bodily pain, within-group values were statistically significant in all three study groups, whereas significant improvements in energy/vitality and health transition question were found in both investigational product groups. Additionally, significant increases in emotional role and social functioning were observed specifically in the high-dose group.

3.6.2 PROMIS-29 questionnaire

As shown in Table 8, there was a statistically significant general improvement in all domains compared to baseline in the groups taking the investigational product. In the placebo group, improvements over time were only observed in the pain interference, pain intensity, and anxiety domains, although the magnitude of improvements was higher in the two doses of the investigational product. Between group statistically significant differences were only found for the item of pain intensity from the second visit, with higher decreases in the investigational product groups. No significant differences were found among the two dosages studied in the pain intensity item.

The high dose group also showed greater increases in physical function at the end of the study compared to placebo ($p < 0.05$).

3.6.3 Emotional wellbeing

Emotional wellbeing was evaluated through different validated questionaries: Beck Depression Inventory (BDI), State–Trait Anxiety Inventory (STAI), and Perceived Stress Scale (PSS). These renowned questionnaires collectively provide valuable in-sights into various dimensions of mental health, offering a nuanced understanding of an individual's emotional state. The results of these questionaries are included in Tables 9, 10.

The Beck questionnaire related to the state of depression revealed a decrease in all groups for each of the measurements conducted in relation to the initial moment. However, only significant differences compared to the start of the study were observed in the two doses of the investigational product. When comparing with placebo, no statistically significant differences were apparent; however, a trend towards significance was observed ($p = 0.149$) (Table 9). When conducting a more in-depth analysis using cut-points of ≥4 for BDI-II to identify

TABLE 4 Percentage of subjects who decreased medication/heat consumption compared to the baseline state for each of the study groups.

Study groups	Medication/heat (% decrease)			Within- group p value	Between-group p value
	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Placebo (n = 42)	4.5%	13.6%	27.3%	0.001	< 0.001
Investigational product					
Low dose (n = 39)	47.8%	78.3%	87%	0.001	
High dose (n = 42)	76%	96%	96%	0.001	

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TABLE 5 Changes in the Cornell musculoskeletal discomfort questionnaire in the three study groups.

Body areas and study groups	Mean ± SD scores				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Neck						
Placebo ($n = 34$)	16.7 ± 25.0	11.0 ± 11.5	10.9 ± 17.7	10.1 ± 11.2	0.688	0.028
Low dose ($n = 34$)	23.4 ± 24.2	11.5 ± 14.1*	10.1 ± 13.6*	7.9 ± 12.3*	0.002	
High dose ($n = 34$)	29.0 ± 26.7	14.0 ± 19.5*	10.9 ± 18.8*	5.0 ± 15.7*	0.001	
Right shoulder						
Placebo ($n = 17$)	16.2 ± 21.9	10.6 ± 10.9	8.8 ± 15.8	9.7 ± 10.7	1.000	0.968
Low dose ($n = 17$)	11.2 ± 23.1	5.4 ± 9.9	5.1 ± 10.0	2.4 ± 4.7	0.396	
High dose ($n = 22$)	13.2 ± 15.8	6.7 ± 12.2	5.4 ± 9.2	1.6 ± 3.5*	0.040	
Left shoulder						
Placebo ($n = 17$)	10.1 ± 21.4	5.7 ± 7.4	4.8 ± 14.7	3.7 ± 7.0	1.000	0.316
Low dose ($n = 14$)	4.7 ± 7.0	4.1 ± 5.8	2.1 ± 3.9	3.0 ± 6.2	1.000	
High dose ($n = 21$)	16.6 ± 20.8	12.3 ± 20.3	9.0 ± 19.6	2.0 ± 3.8*	0.006	
Upper back						
Placebo ($n = 33$)	15.6 ± 19.2	13.0 ± 22.1	7.7 ± 12.3	8.1 ± 11.9	0.227	0.011
Low dose ($n = 31$)	15.7 ± 14.9	8.4 ± 10.4	6.3 ± 16.6	3.3 ± 6.3*	0.007	
High dose ($n = 33$)	27.3 ± 26.4	10.0 ± 14.3*	7.5 ± 9.5*	3.9 ± 9.1*	0.001	
Lower back						
Placebo ($n = 34$)	12.1 ± 16.7	10.8 ± 20.6	9.7 ± 18.5	11.0 ± 22.6	1.000	0.201
Low dose ($n = 33$)	17.4 ± 20.6	12.6 ± 16.6	10.3 ± 16.6	7.1 ± 10.4*	0.049	
High dose ($n = 33$)	16.3 ± 20.3	10.9 ± 11.5	5.2 ± 5.8*	4.0 ± 7.2*	0.018	

SD, standard deviation. *Statistically significant compared to the baseline. Patients with no pain at baseline were excluded from the analysis.

TABLE 6 Changes in the Roland-Morris Disability (RMD) questionnaire in the three study groups.

Study groups	RMD points, mean ± SD				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Placebo ($n = 42$)	3.2 ± 2.1	2.4 ± 1.9*	2.3 ± 2.2*	2.0 ± 2.6*	0.009	0.003
Investigational product						
Low dose ($n = 39$)	3.9 ± 2.6	2.7 ± 2.0*	2.3 ± 1.8*	1.6 ± 1.9*	0.001	
High dose ($n = 42$)	4.9 ± 2.8	3.5 ± 2.5*	2.6 ± 2.4*	2.0 ± 2.5*	0.001	

SD, standard deviation. *Statistically significant compared to baseline.

subjects with minimal symptoms, significant differences in BDI-II scores were observed in investigational product groups compared to placebo ($p = 0.016$) (Table 10). This difference was more pronounced in the high-dose group, where significant differences compared to placebo were observed starting from the second visit ($p < 0.05$).

Although there was a significant reduction in anxiety levels within the high-dose group when compared to the beginning of the study, the investigational products were not significantly superior to placebo in improving anxiety within the overall study population, encompassing both state and trait aspects (Table 9). However, when considering only the subjects who reported an anxiety level of 14 or higher, as assessed by the STAI questionnaire, significant differences in progress were observed between the groups ($p = 0.038$), with the high-dose group exhibiting a noteworthy improvement after 84 days of product consumption ($p < 0.008$) (Table 10).

Finally, in the entire population, the investigational products were not superior to placebo in reducing the perceived stress levels as measured by PSS score ($p = 0.328$). However, in the low and high dose groups of the experimental product, some improvement was seen as compared to the baseline at the end of the study in the low dose group, and from the second visit in the high dose group (Table 9). Furthermore, when analyzing the population with minimal stress (PSS ≥ 16), significant differences in progress were observed between the groups ($p = 0.018$). Specifically, the high-dose group exhibited a significant improvement from the 56th day of product consumption ($p < 0.05$) that was maintained until the end of the study ($p < 0.009$) (Table 10).

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TABLE 7 Changes in quality of life in the three study groups according to the SF-36.

SF-36 domains and study groups	Mean ± SD scores				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Physical functioning						
Placebo ($n = 42$)	84.8 ± 13.3	88.3 ± 13.0	89.3 ± 12.1*	90.2 ± 9.4*	0.006	0.814
Low dose ($n = 39$)	86.4 ± 11.0	89.1 ± 9.7	92.4 ± 7.4*	93.3 ± 6.2*	0.001	
High dose ($n = 42$)	83.2 ± 16.1	85.0 ± 20.4	88.6 ± 14.5*	90.4 ± 14.0*	0.001	
Physical role limitations						
Placebo ($n = 42$)	73.2 ± 18.7	81.9 ± 15.9*	81.0 ± 18.4*	82.4 ± 16.1*	0.006	0.503
Low dose ($n = 39$)	70.4 ± 18.1	80.0 ± 19.0*	82.4 ± 18.0*	85.1 ± 14.9*	0.001	
High dose ($n = 42$)	68.7 ± 21.7	76.0 ± 21.4*	77.5 ± 19.4*	82.7 ± 18.9*	0.001	
Bodily pain						
Placebo ($n = 42$)	47.5 ± 16.1	55.1 ± 16.6*	54.7 ± 18.2*	56.4 ± 17.2*	0.018	0.025
Low dose ($n = 39$)	50.0 ± 15.0	56.3 ± 15.6	60.2 ± 18.6*	65.6 ± 20.1*	0.001	
High dose ($n = 42$)	46.5 ± 16.4	58.4 ± 16.0*	61.1 ± 18.1*	67.8 ± 21.7*	0.001	
General health perception						
Placebo ($n = 42$)	68.3 ± 15.4	69.8 ± 14.2	71.2 ± 14.3	70.6 ± 14.2	1.000	0.998
Low dose ($n = 39$)	66.1 ± 21.1	68.8 ± 19.8	69.4 ± 20.4	70.0 ± 22.1	0.642	
High dose ($n = 42$)	68.0 ± 19.3	70.7 ± 16.1	71.2 ± 16.6	71.7 ± 16.7	0.642	
Energy/vitality						
Placebo ($n = 42$)	55.8 ± 15.8	61.3 ± 17.2	61.8 ± 16.8	62.1 ± 14.4	0.112	0.823
Low dose ($n = 39$)	55.0 ± 16.9	59.8 ± 15.9	63.3 ± 17.0*	64.6 ± 19.7*	0.003	
High dose ($n = 42$)	55.5 ± 17.4	62.4 ± 16.5*	63.8 ± 14.8*	65.6 ± 14.8*	0.001	
Social functioning						
Placebo ($n = 42$)	76.0 ± 21.8	77.7 ± 19.8	79.5 ± 21.5	80.1 ± 21.4	1.000	0.888
Low dose ($n = 39$)	79.2 ± 19.7	84.0 ± 15.7	84.3 ± 20.6	86.5 ± 17.5	0.107	
High dose ($n = 42$)	74.2 ± 21.2	81.3 ± 19.2	80.7 ± 20.7	82.4 ± 19.1*	0.037	
Emotional role						
Placebo ($n = 42$)	80.6 ± 18.4	85.1 ± 18.4	84.5 ± 18.3	84.5 ± 20.0	1.000	0.216
Low dose ($n = 39$)	80.3 ± 20.7	82.3 ± 19.6	85.5 ± 17.2	84.0 ± 23.2	0.350	
High dose ($n = 42$)	73.8 ± 22.9	82.2 ± 22.2*	85.1 ± 19.0*	85.7 ± 19.2*	0.002	
Mental health						
Placebo ($n = 42$)	70.7 ± 15.2	74.5 ± 15.4	73.0 ± 18.5	74.5 ± 18.0	0.800	0.866
Low dose ($n = 39$)	71.7 ± 14.8	73.6 ± 16.7	75.3 ± 15.9	75.5 ± 17.7	0.846	
High dose ($n = 42$)	71.7 ± 20.0	73.6 ± 18.2	73.0 ± 16.3	76.8 ± 17.7	0.257	
Health transition question						
Placebo ($n = 42$)	51.8 ± 20.2	49.4 ± 16.1	52.4 ± 14.4	50.0 ± 12.3	1.000	0.023
Low dose ($n = 39$)	46.2 ± 15.7	54.5 ± 21.4*	53.2 ± 21.6*	56.4 ± 24.1*	0.033	
High dose ($n = 42$)	49.4 ± 21.7	58.7 ± 22.9*	59.5 ± 23.4*	59.5 ± 24.7*	0.027	

SD, standard deviation. *Statistically significant compared to baseline.

3.6.4 Sleep quality: perceived sleep quality index (PSQI) and actigraphy

Sleep quality was assessed both at the perception level and using a tracking de-vice. As shown in Table 11, only the group consuming a high dose of the investigational product revealed a statistically significant improvement in their perceived sleep quality index (PSQI) throughout the study period, starting from the 28th day ($p < 0.05$) until the end of the study ($p < 0.001$). Also, significant differences were observed be-tween experimental and placebo groups ($p = 0.036$), whereas these differences were more noticeable in the higher dose.

The assessment of sleep quality by actigraphy (Table 12) revealed that in general the population of the study had good sleep quality, with

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TABLE 8 Changes in the PROMIS-29 questionnaire in the three study groups.

Domains and study groups	Mean ± SD scores				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Pain interference						
Placebo ($n = 42$)	15.0 ± 3.0	16.5 ± 2.9*	16.8 ± 3.0*	17.2 ± 3.0*	0.001	0.296
Low dose ($n = 39$)	14.6 ± 3.4	16.4 ± 2.9*	17.3 ± 3.0*	18.0 ± 2.4*	0.001	
High dose ($n = 42$)	13.7 ± 3.5	15.6 ± 3.8*	16.6 ± 3.2*	17.5 ± 3.4*	0.001	
Physical function						
Placebo ($n = 42$)	18.4 ± 1.7	18.9 ± 1.9*	19.0 ± 1.7	18.6 ± 2.8	1.000	0.231
Low dose ($n = 39$)	18.7 ± 1.9	19.2 ± 1.1*	19.4 ± 1.3*	19.4 ± 1.0*	0.049	
High dose ($n = 42$)	18.3 ± 2.4	18.9 ± 1.9*	19.0 ± 1.7*	19.4 ± 1.6*	0.004	
Pain intensity						
Placebo ($n = 42$)	5.8 ± 1.4	5.0 ± 1.4*	4.6 ± 1.9*	4.2 ± 1.8*	0.001	0.002
Low dose ($n = 39$)	5.5 ± 1.5	4.3 ± 1.8*	3.6 ± 2.1*	3.2 ± 2.2*	0.001	
High dose ($n = 42$)	5.9 ± 1.5	4.1 ± 1.7*	3.5 ± 2.0*	2.9 ± 2.0*	0.001	
Anxiety						
Placebo ($n = 42$)	15.6 ± 3.4	16.3 ± 2.7	16.6 ± 3.3	17.0 ± 3.1*	0.013	0.402
Low dose ($n = 39$)	15.1 ± 3.3	16.2 ± 3.1*	16.7 ± 3.3*	17.8 ± 2.5*	0.001	
High dose ($n = 42$)	15.0 ± 3.7	16.0 ± 3.4*	16.4 ± 2.9*	17.3 ± 2.8*	0.001	
Depression						
Placebo ($n = 42$)	18.0 ± 2.8	18.4 ± 2.5	18.6 ± 2.4	18.8 ± 2.1	0.507	0.798
Low dose ($n = 39$)	17.7 ± 3.2	17.9 ± 3.3	18.6 ± 2.0*	19.0 ± 1.4*	0.002	
High dose ($n = 42$)	17.8 ± 2.6	18.3 ± 2.6	18.7 ± 1.9	19.1 ± 1.5*	0.003	
Fatigue						
Placebo ($n = 42$)	15.1 ± 2.6	16.2 ± 2.7	16.0 ± 2.8	16.1 ± 3.1	0.118	0.534
Low dose ($n = 39$)	14.5 ± 3.6	15.5 ± 2.8	15.9 ± 3.0*	16.6 ± 3.4*	0.001	
High dose ($n = 42$)	14.2 ± 4.0	14.9 ± 4.2	15.4 ± 3.2*	15.7 ± 3.7*	0.012	
Sleep disturbance						
Placebo ($n = 42$)	13.5 ± 2.8	14.1 ± 2.7	14.0 ± 3.1	14.2 ± 2.9	1.000	0.797
Low dose ($n = 39$)	13.3 ± 3.8	14.3 ± 3.4	14.7 ± 2.9*	14.6 ± 2.9*	0.031	
High dose ($n = 42$)	13.4 ± 3.2	14.3 ± 2.3	14.7 ± 2.5*	15.0 ± 2.8*	0.002	
Satisfaction with social roles						
Placebo ($n = 42$)	15.0 ± 3.7	15.5 ± 3.2	15.4 ± 3.0	15.6 ± 2.8	1.000	0.812
Low dose ($n = 39$)	14.9 ± 3.9	15.5 ± 3.6	16.0 ± 2.9	16.2 ± 3.0*	0.049	
High dose ($n = 42$)	14.5 ± 4.3	14.9 ± 3.7	15.1 ± 3.8	15.9 ± 3.3*	0.035	

SD, standard deviation. *Statistically significant compared to baseline.

low sleep latency, high sleep efficiency (> 90%) with a total sleep time around 7 h, etc. Despite this, it was observed that the groups taking the experimental product significantly reduced latency compared to the study's outset. When comparing the groups, significant differences were observed in relation to the placebo group in the high-dose group ($p < 0.001$) and a trend towards significance was observed between the control and the low-dose groups ($p = 0.107$).

In the other actigraphy evaluated parameters, although a trend towards improvement was observed in sleep efficiency and number of awakenings in favor of the investigational product, it did not reach statistical significance.

3.7 Compliance and safety

The percentage of compliance ranged between 94 and 100% (some subjects re-turned 10 unconsumed capsules). Changes in the level of physical activity were not significant in any study group. At baseline and at the end of study, the mean values were 1.5 ± 0.19 and 1.54 ± 0.23 METs in the placebo group ($p = 0.257$), 1.54 ± 0.02 and 1.57 ± 0.24 METs in the low-dose group ($p = 0.238$), 1.50 ± 0.22 and 1.53 ± 0.24 METs in the high-dose group ($p = 0.221$). In addition, changes in BMI, percentage of fat mass, and SBP and DBP during the study period were not observed.

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TABLE 9 Changes of emotional well-being dimensions in the three study groups.

Questionnaires and study groups	Mean ± SD scores				Within- group *p* value	Between-group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
BDI-II score						
Placebo ($n = 42$)	7.4 ± 4.8	6.0 ± 4.5	6.3 ± 5.0	6.1 ± 5.1	0.281	0.149
Low dose ($n = 39$)	7.3 ± 5.1	5.9 ± 4.7	5.4 ± 5.0*	4.8 ± 5.1*	0.002	
High dose ($n = 42$)	8.8 ± 6.4	7.5 ± 7.2	6.0 ± 6.6*	5.8 ± 5.9*	0.001	
STAI-state, score						
Placebo ($n = 42$)	17.3 ± 9.4	17.6 ± 8.3	17.0 ± 10.2	16.4 ± 10.3	1.000	0.232
Low dose ($n = 39$)	17.2 ± 9.9	16.8 ± 10.9	15.8 ± 10.5	14.6 ± 10.6	0.349	
High dose ($n = 42$)	17.7 ± 10.9	16.3 ± 11.0	15.1 ± 10.7	12.4 ± 9.2*	0.001	
STAI-trait, score						
Placebo ($n = 42$)	19.1 ± 10.0	17.0 ± 8.9	16.2 ± 10.9*	16.2 ± 10.4*	0.038	0.480
Low dose ($n = 39$)	17.4 ± 9.1	16.9 ± 9.2	15.0 ± 9.4	15.0 ± 8.9*	0.049	
High dose ($n = 42$)	19.3 ± 10.7	16.4 ± 8.9*	15.6 ± 10.0*	14.6 ± 10.2*	0.001	
PSS total score						
Placebo ($n = 42$)	20.2 ± 7.7	20.1 ± 7.8	19.5 ± 8.6	18.7 ± 7.8	1.000	0.328
Low dose ($n = 39$)	19.8 ± 8.8	18.6 ± 8.6	17.9 ± 9.0	17.1 ± 9.5*	0.049	
High dose ($n = 42$)	20.9 ± 8.5	18.5 ± 9.3*	18.2 ± 9.3*	16.2 ± 8.9*	0.001	

SD, standard deviation; BDI, beck depression inventory; STAI, state–trait anxiety inventory; PSS, perceived stress scale. *Statistically significant compared to baseline.

TABLE 10 Changes of depression, anxiety, and perceived stress levels in the three study groups among subjects with minimal symptoms at the beginning of the study.

Questionnaires and study groups	Mean ± SD scores				Within- group *p* value	Between-group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
BDI-II score ≥ 4						
Placebo ($n = 35$)	8.5 ± 4.4	6.9 ± 4.5	7.1 ± 5.1	6.8 ± 5.3	0.095	0.016
Low dose ($n = 31$)	8.9 ± 4.4	7.1 ± 4.6	6.5 ± 4.9*	5.8 ± 5.2*	0.001	
High dose ($n = 32$)	10.9 ± 6.1	8.9 ± 7.6*	6.7 ± 7.1*	6.4 ± 6.5*	0.001	
STAI-state, score ≥ 14						
Placebo ($n = 25$)	23.5 ± 6.6	21.8 ± 6.8	22.0 ± 9.7	21.9 ± 9.4	1.000	0.038
Low dose ($n = 21$)	24.6 ± 7.1	23.2 ± 9.7	20.2 ± 8.7	18.8 ± 8.1*	0.009	
High dose ($n = 25$)	24.3 ± 9.0	20.6 ± 11.0	19.0 ± 10.9*	15.6 ± 10.0*	0.001	
PSS score ≥ 16						
Placebo ($n = 31$)	23.6 ± 5.7	23.3 ± 6.3	22.8 ± 7.3	21.8 ± 6.2	1.000	0.018
Low dose ($n = 24$)	25.2 ± 6.8	23.0 ± 7.3	22.0 ± 8.3	21.5 ± 8.1*	0.048	
High dose ($n = 28$)	25.5 ± 6.2	21.8 ± 8.7*	20.1 ± 9.1*	18.0 ± 8.9*	0.001	

SD, standard deviation; BDI, beck depression inventory; STAI, state–trait anxiety inventory; PSS, perceived stress scale. *Statistically significant compared to baseline.

Regarding the safety, the results of physical examination were unrevealing and laboratory tests remained within the normal ranges in the three groups, with no significant changes in the blood count or on the liver or kidney function.

Adverse events of mild to moderate intensity or unrelated to the study product were recorded. Adverse events of mild to moderate intensity included stomach discomfort (placebo 7.1% of patients, low dose 15.4%, high dose 16.7%) and constipation (placebo 2.4% of patients, low dose 4.8%, high dose 4.5%), but none of these symptoms was considered a risk factor for discontinuation of dietary supplementation.

4 Discussion

In this study, the effect of a dietary supplement ingredient comprised of rosemary leaf, ashwagandha root, and sesame seed, administered in low and high doses of 400 and 800 mg/day, was

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TABLE 11 Changes in the Pittsburgh Sleep Quality Index (PSQI) scores in the three study groups.

PSQI score and study groups	Mean ± SD scores				Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 2 28 days	Visit 3 56 days	Visit 4 (final) 84 days		
Placebo ($n = 42$)	6.5 ± 2.5	6.1 ± 2.6	6.2 ± 2.9	6.0 ± 3.2	1.000	0.036
Low dose ($n = 39$)	6.9 ± 3.3	6.5 ± 2.9	6.2 ± 2.2	6.0 ± 2.9	0.230	
High dose ($n = 42$)	7.5 ± 3.8	6.4 ± 3.4*	5.8 ± 3.0*	5.3 ± 3.1*	0.001	

SD, standard deviation; PSQI, Pittsburgh Sleep Quality Index. *Statistically significant compared baseline.

TABLE 12 Results of sleep evaluation by actigraphy in the three study groups at the end of the study as compared with baseline.

Sleep parameters and study groups	Mean ± SD		Within- group *p* value	Between- group *p* value
	Visit 1 baseline	Visit 4 (final) 84 days		
Sleep latency, min				
Placebo ($n = 42$)	3.16 ± 1.02	3.36 ± 0.89	0.203	0.001
Low dose ($n = 39$)	3.37 ± 0.93	3.07 ± 0.82	0.049*	
High dose ($n = 42$)	3.53 ± 0.90	2.81 ± 0.84	0.001*	
Sleep efficiency, %				
Placebo ($n = 42$)	92.0 ± 3.4	91.2 ± 3.6	0.049	0.106
Low dose ($n = 39$)	91.8 ± 2.6	91.8 ± 3.1	0.996	
High dose ($n = 42$)	91.5 ± 3.3	92.0 ± 3.0	0.246	
Total time in bed, min				
Placebo ($n = 42$)	464.0 ± 53.9	461.1 ± 49.4	0.697	0.680
Low dose ($n = 39$)	446.1 ± 62.9	448.7 ± 58.7	0.736	
High dose ($n = 42$)	463.7 ± 61.1	469.9 ± 57.0	0.399	
Total sleep time, min				
Placebo ($n = 42$)	427,0 ± 51,2	420,9 ± 49,8	0.405	0.387
Low dose ($n = 39$)	409,4 ± 58,3	411,9 ± 56,2	0.735	
High dose ($n = 42$)	424,5 ± 60,1	432,5 ± 56,1	0.272	
Wakefulness after sleep onset, min				
Placebo ($n = 42$)	33.9 ± 16.3	36.9 ± 16.5	0.150	0.380
Low dose ($n = 39$)	33.4 ± 13.7	33.7 ± 14.0	0.875	
High dose ($n = 42$)	35.6 ± 14.8	34.6 ± 14.0	0.622	
Number of awakenings				
Placebo ($n = 42$)	14.5 ± 6.0	15.5 ± 6.8	0.190	0.204
Low dose ($n = 39$)	14.7 ± 5.5	14.8 ± 5.5	0.929	
High dose ($n = 42$)	14.4 ± 5.2	13.5 ± 6.0	0.224	
Awakenings, mean number of min				
Placebo ($n = 42$)	2.35 ± 0.78	2.57 ± 0.95	0.123	0.419
Low dose ($n = 39$)	2.36 ± 0.84	2.23 ± 0.69	0.808	
High dose ($n = 42$)	2.62 ± 1.19	2.79 ± 1.28	0.230	

SD, standard deviation; min, minutes. *Statistically significant compared to baseline.

assessed on a population of individuals with persistent myofascial back pain for 12 weeks. For that purpose, a randomized, double-blind placebo controlled clinical trial was conducted. The results of the study showed that the botanical extract blend was effective in reducing the intensity of back myofascial pain, to a significantly greater extent compared to placebo. The improvement in back pain was detected using subjective scoring with a VAS scale during the visits, which corroborated the results observed by the weekly VAS assessment performed at home. Significant improvements vs. placebo was detected as early as during the first week of intake Although a slightly better analgesic efficacy was observed in the high dose group, no significant differences were observed during any visit when comparing the two

experimental doses. This finding suggests that the product effectively reduces pain at both doses used. In addition, the reduced pain sensitivity resulted in a significant reduction in analgesic medication among subjects taking the botanical blend, compared to the placebo group. This effect increased consistently over the entire study period.

The analgesic effect of the investigational product is consistent with the pain relieving properties described for the individual components. In relation to *Rosmarinus officinalis* L., different reviews have highlighted various medicinal properties, including antitumoral, anti-inflammatory, analgesic, neurodegenerative, endocrinal, antiinfective and antioxidant effects (28, 29). *R. officinalis* is mainly composed of polyphenols (such as apigenin, diosmin, luteolin, and phenolic acids especially rosmarinic acid) and terpenes (such pirosmanol, carnosol, carnosic acid, ursolic acid and oleanolic acid), which account for the beneficial therapeutic applications and pleiotropic use of rosemary. It has been shown that polyphenols found in plant extracts have antinociceptive affects, with attenuation of neuropathic pain in animal models as well as nociceptive and inflammatory pain (30). Moreover, the dipertenoids carnosol and carnosic acid exert anti-inflammatory and analgesic activities, interfering with the multiple signaling pathways that are deregulated during inflammation and underlying mechanisms of nociceptive pain (31).

Other components of the investigational product were ashwagandha root and sesame seed. Studies have shown that ashwagandha can help relieve persistent pain. For example, a randomized double-blind placebo-controlled trial using a standardized aqueous extract of roots plus leaves of *W. somnifera* (125 and 250 mg) administered for 12 weeks in patients with knee joint pain and discomfort, reported a significant pain reduction at 4 weeks compared to placebo (32). Many other benefits in a wide range of conditions related to immunomodulatory, cardioprotective, neuroprotective, antiaging, anti-stress/adaptogenic, anti-cancer, and anti-diabetic pharmacological activities of this phytochemical have been reported (33). On the other hand, sesamin, an active compound present in *Sesamum indicum*, has been shown to attenuate joint pain in osteoarthritis (34, 35).

Besides improvements in pain intensity according to VAS scores, objective measurements based on the PROMIS-29 and Cornell questionnaires showed overall better results in subjects treated with the investigational product. In the PROMIS-29, pain intensity decreased to a greater extent in both groups (low and high dose) of the investigational product compared to placebo. Although the reduction was slightly greater in the group that took the highest dose, no significant differences were observed in any of the visits when comparing the two doses. In the domain of physical function improvements, the results were also higher in the investigational product groups com-pared to the placebo group at the end of the study. This result was strengthened by the fact that clinically relevant changes in the Pain Disability Roland Morris Questionnaire were found in the groups treated with the investigational ingredient.

In the Cornell questionnaire, a significant improvement in pain relief in the cervical and upper back areas was observed after treatment with the investigational product compared to placebo, in particular in the high dose group. However, due to the limited number of subjects with shoulder pain, the results were not statistically significant, especially in the group that received a low dose of the investigational product. Thus, further studies are required with a larger sample size of individuals suffering from musculoskeletal shoulder pain to draw definitive conclusions.

Pain reduction might be accompanied by an improvement in QoL. In general, subjects assigned to the investigational product, especially those in the high dose group, showed significantly greater improvements from baseline to the end of the study in the domains of the SF-36 questionnaire of bodily function and health transition than those treated with placebo. There are numerous studies demonstrating that herb/botanical supplements may improve the quality of life in patients with different conditions (36). Previous studies have established connections between pain perception and emotional states (37). In addition, numerous studies have shown that patients with chronic musculoskeletal pain including lower back pain, osteoarthritis, rheumatoid arthritis, and fibromyalgia, tend to have a higher incidence of depression, anxiety, and sleep disturbance (38–40). This highlights the importance of a comprehensive approach to pain management.

The results of our study showed that although the investigational product did not exhibit superiority over the placebo in reducing depression symptoms, anxiety level and stress across the entire study population, a subgroup analysis revealed promising outcome. In a secondary analysis selecting subjects with minimal symptoms (those with scores of $\geq$4 for BDI-II, $\geq$ 14 for STAI-state, and $\geq$ 16 for PSS) subjects treated with the investigational product showed significant improvements in the levels of depression, anxiety, and perceived stress, which was particularly noticeable in the high dose group. Therefore, improvements seen after consumption of the investigational product in subjects with minor symptoms further support the beneficial effect of the product, especially at high doses. This aligns with previous studies emphasizing the bidirectional relationship between pain and emotional wellbeing. In addition to the reduction of pain facilitated by the investigational product, the improvements in emotional wellbeing observed in our study could be attributed to the presence in the investigational product of *Withania somnifera*, a plant with recognized adaptogenic properties. *Withania somnifera* has been used in traditional medicine to treat a variety of conditions, including anxiety and stress-related disorders (41).

On the other hand, the subgroup-specific improvements in depression, anxiety and stress levels highlight the importance of considering baseline conditions when assessing the efficacy of the investigational product and future studies should delve deeper into cohorts characterized by more pronounced emotional distress.

Additionally, there is evidence that pain and sleep quality are interconnected (42), and it is known that back pain increases the presence of sleep disturbances, which may trigger disability and depressive symptoms. At the end of the study, the high dose of the investigational product resulted in an improvement in the quality of sleep evaluated by the PSQI compared to the lower dose and the placebo groups. This was confirmed in the sleep quality assessment by actigraphy, particularly in the sleep latency domain. Herbal medicinal products are widely considered natural alternatives to common medication to help with sleep disorders and treatment of insomnia (43, 44). However, poor methodology of many studies restricts any clear conclusion. The present findings should be interpreted taking into account some limitations of the study, such as the reduced sample size and the fact that the dietary supplement was not administered beyond 12 weeks. However, the validity of the present findings is supported by the design of the study as a randomized double-blind placebo controlled trial and the number of different variables that have been evaluated using validated instruments to achieve the study objectives. Moreover, two daily

doses (400 and 800 mg) of the botanical extract supplement were evaluated to determine whether there were significant differences in efficacy. Two studies included in the systematic review of Oltean et al. (15) evaluated low and high doses of herbal products in patients with exacerbated episodes of back pain (45, 46). In a randomized double-blind controlled study of 197 subjects with exacerbations of chronic low back pain, an oral *Harpagophytum* extract containing 50 and 100 mg of the marker harpagoside was administered, and the benefits appeared to be greater than placebo in the two dose groups ($p = 0.027$) (45). In the second randomized controlled study of 210 patients with an exacerbation of back pain assigned to receive an oral willow bark extract with either 120 mg (low dose) or 240 mg (high dose) of salicin or placebo, the percentages of pain-free patients were 39% in the high-dose extract, 21% in the low-dose extract, and 6% in the placebo group ($p < 0.001$) (46). In the present study, the high dose of the investigational product appeared to have more pronounced effects than the lower dose, although without statistically significant differences, improvement of some domains of QoL, emotional wellbeing, and perceived sleep quality. Overall, the safety of the product and its effectiveness in reducing pain provide the basis for further studies of prolonged administration in a larger study population of subjects with musculoskeletal back pain.

5 Conclusion

Dietary supplementation for 12 weeks of a blend of polyphenolic standardized extracts of rosemary, ashwagandha, and sesame was effective in reducing the intensity of pain in subjects with chronic myofascial cervical and back pain. The effect of the investigational product was significantly higher compared to placebo at both dosages studied.

Furthermore, the positive effects of the investigational product on emotional wellbeing were demonstrated among subjects with mild manifestations, particularly with the high dose (800 mg daily). Beyond pain relief, the investigational product demonstrated benefits in enhancing the quality of life (QoL) and improving sleep quality among the study participants. The investigational product was safe and well tolerated, which justifies the design of future controlled clinical trials with a larger sample size and a more prolonged administration period.

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.

Ethics statement

The studies involving humans were approved by Comité de Ética de la Investigación de la UCAM. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SP-P: Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. JM-C: Investigation, Software, Validation, Writing – original draft, Writing – review & editing. JT: Investigation, Validation, Writing – original draft, Writing – review & editing. AL-R: Investigation, Writing – review & editing. JM: Investigation, Writing – review & editing. MM-C: Investigation, Writing – original draft, Writing – review & editing. MD: Investigation, Writing – review & editing. EV: Investigation, Writing – review & editing. VÁ-G: Investigation, Writing – review & editing. NC: Visualization, Writing – review & editing. PN: Visualization, Writing – review & editing. MC: Visualization, Writing – review & editing. FJL-R: Data curation, Formal Analysis, Methodology, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

NC, PN, MC, and FJL-R were employed by the Monteloeder S.L.

The remaining 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/fnut.2024.1403108/full#supplementary-material

References

1. Freburger JK, Holmes GM, Agans RP, Jackman AM, Darter JD, Wallace AS, et al. The rising prevalence of chronic low back pain. *Arch Intern Med*. (2009) 169:251–8. doi: 10.1001/archinternmed.2008.543

2. Carey TS, Evans A, Hadler N, Kalsbeek W, McLaughlin C, Fryer J. Care-seeking among individuals with chronic low back pain. *Spine*. (1976) 20:312–7. doi: 10.1097/00007632-199502000-00009

3. Fritz JM, Childs JD, Wainner RS, Flynn TW. Primary care referral of patients with low back pain to physical therapy: impact on future health care utilization and costs. *Spine*. (2012) 37:2114–21. doi: 10.1097/BRS.0b013e31825d32f5

4. Fatoye F, Gebrye T, Ryan CG, Useh U, Mbada C. Global and regional estimates of clinical and economic burden of low back pain in high-income countries: a systematic review and meta-analysis. *Front Public Health*. (2023) 11:1098100. doi: 10.3389/fpubh.2023.1098100

5. Wu A, March L, Zheng X, Huang J, Wang X, Zhao J, et al. Global low back pain prevalence and years lived with disability from 1990 to 2017: estimates from the global burden of disease study 2017. *Ann Transl Med*. (2020) 8:299. doi: 10.21037/atm.2020.02.175

6. de Souza IMB, Sakaguchi TF, Yuan SLK, Matsutani LA, do Espírito-Santo AS, CAB P, et al. Prevalence of low back pain in the elderly population: a systematic review. *Clinics*. (2019) 74:e789. doi: 10.6061/clinics/2019/e789

7. Wong CK, Mak RY, Kwok TS, Tsang JS, Leung MY, Funabashi M, et al. Prevalence, incidence, and factors associated with non-specific chronic low back pain in community-dwelling older adults aged 60 years and older: a systematic review and meta-analysis. *J Pain*. (2022) 23:509–34. doi: 10.1016/j.jpain.2021.07.012

8. World Health Organization. Ageing and health. Available at: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health (Accessed September 3, 2023).

9. Chou R, Shekelle P. Will this patient develop persistent disabling low back pain? *JAMA*. (2010) 303:1295–302. doi: 10.1001/jama.2010.344

10. Hashmi JA, Baliki MN, Huang L, Baria AT, Torbey S, Hermann KM, et al. Shape shifting pain: chronification of back pain shifts brain representation from nociceptive to emotional circuits. *Brain*. (2013) 136:2751–68. doi: 10.1093/brain/awt211

11. World Health Organization. Chronic low back pain. Available at: https://www.who.int/news-room/fact-sheets/detail/low-back-pain (Accessed September 4, 2023).

12. Wand BM, O'Connell NE. Chronic non-specific low back pain - sub-groups or a single mechanism? *BMC Musculoskelet Disord*. (2008) 9:11. doi: 10.1186/1471-2474-9-11

13. Chou R, Deyo R, Friedly J, Skelly A, Weimer M, Fu R, et al. Systemic pharmacologic therapies for low Back pain: a systematic review for an American College of Physicians Clinical Practice Guideline. *Ann Intern Med*. (2017) 166:480–92. doi: 10.7326/M16-2458

14. Chou R, Deyo R, Friedly J, Skelly A, Hashimoto R, Weimer M, et al. Nonpharmacologic therapies for low Back pain: a systematic review for an American College of Physicians Clinical Practice Guideline. *Ann Intern Med*. (2017) 166:493–505. doi: 10.7326/M16-2459

15. Oltean H, Robbins C, van Tulder MW, Berman BM, Bombardier C, Gagnier JJ. Herbal medicine for low-back pain. *Cochrane Database Syst Rev*. (2014) 2014:CD004504. doi: 10.1002/14651858.CD004504.pub4

16. Gagnier JJ. Evidence-informed management of chronic low back pain with herbal, vitamin, mineral, and homeopathic supplements. *Spine J*. (2008) 8:70–9. doi: 10.1016/j.spinee.2007.10.011

17. Askew RL, Cook KF, Revicki DA, Cella D, Amtmann D. Evidence from diverse clinical populations supported clinical validity of PROMIS pain interference and pain behavior. *J Clin Epidemiol*. (2016) 73:103–11. doi: 10.1016/j.jclinepi.2015.08.035

18. Cook KF, Jensen SE, Schalet BD, Beaumont JL, Amtmann D, Czajkowski S, et al. PROMIS measures of pain, fatigue, negative affect, physical function, and social function demonstrated clinical validity across a range of chronic conditions. *J Clin Epidemiol*. (2016) 73:89–102. doi: 10.1016/j.jclinepi.2015.08.038

19. Hedge A, Morimoto S, McCrobie D. Effects of keyboard tray geometry on upper body posture and comfort. *Ergonomics*. (1999) 42:1333–49. doi: 10.1080/001401399184983

20. Bombardier C, Hayden J, Beaton DE. Minimal clinically important difference. Low back pain: outcome measures. *J Rheumatol*. (2001) 28:431–8.

21. Kovacs FM, Llobera J, Gil Del Real MT, Abraira V, Gestoso M, Fernández C, et al. Validation of the Spanish version of the Roland-Morris questionnaire. *Spine*. (1976) 27:538–42. doi: 10.1097/00007632-200203010-00016

22. Alonso J, Prieto L, Antó JM. The Spanish version of the SF-36 health survey (the SF-36 health questionnaire): an instrument for measuring clinical results. *Med Clin*. (1995) 104:771–6.

23. Vilagut G, Ferrer M, Rajmil L, Rebollo P, Permanyer-Miralda G, Quintana JM, et al. The Spanish version of the short form 36 health survey: a decade of experience and new developments. *Gac Sanit*. (2005) 19:135–50. doi: 10.1157/13074369

24. Sanz J, Perdigón AL, Vázquez C. Adaptación española del Inventario para la Depresión de Beck-II (BDI-II). Propiedades psicométricas en la población general. *Ciencia Y Salud*. (2003) 14:249–80.

25. Buela-Casal G, Guillén-Riquelme A. Short form of the Spanish adaptation of the state-trait anxiety inventory. *Int J Clin Health Psycho*. (2017) 17:261–8. doi: 10.1016/j.ijchp.2017.07.003

26. Remor E, Carrobles JA. Spanish version of the perceived stress scale (PSS-14): psychometric study in a HIV+ sample. *Ansiedad Y Estrés*. (2001) 7:195–201.

27. Hita-Contreras F, Martínez-López E, Latorre-Román PA, Garrido F, Santos MA, Martínez-Amat A. Reliability and validity of the Spanish version of the Pittsburgh sleep quality index (PSQI) in patients with fibromyalgia. *Rheumatol Int*. (2014) 34:929–36. doi: 10.1007/s00296-014-2960-z

28. Ghasemzadeh Rahbardar M, Hosseinzadeh H. Therapeutic effects of rosemary (*Rosmarinus officinalis* L.) and its active constituents on nervous system disorders. *Iran J Basic Med Sci*. (2020) 23:1100–12. doi: 10.22038/ijbms.2020.45269.10541

29. Andrade JM, Faustino C, Garcia C, Ladeiras D, Reis CP, Rijo P. *Rosmarinus officinalis* L.: an update review of its phytochemistry and biological activity. *Future Sci OA*. (2018) 4:FSO283. doi: 10.4155/fsoa-2017-0124

30. Boadas-Vaello P, Vela JM, Verdu E. New pharmacological approaches using polyphenols on the physiopathology of neuropathic pain. *Curr Drug Targets*. (2017) 18:160–73. doi: 10.2174/1389450117666160527142423

31. Maione F, Cantone V, Pace S, Chini MG, Bisio A, Romussi G, et al. Anti-inflammatory and analgesic activity of carnosol and carnosic acid in vivo and in vitro and in silico analysis of their target interactions. *Br J Pharmacol*. (2017) 174:1497–508. doi: 10.1111/bph.13545

32. Ramakanth GS, Uday Kumar C, Kishan PV, Usharani P. A randomized, double blind placebo controlled study of efficacy and tolerability of Withaina somnifera extracts in knee joint pain. *J Ayurveda Integr Med*. (2016) 7:151–7. doi: 10.1016/j.jaim.2016.05.003

33. Mukherjee PK, Banerjee S, Biswas S, Das B, Kar A, Katiyar CK. Withania somnifera (L.) Dunal - Modern perspectives of an ancient Rasayana from Ayurveda. *J Ethnopharmacol*. (2021) 264:113157. doi: 10.1016/j.jep.2020.113157

34. Hsu DZ, Chu PY, Jou IM. Daily sesame oil supplement attenuates joint pain by inhibiting muscular oxidative stress in osteoarthritis rat model. *J Nutr Biochem*. (2016) 29:36–40. doi: 10.1016/j.jnutbio.2015.10.007

35. Askari A, Ravansalar SA, Naghizadeh MM, Mosavat SH, Khodadoost M, Jazani AM, et al. The efficacy of topical sesame oil in patients with knee osteoarthritis: a randomized double-blinded active-controlled non-inferiority clinical trial. *Complement Ther Med*. (2019) 47:102183. doi: 10.1016/j.ctim.2019.08.017

36. Wu X, Chung VCH, Lu P, Poon SK, Hui EP, Lau AYL, et al. Chinese herbal medicine for improving quality of life among nonsmall cell lung cancer patients: overview of systematic reviews and network meta-analysis. *Medicine*. (2016) 95:e2410. doi: 10.1097/MD.0000000000002410

37. Yao C, Zhang Y, Lu P, Xiao B, Sun P, Tao J, et al. Exploring the bidirectional relationship between pain and mental disorders: a comprehensive Mendelian randomization study. *J Headache Pain*. (2023) 24:82. doi: 10.1186/s10194-023-01612-2

38. Wong JJ, Tricco AC, Côté P, Liang CY, Lewis JA, Bouck Z, et al. Association between depressive symptoms or depression and health outcomes for low back pain: a systematic review and meta-analysis. *J Gen Intern Med*. (2022) 37:1233–46. doi: 10.1007/s11606-021-07079-8

39. Börsbo B, Peolsson M, Gerdle B. Catastrophizing, depression, and pain: correlation with and influence on quality of life and health - a study of chronic whiplash-associated disorders. *J Rehabil Med*. (2008) 40:562–9. doi: 10.2340/16501977-0207

40. Beyazal MS, Tüfekçi A, Kırbaş S, Topaloğlu MS. The impact of fibromyalgia on disability, anxiety, depression, sleep disturbance, and quality of life in patients with migraine. *Noro Psikiyatr Ars*. (2018) 55:140–5. doi: 10.5152/npa.2016.12691

41. Speers AB, Cabey KA, Soumyanath A, Wright KM. Effects of *Withania somnifera* (Ashwagandha) on stress and the stress- related neuropsychiatric disorders anxiety, depression, and insomnia. *Curr Neuropharmacol*. (2021) 19:1468–95. doi: 10.2174/1570159X19666210712151556

42. Andersen ML, Araujo P, Frange C, Tufik S. Sleep disturbance and pain: a tale of two common problems. *Chest*. (2018) 154:1249–59. doi: 10.1016/j.chest.2018.07.019

43. Hosseini A, Mobasheri L, Rakhshandeh H, Rahimi VB, Najafi Z, Askari VR. Edible herbal medicines as an alternative to common medication for sleep disorders: a review article. *Curr Neuropharmacol*. (2023) 22:1205–32. doi: 10.2174/1570159X21666230621143944

44. Yeung WF, Chung KF, Poon MM, Ho FY, Zhang SP, Zhang ZJ, et al. Chinese herbal medicine for insomnia: a systematic review of randomized controlled trials. *Sleep Med Rev*. (2012) 16:497–507. doi: 10.1016/j.smrv.2011.12.005

45. Chrubasik S, Junck H, Breitschwerdt H, Conradt C, Zappe H. Effectiveness of Harpagophytum extract WS 1531 in the treatment of exacerbation of low back pain: a randomized, placebo-controlled, double-blind study. *Eur J Anaesthesiol*. (1999) 16:118–29. doi: 10.1046/j.1365-2346.1999.00435.x

46. Chrubasik S, Eisenberg E, Balan E, Weinberger T, Luzzati R, Conradt C. Treatment of low back pain exacerbations with willow bark extract: a randomized double-blind study. *Am J Med*. (2000) 109:9–14. doi: 10.1016/S0002-9343(00)00442-3

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Network analysis combined with experimental assessment to explore the therapeutic mechanisms of New Shenqi Pills formula targeting mitochondria on senile diabetes mellitus

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Background: The escalation of global population aging has accentuated the prominence of senile diabetes mellitus (SDM) as a consequential public health concern. Oxidative stress and chronic inflammatory cascades prevalent in individuals with senile diabetes significantly amplify disease progression and complication rates. Traditional Chinese Medicine (TCM) emerges as a pivotal player in enhancing blood sugar homeostasis and retarding complication onset in the clinical management of senile diabetes. Nonetheless, an evident research gap persists regarding the integration of TCM's renal tonification pharmacological mechanisms with experimental validation within the realm of senile diabetes therapeutics.

Aims: The objective of this study was to investigate the mechanisms of action of New Shenqi Pills (SQP) in the treatment of SDM and make an experimental assessment.

Methods: Network analysis is used to evaluate target pathways related to SQP and SDM. Mitochondrial-related genes were obtained from the MitoCarta3.0 database and intersected with the common target genes of the disease and drugs, then constructing a protein-protein interaction (PPI) network making use of the GeneMANIA database. Representative compounds in the SQP were quantitatively measured using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to ensure quality control and quantitative analysis of the compounds. A type 2 diabetes mice (C57BL/6) model was used to investigate the pharmacodynamics of SQP. The glucose lowering efficacy of SQP was assessed through various metrics including body weight and fasting blood glucose (FBG). To elucidate the modulatory effects of SQP on pancreatic beta cell function, we measured oral glucose tolerance test (OGTT), insulin histochemical staining and tunel apoptosis detection, then assessed the insulin-mediated phosphoinositide 3-kinase (PI3K)/protein kinase A (Akt)/glycogen synthase kinase-3β (GSK-3β) pathway in diabetic mice via Western blotting. Additionally, we observe the structural changes of the nucleus, cytoplasmic granules and mitochondria of pancreatic islet β cells.

Results: In this investigation, we identified a total of 1876 genes associated with senile diabetes, 278 targets of SQP, and 166 overlapping target genes, primarily

enriched in pathways pertinent to oxidative stress response, peptide response, and oxygen level modulation. Moreover, an intersection analysis involving 1,136 human mitochondrial genes and comorbidity targets yielded 15 mitochondria-related therapeutic targets. Quality control assessments and quantitative analyses of SQP revealed the predominant presence of five compounds with elevated concentrations: Catalpol, Cinnamon Aldehyde, Rehmanthin D, Trigonelline, and Paeonol Phenol. Vivo experiments demonstrated notable findings. Relative to the control group, mice in the model group exhibited significant increases in body weight and fasting blood glucose levels, alongside decreased insulin secretion and heightened islet cell apoptosis. Moreover, β-cells nuclear condensation and mitochondrial cristae disappearance were observed, accompanied by reduced expression levels of p-GSK-3β protein in islet cells ($p < 0.05$ or $p < 0.01$). Conversely, treatment groups administered SQP and Rg displayed augmented expressions of the aforementioned protein markers ($p < 0.05$ or $p < 0.01$), alongside preserved mitochondrial cristae structure in islet β cells.

Conclusion: Our findings suggest that SQP can ameliorate diabetes by reducing islet cell apoptosis and resist oxidative stress. These insulin-mediated PI3K/AKT/GSK-3β pathway plays an important regulatory role in this process.

KEYWORDS

senile diabetes, New Shenqi Pills, network analysis, vivo experiments, oxidative stress, mitochondrial cristae structure

1 Introduction

Presently, there is a notable global demographic shift toward an aging population, with projections indicating that the proportion of elderly individuals worldwide will rise from the current 15%–25% by 2050. This demographic trend underscores the increasing significance of addressing senile diabetes mellitus (SDM) as a focal point in diabetes prevention and management strategies. Among the elderly, diabetes often manifests with insulin secretion deficits and a heightened susceptibility to hypoglycemia, alongside a spectrum of comorbidities affecting the cardiovascular, cerebrovascular, and renal systems, compounded by conditions such as hypertension and hyperlipidemia. Recent literature underscores the complexity of managing diabetes in older adults, highlighting the challenge of achieving therapeutic efficacy with monotherapeutic approaches. Consequently, there is a growing imperative for the implementation of comprehensive, multidisciplinary management strategies to mitigate disability and mortality rates in elderly diabetic populations. Consequently, there is burgeoning interest in exploring Traditional Chinese Medicine (TCM) formulations with multitarget therapeutic profiles and integrating TCM with conventional western medical modalities as promising avenues for addressing elderly diabetes.

One of the causes of SDM is considered to be spleen and kidney deficiency in TCM theory. The treatment strategy is often based on syndrome differentiation and treatment, focusing on strengthening the spleen and kidneys. Notable among the TCM preparations are Shenqi Pills, known for their kidney-tonifying and qi-tonifying effects. The formula of New Shenqi Pills (SQP), based on the traditional "Jingui Shenqi Pills", has evolved to include Codonopsis (Campanulaceae; Codonopsis pilosula), Astragalus (Faboideae; Astragalus membranaceus), Purslane (Portulacaceae; Portulaca oleracea), and Fenugreek (Fabaceae Papilioideae; Trigonella foenum-graecum L.). Clinical studies have shown that Shenqi Pills have good hypoglycemic effects in the treatment of diabetes. Pharmacological studies and related animal experimental studies have shown that Shenqi Pills can regulate glucose homeostasis and improve insulin resistance by intervening in inflammation-related pathways. The relevant mechanism may be related to affecting the expression of PI3K/AKT pathway. However, the mechanism of action of Shenqi Pills on SDM has not been clearly verified. Based on this, we modified Shenqi pills and investigated their mechanism of action in the treatment of SDM. Given the multifaceted nature of TCM components and targets, elucidating their potential therapeutic mechanisms remains a formidable challenge that needs to be explored through network analysis and experimental exploration.

Network analysis offers a systematic framework for elucidating drug-disease interactions through the delineation of compound-protein/gene-disease networks. By probing these networks, one can discern the modulatory effects of SQP on SDM, while concurrently forecasting the impacts of pharmacological constituents on pivotal targets and associated pathways. Moreover, the employment of high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) facilitates the precise quantification of molecular entities. This analytical approach is further reinforced by *in vivo* investigations employing murine models, which serve to validate the therapeutic efficacy of SQP via serological assays, Western blotting, and electron microscopy analyses (Figure 1). This integrative methodology furnishes substantive empirical insights into the therapeutic potential of SQP in SDM management, fostering the advancement of multi-target therapeutic strategies.

2 Materials and methods

2.1 Chemicals and reagents

The SQP granules were purchased from the Pharmacy Department of Guang'anmen Hospital (Beijing, China, May to

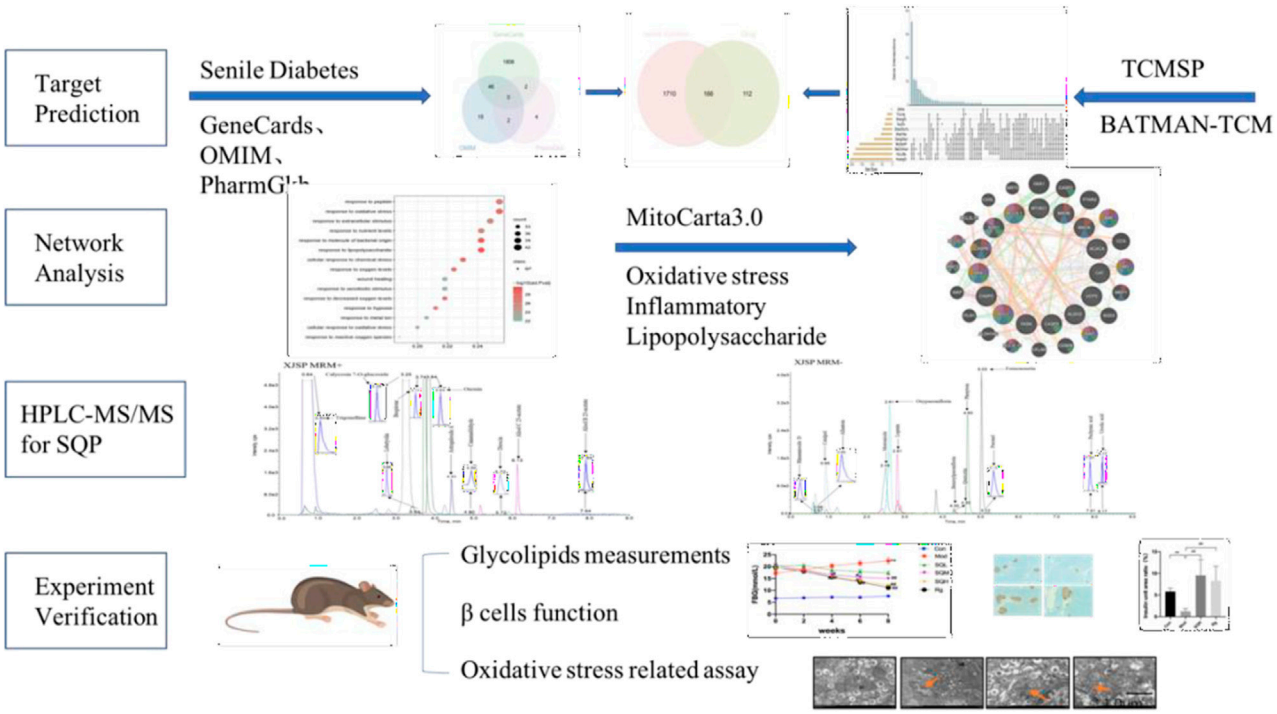

FIGURE 1
Flowchart of ideas for this study..

TABLE 1 SQP Dosage (dosage per dose of medication).

Botanical drug	Unit (g)	Botanical drug	Unit (g)
Rehmannia glutinosa	24	Peony bark	9
Rhizoma dioscoreae	12	Cinnamomi ramulus	3
Cornus officinalis	12	Codonopsis pilosula	9
Poria	9	Astragalus membranaceus	12
Alisma orientalis	9	Trigonella foenum-graecum	6
Purslane	9		

August 2023). Drug composition: *Rehmannia glutinosa* [Scrophulariaceae Rehmannia genus; *Radix Rehmanniae root tuber*], *Rhizoma dioscoreae* [Dioscoreaceae; *Dioscorea dried rhizome*], *Cornus officinalis* [Dogwood family; *Dogwood dry ripe pulp*], *Poria* [Polyporaceae; *Poria cocos dried sclerotia*], *Alisma orientalis* [Alismataceae; *Alisma dried tubers*], *Peony bark* [Ranunculaceae; *Peony dry root bark*], *Cinnamomi Ramulus* [Lauraceae; *Dried twigs of cinnamon*], *Codonopsis pilosula* [Platycodonaceae; *Dried root of Codonopsis pilosula*], *Astragalus membranaceus* [Leguminosae; *Dried roots of Astragalus membranaceus*], *Trigonella foenum-graecum* [Leguminosae; *Fenugreek dry mature seeds*], *Purslane* [Portulacaceae; *Dry aerial parts of purslane*] (Table 1). Rosiglitazone (Chengdu Hengrui Pharmaceutical Co. Ltd., China. H20030569). High-fat diet including 58 kcal% fat (mostly derived from saturated fatty acids), 25 kcal% protein, and 17 kcal% carbohydrates (D12492) was obtained from Research Diets (New Brunswick, NJ, United States of America). STZ (S0130; CAS Number: 18,883–66–4) was obtained from Sigma (Livonia, MI, United States of America); LY294002 (ab120243; CAS number: 154,447–36–6), a PI3K inhibitor that can suppress phosphorylated (p)Akt expression, GSK-3β(ab93926) were obtained from Abcam (Cambridge, United Kingdom). GAPDH (ab8245), PI3K (4257), pPI3K (4228S), AKT (4691), pAKT (4060S), and pGSK -3β(ser-9) (5558T) were obtained from CST (Boston, MA, United States of America); High glucose/palmitic acid (Glu/PA; KT002) was obtained from Kunchuang (Xi'an, China); an annexin V-FITC/Pro-pidium iodide (PI) detection kit (KGA108) was obtained from KeyGEN BioTECH (Beijing, China).

2.2 Access to SQP and SDM targets

Use "senile diabetes" as the keyword to search the GeneCards database (https://www.genecards.org/), set Score≥1, OMIM database (https://omim.org/), and PharmGkb database (https://www.pharmgkb.org/), remove duplicates and then take the union to obtain the disease genes of SDM.

Search the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, https://old.tcmsp-e.com/index.php) to collect SQP Chinese yam, dogwood, poria, Alisma, paeonol, cassia twig, astragalus, dangshen, For medicinal ingredients such as fenugreek and purslane, search the BATMAN-TCM database (http://bionet.ncpsb.org.cn/batman-tcm/) to collect the main chemical ingredients of raw materials that are not included in the TCMSP database and take them orally Bioavailability (OB) ≥

30% and drug-likeness (DL) ≥ 0.18 are the screening conditions. Combined with literature research, ingredients that do not meet the screening conditions but have clear pharmacological effects are included as candidates. Use the TCMSP database to search for verified component targets in the active ingredients obtained, import the obtained targets into the Uniprot database (https://www.uniprot.org/), limit the species to human, and correct the target gene name to its official name. The common target genes of SDM and SQP were obtained through R software.

2.3 Analysis of intersectional target pathways and functional enrichment

Use the R software "clusterProfiler" package, set the species to human origin (*Homo sapiens*), and conduct Biological Process (BP) Gene Ontology (GO) enrichment analysis on the common target genes of SDM and SQP. The corrected p-value < 0.05 was used as the filtering condition.

2.4 Access to mitochondria-related gene

Mitochondria-related genes were obtained through the MitoCarta3.0 database (https://www.broadinstitute.org/mitocarta/mitocarta30-inventory-mammalian-mitochondrial-proteins-and-pathways). The database performs mass spectrometry analysis of mitochondria isolated from 14 tissues, evaluates protein localization by large-scale GFP tagging/microscopy, and integrates the results using a Bayesian algorithm with six other genomic datasets of mitochondrial localization. Contains 1,136 human mitochondrial genes and 1,140 mice mitochondrial genes[7].

2.5 Construction of mitochondria related genes-disease target protein interaction network

The mitochondria-related genes were intersected with the comorbid disease targets of SDM and SQP to obtain mitochondria-related drug treatment targets. The GeneMANIA database (http://genemania.org/) was used to select the top five functions.

2.6 Animal experiment

2.6.1 Quality control of SQP using high-performance liquid chromatography/mass spectrometry (HPLC-MS/MS)

HPLC-MS/MS (Agilent, Santa Clara, CA, United States of America) was used to quantitatively determine the levels of representative compounds in SQP. Mass spectrometry measurements were performed using a Sciex API 4000 Qtrap MS system equipped with a Turbo Ionspray interface (Applied Biosystems, Foster City, CA, United States of America). The samples were analyzed in either positive or negative electrospray ionization mode and monitored in the multiple reactions monitoring mode. High-purity nitrogen was used as the curtain gas, ion source gas 1, and ion source gas 2, with ow rates of 30, 60, and 60 psi, respectively. The spray voltage was ±4.5 kV, and the capillary temperature was set to 600°C.

2.6.2 Experimental models and pharmacotherapy

Male C57BL/6 mice (n = 70)aged 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Company (certificate number SCXK 2019–0010, Beijing, China). They were randomly divided into a control (con) group (n = 10)and a high-fat diet (HFD) group (n = 60). All animals were housed in groups of four to six animals and maintained under a 12 h light–dark cycle at 20°C–24°C with free access to food and water. Mice in the HFD group were given a one-time intraperitoneal injection of 30 mg/kg STZ and blood was collected from the tail tip 72 h later to measure fasting blood glucose (FBG). We selected mice with FBG ≥16.7 mmol/L as successful SDM modeling mice, and divided them into six groups according to body weight using balanced random sampling: model, (Mod), shenqi low (SQL), shenqi middle (SQM), shenqi high (SQH), rosiglitazone (Rg) and SQH + PI3K inhibitor LY294002 (S + LY; 29.6 g/kg SQP+25 mg/kg LY294002 intraperitoneally, 20 min before SQP gavage) groups.

According to a mouse-to-human drug conversion ratio of 9:1, the dose (g/kg) of SQP for each mice was calculated as follows: 9 × the daily adult dose (114 g of crude drug)/the average adult weight (70 kg) = 14.8 g (crude drug)/kg. We designated this as the medium-dose group according to a previous study. The low-dose group was set at 7.4 g/kg, and the high-dose group was set at 29.6 g/kg. The modern drug group was given rosiglitazone 0.52 mg/kg/d by gavage, and the normal group was given an equal volume of normal saline by gavage, once a day for eight consecutive weeks (Figure 2).

2.6.3 Serum parameter measurements

FBG was measured at 0, 2, 4, 6, and 8 weeks after administration. At the end of 8 weeks, the levels of glycated albumin (GA), cholesterol (CHO), triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and free fatty acids (FFA) were measured.

2.6.4 β cells function

Oral Glucose Tolerance Test Mice in each group were fasted for 10 h at the end of 8 weeks after administration, and blood glucose values were measured again at 30, 60, and 120 min after glucose loading. Oral Glucose Tolerance Test (OGTT) curves were drawn based on the blood glucose values at each time point.

Insulin secretion assay The Fasting insulin (FINS) value of mice in each group at the end of 8 weeks was measured using an iodine [^{125}I] insulin radioimmunoassay kit.

Prepare paraffin-embedded sections, dewax and hydrate the sections, inactivate endogenous enzymes, and microwave antigen retrieval. DAB chromogenic solution and hematoxylin staining solution were added in sequence, and 1% hydrochloric acid alcohol differentiation solution was used. After dehydration, transparency, and sealing, the stained sections were placed under an automatic scanner for scanning and observation.

β cells tunel apoptosis detection The paraffin sections were dewaxed, and proteinase K working solution was added to

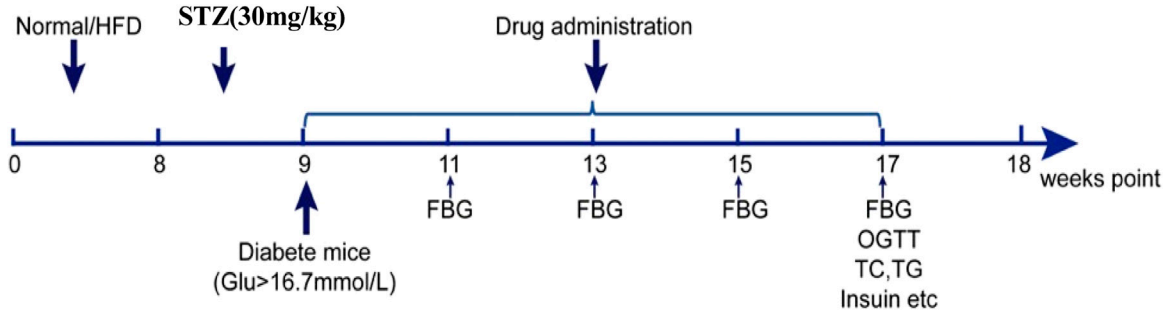

FIGURE 2
SDM mice modeling and medication timeline.

cover the tissue. After breaking the membrane, buffer was added to cover the tissue. After incubation at 37°C for 2 h, the nuclei were counterstained with DAPI. After sealing, the cells were observed under a fluorescence microscope and images were collected.

2.6.5 β cells oxidative stress related assay

Transmission electron microscopy Observe the structural changes of the nucleus, cytoplasmic granules and mitochondria of pancreatic islet β cells. After the pancreatic tissue is peeled off, cut the pancreatic tail tissue into a size of 1 mm^3 and fix it in 2.5% glutaraldehyde overnight. Change the PBS buffer the next day, fix it with osmic acid, perform gradient dehydration, infiltration and embedding, trimming, sectioning and staining, use transmission electron microscopy to observe mitochondrial structure and collect images.

Western blots Take the mice pancreatic tissue and quantitatively measure the protein concentration using the BCA method. After loading the sample for electrophoresis and electrotransfer, add the primary antibody and incubate at 4°C overnight. After washing the membrane, incubate the secondary antibody at room temperature for 120 min. After washing the membrane, use exposure solution to develop color. Imaging with a chemiluminescence imaging system, and using ImageJ software to count the gray value of the protein band and the corresponding gray value of the internal reference protein band. The gray value target band/gray value internal reference band is the relative content of the target protein contained in each sample. At least three samples were measured for each target gene.

2.7 Statistical analysis

SPSS 26.0 software was used for statistical analysis of the results, and GraphPad Prism eight software was used for graphing. The data obtained from the experiment are measurement data. Firstly, the normal distribution test is carried out. The independent sample *t*-test was used for comparison between two groups of data, and Levene's method was used to test the homogeneity of variances in the data. If the variances were homogeneous, one-way analysis of variance was used to compare data between multiple groups. $p < 0.05$ means the difference is statistically significant, $p < 0.01$ means the difference is statistically significant.

3 Results

3.1 Results of database analysis

3.1.1 Network analysis research shows that the target genes of SQP treatment of SDM related to oxidative stress response

SDM disease genes were obtained from the GeneCars database, OMIM database, and PharmGkb database, and 1854, 139, and 64 disease genes were obtained respectively. After merging the three, 1876 SDM disease genes were obtained by removing duplicates. Drug target genes were obtained through TCMSP and BATMAN-TCM databases, and a total of 278 SQP target genes were obtained. After obtaining the SDM and SQP target genes, they were intersected to obtain a total of 166 common target genes (Figures 3A–C).

In order to elucidate the various biological functions and mechanisms of SQP in alleviating SDM, GO enrichment analysis was performed on 166 common target genes, using $p < 0.05$ as the selection condition. The BP results showed that the key cross-target genes of SQP in treating SQM were mainly enriched. It focuses on related biological processes such as oxidative stress response, inflammatory response, and response to lipopolysaccharide (Figure 4).

3.1.2 Comorbid target genes are related to mitochondria combining with MitoCarta3.0 database

Combined with the results of GO enrichment analysis, we concluded that SQP may play a role in the treatment of SDM by improving the biological processes of oxidative stress and chronic inflammation. In order to furtherly verify the relevant biological molecular mechanisms, we obtained mitochondria-related genes through the MitoCarta3.0 database, and intersect with the comorbid targets of SDM and SQP, and use the GeneMANIA database to select the top five functions of the intersection genes, which are mitochondrial outer membrane, protein embedded in mitochondrial membrane, apoptosis signaling pathway, and positive regulation of apoptosis signaling pathways, regulation of mitochondrial membrane permeability, etc. The interacting targets were imported into the STRING database, and the network was further visualized and analyzed by Cytoscape 3.8.2 to construct a protein interaction map (Figure 5).

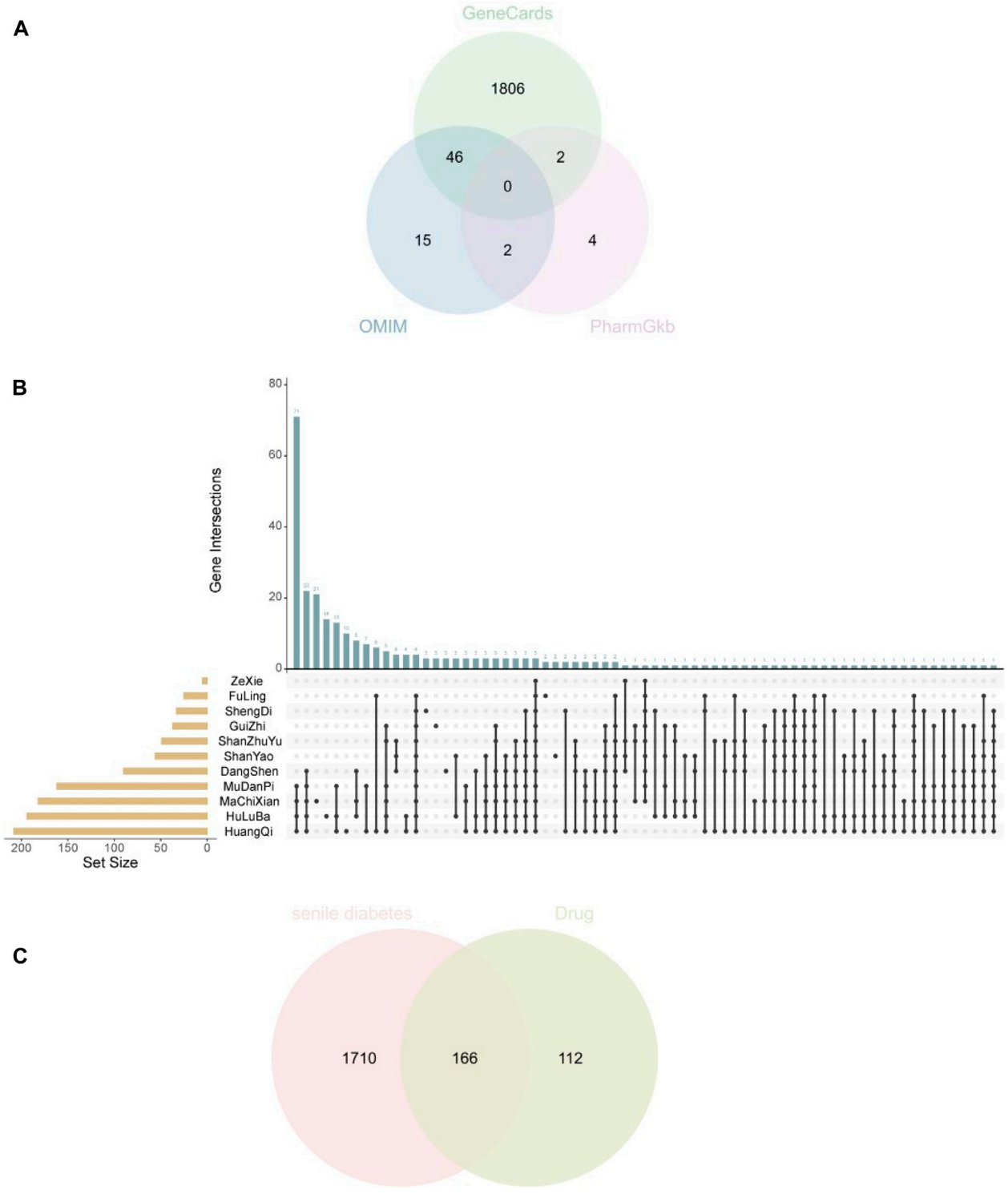

FIGURE 3
(A) Three databases obtain the disease genes of diabetes in the elderly **(B)** Drug target genes of Shenqi Pills **(C)** Common target genes of SDM and SQP.

3.2 Results of animal experiment

3.2.1 The main component of SQP has a protective effect on pancreatic β cells

HPLC-MS/MS analysis was performed to determine the 21 compounds in SQP: rhmannioside D, catalpol, loganin, morroniside, ursolic acid, cinnamaldehyde, trigonelline, lobetyolin, alisol B 23-acetate, alisol C 23-acetate, allantoin, dioscin, calycosin 7-O-glucoside, formononetin, ononin, benzoylpaeoniflorin, oxypaeoniflorin, paeonol, astragaloside A, quercetin and pachymic acid content in the SQP granules. The chromatographic pro les of SQP are shown in Figures 6A, B. Mass

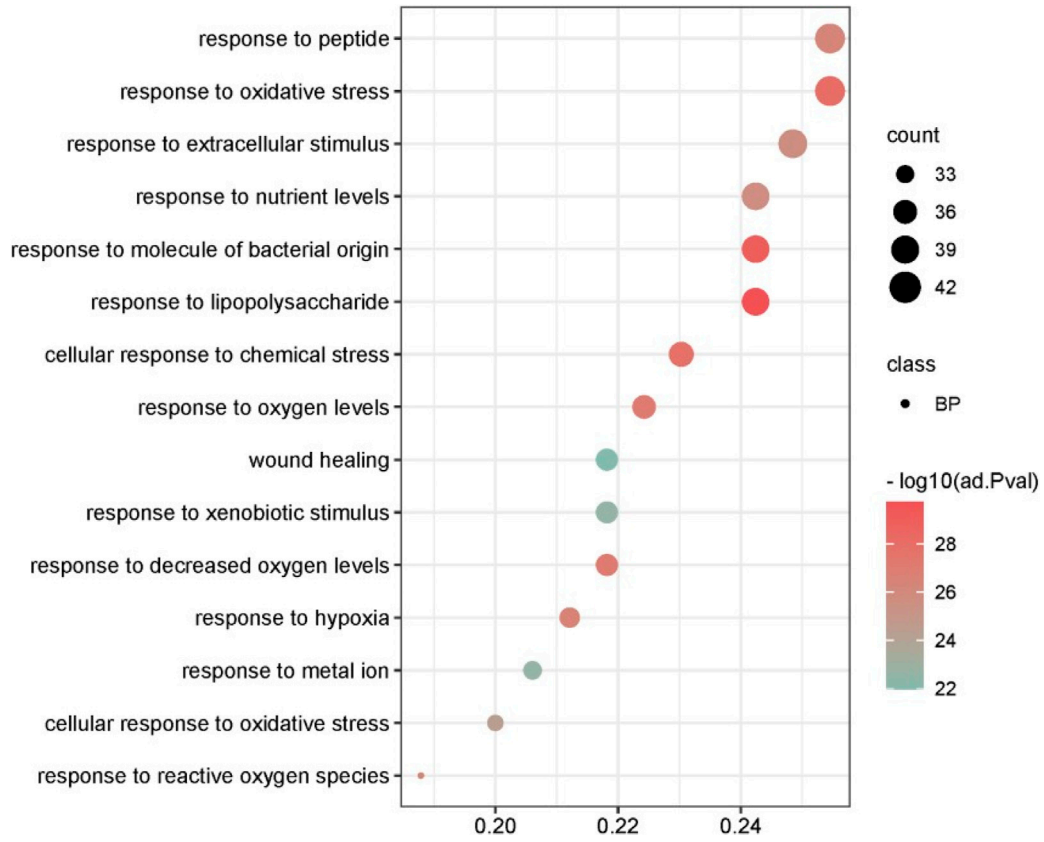

FIGURE 4
GO enrichment analysis of BP-related common target genes of SDM and SQP.

spectrometry analysis of SQP showed that the top five compounds with higher concentrations in SQP were: catalpol, cinnamic aldehyde, digoxin D, trigonelline and paeonol, followed by iridoid glycosides (Table 2).

3.2.2 SQP reduced body weight, and improved glucose homeostasis in diabetic mice

Weight after the model is stable and before formal intervention, the fasting body weight is measured and set as 0w body weight. After 8 weeks of drug intervention, the body weight of mice in the Mod, SQL, and SQM increased ($p < 0.05$), with the Mod increasing most significantly ($p < 0.01$). At the end of 8 weeks, the body weight of mice in the drug group was compared with the Mod. There is a statistical difference ($p < 0.05$), among which SQH has the lowest weight ($p < 0.01$) (Table 3; Figure 7).

FBG Compared with the Con, the FBG levels of mice in the Mod were increased from 0W to 8W ($p < 0.01$). Compared with the Mod, there was no significant difference in the FBG levels of mice in each drug intervention group before administration ($p > 0.05$). Starting from 4 weeks after administration, FBG in the SQM and SQH decreased compared with the Mod ($p < 0.05$). After 8 weeks of medication, FBG in the SQM and SQH was significantly lower than the Mod ($p < 0.01$) (Table 4; Figure 8).

3.2.3 SQP improves islet cell function

Compared to Con, the blood glucose levels in Mod were significantly elevated at all time points ranging from 0 to 120 min during OGTT ($p < 0.01$). Notably, the most pronounced increase in blood glucose levels in the Mod group occurred at 30 min post-glucose administration ($p < 0.05$). Subsequently, at the 60-min mark post-administration, a declining trend in blood glucose levels was observed across all drug intervention groups. Specifically, statistically significant differences were noted between SQM, SQH, and Mod ($p < 0.01$). Furthermore, after 120 min of glucose administration, the blood sugar levels of SQM, SQH, and Rg, statistically significant differences were observed (Table 5, $p < 0.05$).

FINS Compared with Con, the FINS level in the Mod was significantly lower ($p < 0.01$). Compared with Mod, the FINS levels of SQM and SQH increased ($p < 0.05$), and there was no statistical difference between SQL and Mod ($p > 0.05$) (Table 6; Figure 9A, B).

Insulin immunohistochemical staining Con, Mod, SQH (according to the results of mice fasting insulin levels) and Rg mice pancreatic tissue were selected for insulin immunohistochemical staining. The results showed that under high magnification, Con insulin secretion granules were of equal size, and Mod was smaller. Insulin secretory granules decreased compared with Con ($p < 0.01$). SQH insulin secretion granules became larger, although the sizes varied, but the proportion of insulin volume per unit area was larger than that of the Mod ($p < 0.01$). The size of Rg insulin secretion granules was unequal, and it was also larger than that of Mod ($p < 0.01$). Apply ImageJ software to analyze statistical results (Figures 10A, B).

Pancreatic β-cells apoptosis The results of tunel apoptosis of pancreatic islet β-cells showed that compared with Con, the

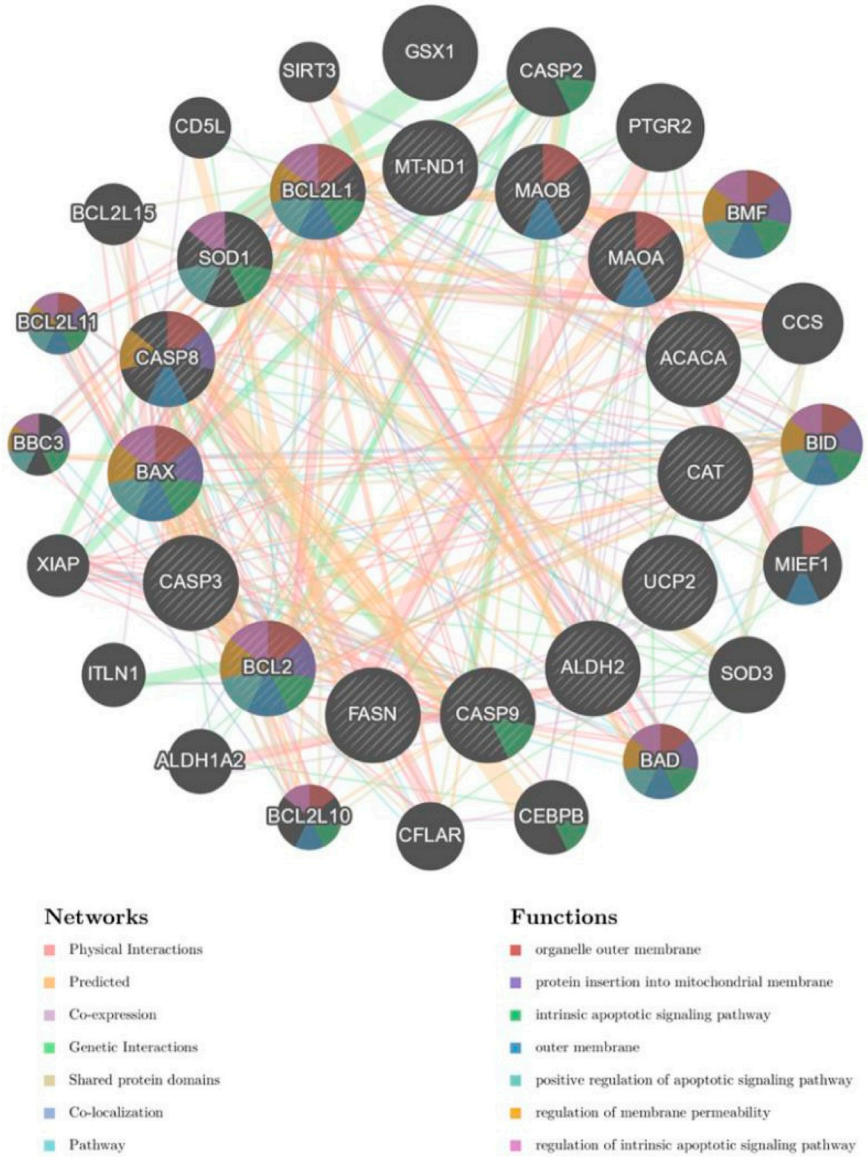

FIGURE 5
Mitochondria-related drug therapeutic target functions. (Nodes in the network diagram represent potential target genes, and connections between nodes refer to interactions between proteins. The node's size and color will be scaled using the "degree" value.

apoptosis rate of islet β-cells in Mod was significantly increased ($p < 0.01$). Compared with Mod, the apoptosis rates of pancreatic islet β cells in SQH and Rg were significantly reduced ($p < 0.01$). Apply ImageJ software to analyze statistical results (Figures 11A, B).

3.2.4 SQP improves lipid metabolism, protects mitochondrial morphology, and reduces oxidative stress levels

GA Compared with the Con, the GA level in the Mod increased ($p < 0.05$); compared with the Mod, the GA level in the SQM, SQH and Rg decreased ($p < 0.05$ or $p < 0.01$). There was no statistically significant difference in GA between medication intervention groups ($p > 0.05$). Four items of blood lipids Compared with the Con, the levels of TG and LDL in the Mod increased ($p < 0.01$). Compared with the Mod, the levels of TG and LDL in each dose group of SQP and the Rg were reduced ($p < 0.05$ or $p < 0.01$). There was no statistically significant difference between the CHO and HDL medication intervention groups and the Mod ($p > 0.05$)。FFA Compared with the Con, the FFA level in the Mod increased ($p < 0.01$). Compared with the Mod, the FFA levels in each dose group of SQP and the Rg were reduced ($p < 0.05$ or $p < 0.01$), and the GA differences between each medication intervention group were no statistical significance ($p > 0.05$) (Table7; Figures 12A–F).

The transmission electron microscopy results of the ultrastructural observation of the mitochondria of pancreatic islet β cells showed that the nucleus of Con islet β cells was large and round or oval in shape. There were a large number of secretory granules in the cytoplasm, most of which were spherical, with

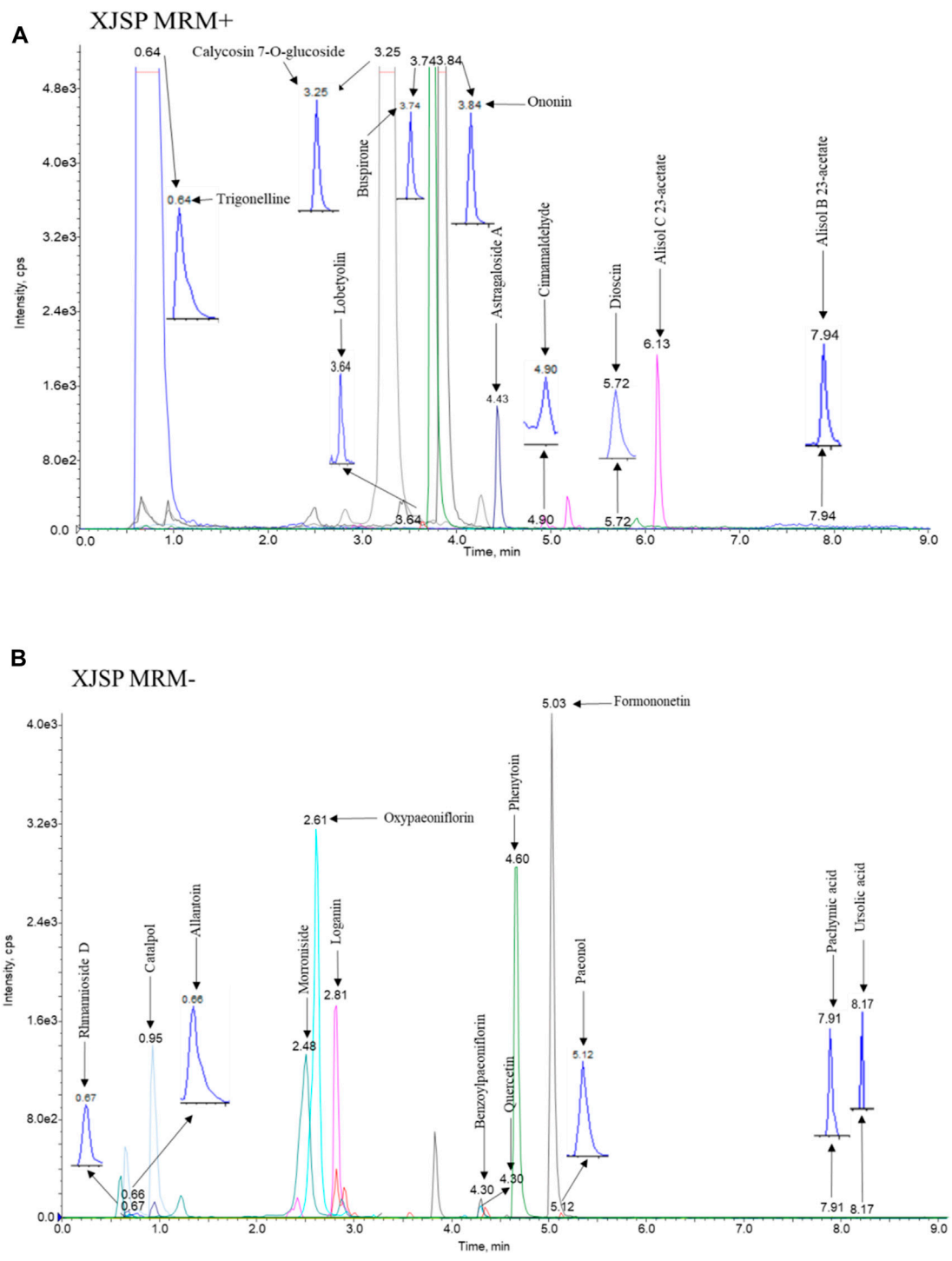

FIGURE 6
Representative chromatograms of 21 compounds in SQP. **(A)** Positive ion mode chromatogram **(B)** Negative ion mode chromatogram.

membranes on the outside and inside the granules. Cores with different electron densities are mostly round, with a large gap between the membrane and the core, bright and clear. Mitochondria have regular shapes and clear structures. Compared with Con, the Mod cells nucleus shrunk and showed an irregular shape, the number of cytoplasmic granules was reduced and unevenly distributed, the inner core shape of the granules was irregular, and the granules were not clear. The mitochondria were shrunken, the mitochondrial cristae were broken, and they gathered toward the base. Compared with Mod, the nuclei of SQH and Rg cells are enlarged, the nuclei are round, the number of cytoplasmic granules is increased, and the shape of the inner core of the granules is slightly regular. The mitochondria are regular in shape, and mitochondrial cristae are present (Figures 13A–C).

TABLE 2 Actual measured concentrations of 21 compounds in SQP.

Compounds	Concentrations (μg/g)			
	SQP 1–1	SQP 1–2	SQP 2–1	SQP 2–2
Rhmannioside D	490	458	361	449
Catalpol	5100	4730	4200	3990
loganin	308	292	323	264
Monogside	291	289	293	283
Ursolic acid	1.83	2.30	0.656	0.930
Cinnamaldehyde	615	741	864	964
Trigonelline	266	280	489	508
Lobetyolin	32.1	31.5	37.7	35.9
Alisol B 23-acetate	35.0	31.6	52.2	64.3
Alisol C 23-acetate	5.83	7.04	10.9	13.9
Allantoin	201	196	413	313
Dioscin	24.8	17.2	17.7	16.0
Calycosin 7-*O*-glucoside	52.3	55.4	61.0	64.5
Formononetin	10.6	9.95	18.9	18.4
Ononin	50.3	59.1	107	122
Benzoylpaeoniflorin	330	320	267	253
Oxypaeoniflorin	254	240	189	189
Paeonol	308	366	328	252
Astragaloside A	117	141	133	158
Quercetin	3.29	2.36	3.12	2.19
Poria acid	17.6	16.8	4.48	6.47

TABLE 3 Body weight in each group at different time points ($\bar{x} \pm s, g, week$).

Group	0w	2w	4w	6w	8w
Con	26.1 ± 0.6	27.1 ± 0.6	28.4 ± 0.6	29.2 ± 0.8	29.3 ± 1.4
Mod	25.2 ± 1.5	25.5 ± 1.3	26.1 ± 1.5	28.3 ± 1.7	31.7 ± 1.4*
SQL	24.2 ± 0.9	25.0 ± 0.4	25.2 ± 0.7	26.2 ± 0.9	27.7 ± 1.0$^{\#}$
SQM	24.3 ± 1.1	25.0 ± 1.1	25.5 ± 1.1	27.2 ± 1.0	28.3 ± 0.9$^{\#}$
SQH	24.1 ± 0.9	24.5 ± 0.5	25.5 ± 0.7	26.1 ± 0.7$^{\#}$	26.4 ± 0.7$^{\#\#}$
Rg	25.0 ± 0.8	25.3 ± 0.7	25.5 ± 0.6	26.3 ± 0.4$^{\#}$	27.1 ± 0.4$^{\#}$

Compared to Con.
*$p < 0.05$,**$p < 0.01$; Compared to Mod.
$^{\#}p < 0.05$;$^{\#\#}p < 0.01$.

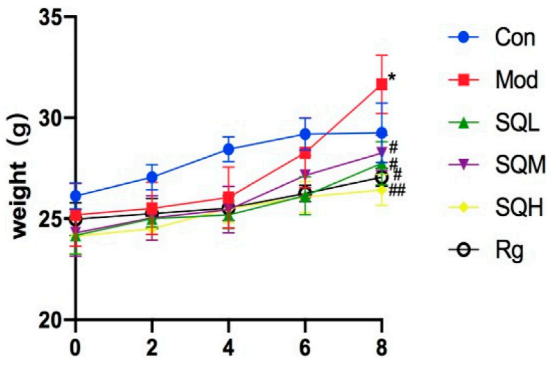

FIGURE 7
Body weight of mice in each group at different time points. (Compared with Con, *$p < 0.05$, **$p < 0.01$; Compared with Mod, $^{\#}p < 0.05$; $^{\#\#}P<0.01$).

The expression of PI3K-Akt-GSK-3β signaling axis protein WB results show that compared with Con, the protein expression of PI3K/p-PI3K, Akt/p-Akt and p-GSK-3β in Mod decreased ($p < 0.05$ or $p < 0.01$), and the expression of GSK-3β increased ($p < 0.01$). Compared with Mod, the protein expressions of PI3K/p-PI3K, Akt/p-Akt and p-GSK-3β were increased in SQM, SQH and Rg ($p < 0.05$ or $p < 0.01$). Compared with SQH, the PI3K blocker group (LY294002, hereinafter referred to as the LY group) had reduced protein expressions of PI3K/p-PI3K, Akt/p-Akt and p-GSK-3β ($p < 0.05$ or $p < 0.01$), and increased GSK- 3β expression ($p < 0.05$) (Figures 14A–C).

TABLE 4 FBG in each group at different time points ($\bar{x} \pm s$, *week*).

Group	0w	2w	4w	6w	8w
Con	6.7 ± 0.4	6.9 ± 0.4	7.2 ± 0.3	7.1 ± 0.2	7.6 ± 0.3
Mod	17.3 ± 0.7**	18.6 ± 0.8**	20.3 ± 0.9**	21.4 ± 1.1**	22.4 ± 1.3**
SQL	20.2 ± 1.1**	20.4 ± 0.7**	18.6 ± 0.9**	17.9 ± 0.8**	17.3 ± 0.7**
SQM	20.5 ± 0.7**	18.6 ± 1.0**	16.5 ± 0.7**#	15.5 ± 0.8**#	15.0 ± 0.6**##
SQH	19.5 ± 0.7**	18.6 ± 0.5**	15.9 ± 0.8**#	14.0 ± 0.8**#	12.2 ± 0.5**##
Rg	19.6 ± 0.9**	18.0 ± 0.6**	15.5 ± 0.6**#	13.6 ± 0.9**#	11.2 ± 0.9**##

Compared to Con.
$p < 0.05$,**$p < 0.01$; Compared to Mod.
$p < 0.05$;##$p < 0.01$.

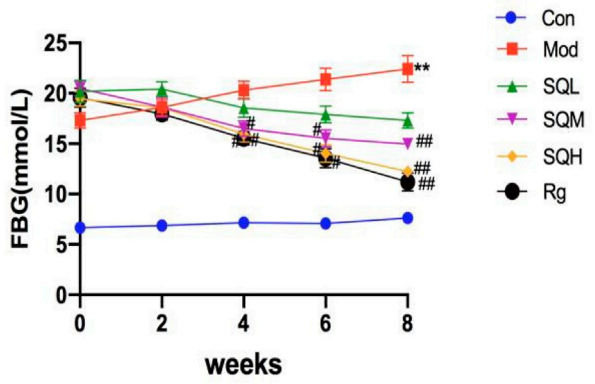

FIGURE 8
FBG of mice in each group at different time points.

TABLE 5 Effect of SQP on glucose tolerance ($\bar{x} \pm s$).

Group	Glucose (mmol/L)			
	0min	30min	60min	120min
Con	7.6 ± 0.4	11.8 ± 0.8	9.6 ± 0.8	6.3 ± 0.5
Mod	22.9 ± 1.3**	30.0 ± 1.2**	23.8 ± 3.3**	20.2 ± 1.4**
SQL	17.6 ± 0.7*#	23.1 ± 2.1*#	18.7 ± 3.2*	17.7 ± 5.5*
SQM	14.9 ± 0.6*#	17.8 ± 0.8*##	13.2 ± 1.6#	10.5 ± 1.1#
SQH	11.9 ± 0.5*#	15.7 ± 0.2##	12.6 ± 1.2#	9.5 ± 0.2#
Rg	11.4 ± 1.0*#	15.8 ± 1.1##	12.1 ± 0.9#	10.0 ± 0.5#

Compared to Con.
$p < 0.05$,**$p < 0.01$; Compared to Mod.
$p < 0.05$;##$p < 0.01$.

TABLE 6 Effects of SQP on FINS, HOMA-β, and ISI ($\bar{x} \pm s$).

Group	FINS (μIU/mL)	HOMA-β
Con	43.25 ± 9.22	208.64 ± 40.43
Mod	22.87 ± 5.19**	23.39 ± 4.05**
SQL	22.12 ± 6.25	31.45 ± 9.42
SQM	29.72 ± 1.65#	52.14 ± 4.25#
SQH	34.43 ± 6.34#	82.19 ± 17.49#
Rg	34.15 ± 4.64#	87.57 ± 12.59#

Compared to Con.
$p < 0.05$,**$p < 0.01$; Compared to Mod.
$p < 0.05$.

4 Discussion

Given the multifaceted complications associated with Subclinical Diabetic Mellitus (SDM), including atypical symptoms, a high risk of hypoglycemia, and suboptimal patient self-management, standardized management of SDM constitutes a crucial component of clinical intervention (Lin et al., 2016; Yu et al., 2020; Yu et al., 2022). Particularly noteworthy is the frequent coexistence of SDM with various chronic conditions, underscoring the importance of actively pursuing comprehensive diagnostic and therapeutic measures.

As a component of alternative medicine, Chinese herbal medicine has been widely clinically accepted because of its safety and multi-target effects (Xu et al., 2015; Wang et al., 2018). According to the principles of traditional Chinese medicine, SDM is mainly characterized by deficiency, among which the "pi" and "shen" are the key organs (Chen and He, 2020). Shenqi pills have always been used as a basic prescription to nourish the "shen" and replenish "qi". An improved version of Shenqi Pills (SQP) omits the pungent aconite to reduce potential damage to body fluids and enhance "pi" function. Noteworthy ingredients include codonopsis pilosula and astragalus membranaceus to nourish qi, trigonella foenum-graecum to nourish the "shen" and warm "yang qi", purslane to strengthen the "pi" and remove dampness. Clinical studies have confirmed that Shenqi Pills can improve the symptoms of polydipsia and polyuria and maintain blood sugar stability in SDM patients. However, its precise mechanism of action remains elusive. Therefore, this study is dedicated to identifying target genes related to SQP and SDM through gene database exploration, and conducting GO enrichment analysis through network review to elucidate its putative mechanism.

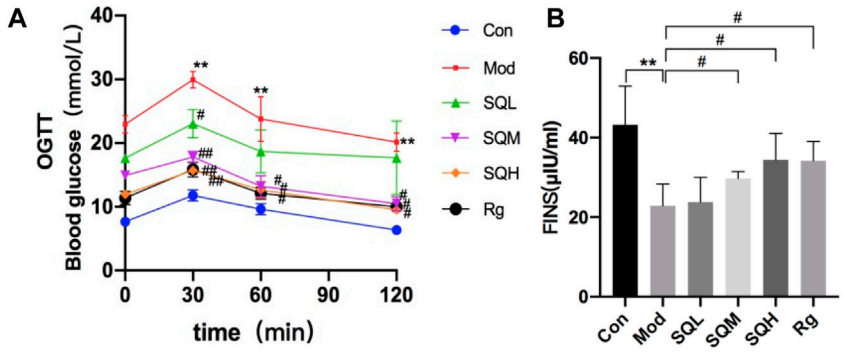

FIGURE 9
Effect of SQP on OGTT and FINS in mice. **(A)** OGTT in mice **(B)** FINS in mice. (Compared with Con, $^{**}p < 0.01$; Compared with Mod, $^{\#}p < 0.05$).

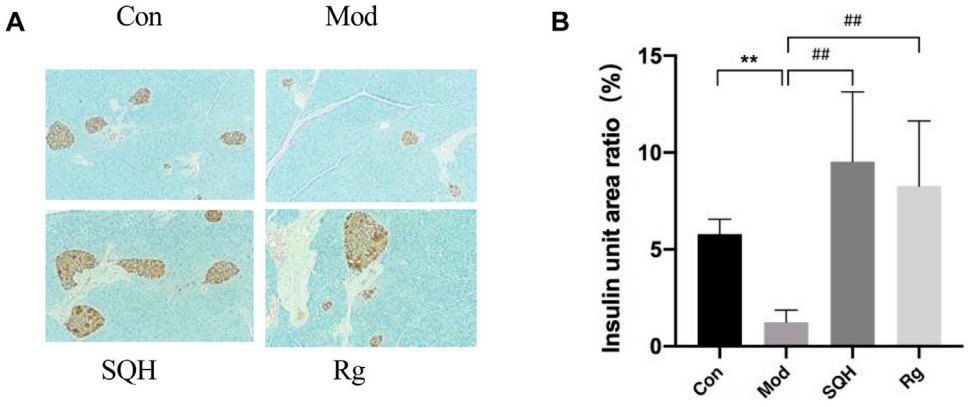

FIGURE 10
Effects of insulin secretion levels in pancreatic tissue of mice (400×) **(A)** Histochemical staining results of insulin in pancreatic tissue of mice under high magnification **(B)** Statistics of insulin levels per unit area of pancreatic tissue in mice. (Compared with Con, $^{**}p < 0.01$; Compared with Mod, $^{\#\#}p < 0.01$) A.

Furthermore, we designed in experiments to confirm the feasibility of the theoretical framework.

Mass spectrometry analysis of the SQP formulation revealed Catalpol as the main component, belonging to the iridoid glycosides class. Iridoid glycosides are known to enhance lipid metabolism, alleviate insulin resistance, and stimulate insulin secretion, thereby exerting a hypoglycemic effect (Bhattamisra et al., 2019; Bhattamisra et al., 2021). This pharmacological insight furnishes experimental validation for subsequent SQP investigations. In this investigation, a comprehensive screening process involving various databases and subsequent Venn analysis identified 166 potential targets of SQP pertinent to SDM treatment. Visualization of these findings was facilitated through PPI diagrams. To illuminate the multifaceted mechanisms underlying SQP's efficacy in SDM management, we conducted GO enrichment analysis on intersecting target genes. The outcomes revealed a predominant enrichment of target genes in key biological processes, notably oxidative stress response, inflammatory response, and lipopolysaccharide responsiveness. Oxidative stress stands as a significant risk factor in SDM pathology, capable of activating multiple pathways leading to cellular damage. Notably, the excessive generation of mitochondrial superoxide anions induced by elevated sugar and fat levels serves as a primary trigger for cellular injury (Andreas et al., 2019; Han et al., 2021; Pruett et al., 2022). The pivotal role of oxidative stress-induced mitochondrial dysfunction in SDM pathogenesis has garnered widespread attention (Yin and Ding, 2013; Andrieux et al., 2021; Sulkshane et al., 2021). Given mitochondria's central role in cellular oxidative stress, we identified mitochondria-related genes via the MitoCarta3.0 database. Subsequently, we intersected these genes with the co-regulated targets of SDM and SQP, leveraging the GeneMANIA database to discern the top five functions of the resultant intersection genes: mitochondrial outer membrane and embedded mitochondrial membrane proteins, apoptosis signaling pathway involvement, positive regulation of apoptosis signaling pathway, and regulation of mitochondrial membrane permeability. These findings suggest that SQP's therapeutic action in SDM may encompass enhancement of biological processes such as oxidative stress

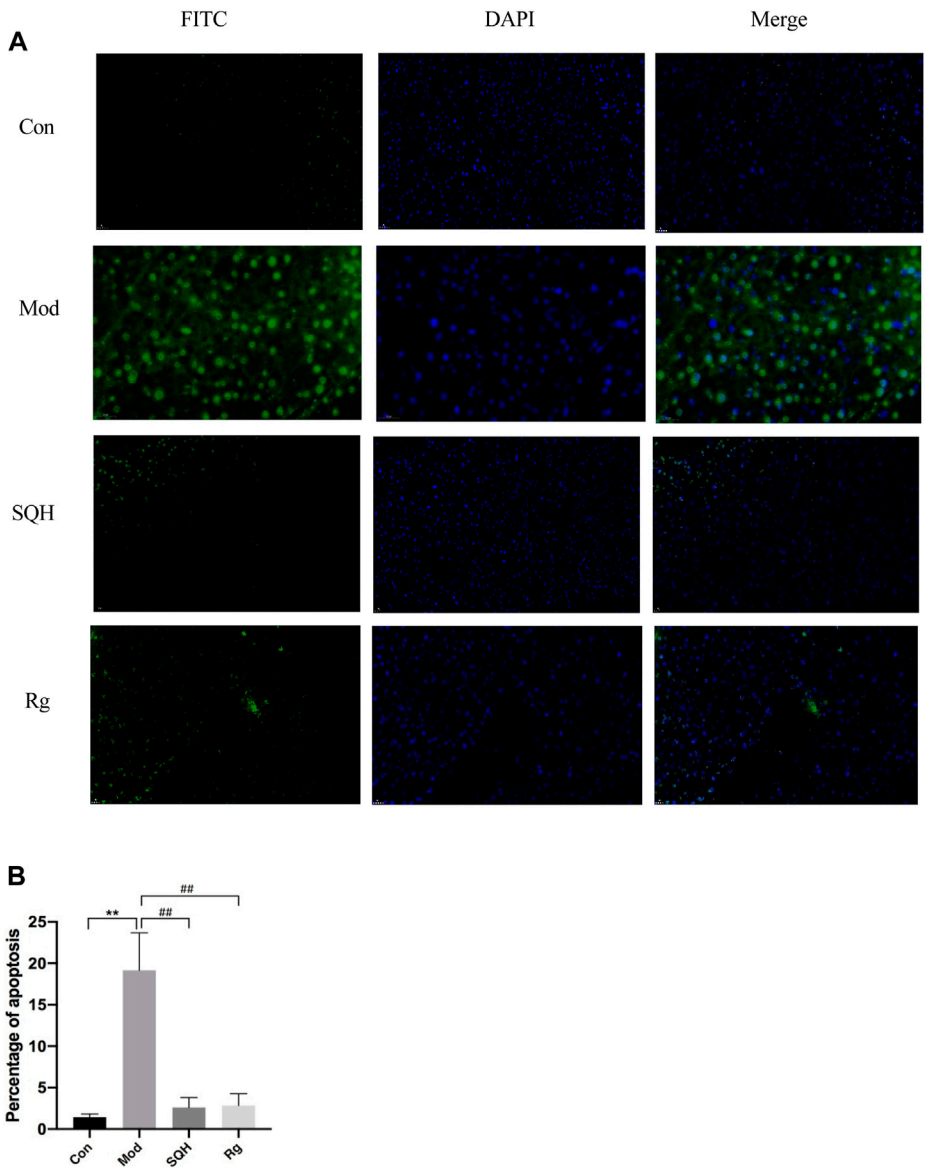

FIGURE 11
Apoptosis rate of pancreatic β cells in mice (400×) **(A)** Staining results of pancreatic β-cells in mice under a fluorescence microscope **(B)** Statistics of apoptosis of pancreatic β-cells in mice. (Compared with Con, $^{**}p < 0.01$; Compared with Mod, $^{\#\#}p < 0.01$).

TABLE 7 GA, four blood lipids, and free fatty acid levels in each group ($\bar{x} \pm s$, *mmol/L*).

Group	GA (nmol/L)	CHO	TG	LDL-C	HDL-C	FFA
Con	86.06 ± 12.73	2.93 ± 0.27	1.91 ± 0.38	0.29 ± 0.02	2.40 ± 0.25	1.53 ± 0.31
Mod	111.31 ± 6.21*	5.94 ± 0.79**	4.25 ± 1.01**	0.51 ± 0.06*	4.43 ± 0.69**	2.49 ± 0.53**
SQL	91.84 ± 7.91	5.70 ± 0.95	2.82 ± 1.66$^{\#}$	0.38 ± 0.10$^{\#}$	4.34 ± 0.41	1.86 ± 0.58$^{\#}$
SQM	88.18 ± 5.87$^{\#}$	5.61 ± 0.98	3.13 ± 0.54$^{\#}$	0.37 ± 0.05$^{\#}$	4.38 ± 0.71	1.83 ± 0.17$^{\#}$
SQH	65.79 ± 9.16$^{\#\#}$	5.35 ± 0.54	1.63 ± 0.31$^{\#\#}$	0.36 ± 0.04$^{\#}$	4.19 ± 0.50	1.61 ± 0.21$^{\#\#}$
Rg	88.23 ± 20.52$^{\#}$	5.68 ± 0.77	2.94 ± 0.62$^{\#}$	0.34 ± 0.06$^{\#}$	4.16 ± 0.43	1.84 ± 0.25$^{\#}$

Compared to Con.
$^{*}p < 0.05$,$^{**}p < 0.01$; Compared to Mod.
$^{\#}p < 0.05$.

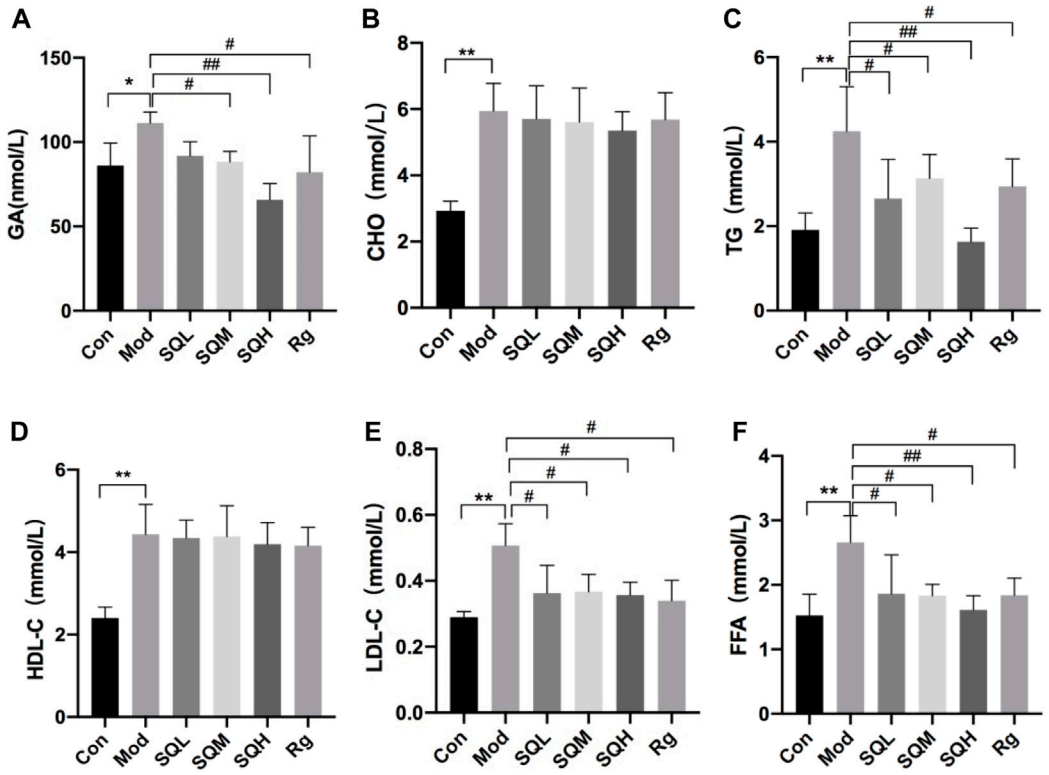

FIGURE 12
GA, CHO, TG, HDL-C, LDL-C and FFA levels of mice in each group **(A)** GA in mice; **(B–E)** Four blood lipids in mice; **(F)** FFA in mice. (Compared with Con, $^{*}p < 0.05$, $^{**}p < 0.01$; Compared with Mod, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$).

and inflammatory response, with pertinent mechanisms potentially involving alterations in mitochondrial morphological structure and the expression of apoptosis pathway proteins.

To further corroborate our preceding findings, we devised *in vivo* experiments aimed at elucidating the impact of oxidative stress on glucose and lipid metabolism dysregulation in mice, alongside associated pathways. Employing a combination of high-sugar and high-fat diet alongside streptozotocin (STZ) induction, we established an SDM model in C57BL/6 mice. Given recent research correlating cellular aging with metabolic reprogramming pathways encompassing autophagy, oxidative stress, epigenetic modifications, and chronic inflammation, we procured 6-week-old mice to undergo a transition from adaptive feeding to a high-sugar and high-fat regimen spanning 9 weeks (Finkel and Oxidants, 2000; Klionsky et al., 2021; Li et al., 2023; Maldonado et al., 2023). Subsequently, SQP intervention was administered for 8 weeks, culminating in mice aged 23 weeks at the study's conclusion. The utilization of a high-sugar and high-fat diet aimed to foster an internal milieu conducive to oxidative stress and chronic inflammation, thus inducing an SDM mouse model (Ferrucci and Fabbri, 2018). Post-intervention assessments revealed a controlled weight gain trajectory in mice receiving SQP. Serum analyses indicated reduced fasting blood sugar and serum albumin levels in the TCM group compared to the control, indicative of SQP's potential to ameliorate weight gain induced by the high-sugar and high-fat diet while mitigating its impact on blood sugar levels (Li et al., 2019; Lytrivi et al., 2020a; Geidl-Flueck et al., 2021). Chronic inflammatory responses are known to precipitate dyslipidemia and elevated FFA levels, complicating glucose metabolism and insulin resistance. Notably, SQP intervention led to significant reductions in serum TG, LDL-C, and FFA levels, suggesting a potential role in attenuating insulin resistance by dampening inflammatory responses. Moreover, the high-sugar and high-fat diet-induced oxidative stress milieu impacts pancreatic β-cells apoptosis. OGTT and fasting insulin FINS measurements post-SQP intervention revealed improved pancreatic islet function in SDM mice. Immunohistochemical analysis of insulin secretion demonstrated enhanced pancreatic β-cells functionality following SQP administration. Additionally, tunel staining highlighted a reduction in apoptotic β cells under high-sugar and high-fat conditions, further underscoring SQP's potential to mitigate β-cells apoptosis in such environments.

Regarding the verification of the mechanism of inflammation and oxidative stress on β-cells apoptosis, we used electron microscopy to observe the ultrastructure of β-cells (Lytrivi et al., 2020b). The results showed that SQP can protect the integrity of β-cells mitochondrial structure and morphology, and protect the existence of mitochondrial cristae morphology, confirming the above theoretical results of intersection target genes in theory. Glycogen synthase kinase-3

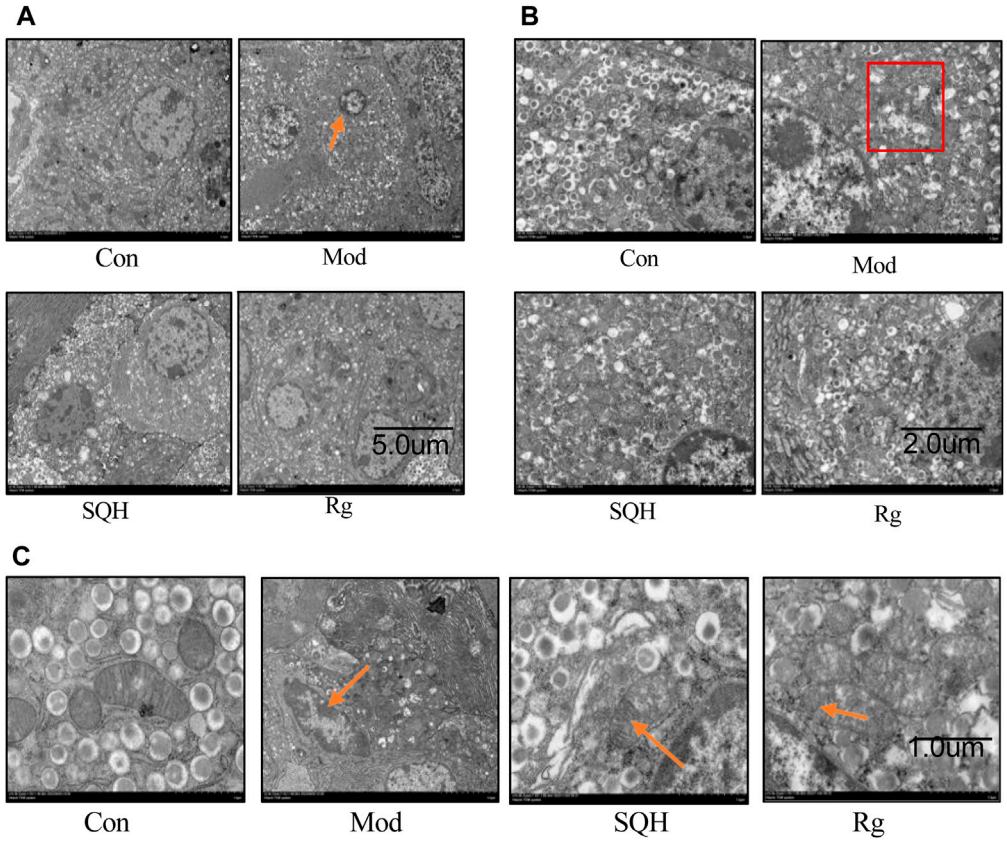

FIGURE 13
Transmission electron microscopy observation of the ultrastructure of pancreatic β cells. **(A)** Nucleus structure of pancreatic islet β cells **(B)** Cytoplasmic granule structure of pancreatic islet β cells **(C)** Mitochondrial structure of pancreatic islet β cells (The arrows in **(A)** point to the pyknotic nuclei of islet β cells. The boxes in **(B)** indicate reduced cytoplasmic granules. The arrows in the Mod in **(C)** point to mitochondrial cristae with broken morphology, and the arrows in the SQH and Rg point to the presence of normal mitochondrial cristae morphology.

reduces glycogen synthesis by inhibiting glycogen synthase in the insulin signaling pathway and leads to insulin resistance (Akhtar and Sah, 2020). GSK-3β is a multifunctional serine/threonine kinase that belongs to the glycogen synthase kinase subfamily and is involved in inflammation, endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis pathways. Considered a negative regulator of β-cells mass in type 2 diabetes, p-GSK exerts anti-apoptotic effects. Both *in vivo* and *in vitro* experimental investigations have delineated the pivotal role of GSK-3 activity in governing mitochondrial function within skeletal muscle cells. Specifically, studies involving GSK-3 knockout (KO) have demonstrated a reduction in mitochondrial oxidative damage coupled with an augmentation of mitochondrial biogenesis (Wang et al., 2020; Cui et al., 2022; Cao et al., 2023). Its inactivation is usually caused by phosphorylation of substrates. GSK-3β in islet β cells is related to oxidative stress-induced apoptosis (Kim et al., 2010). The PI3K/Akt signaling pathway predominantly governs the phosphorylation of glycogen synthase kinase-3β (GSK-3β) at the serine nine site (Ser9). Upon activation, Akt acts as an upstream regulator of GSK-3β. Activation of Akt leads to its interaction with GSK-3β, resulting in the phosphorylation of the serine nine residue and subsequent inactivation of GSK-3β. This inactivation contributes to an anti-apoptotic effect (Qian et al., 2021; Nagao et al., 2022). Therefore, this study selected the PI3K/Akt/GSK-3β signaling pathway to investigate the impact of oxidative stress on pancreatic islet β cells. The findings indicate that a high-sugar and high-fat diet can elevate the expression of GSK-3β in pancreatic islet cells. However, intervention with SQP results in increased expression of p-GSK-3β, which inversely correlates with apoptosis, along with a reduction in GSK-3β expression. Furthermore, by enhancing the expression of upstream PI3K/Akt and employing a PI3K blocker, the study successfully blocked the aforementioned effects of SQP, confirming that SQP may mitigate oxidative stress-induced β-cell apoptosis via modulation of the PI3K/Akt/GSK-3β signaling pathway.

Hence, through a combination of network analysis and experimental validation, the study substantiates the efficacy of SQP in treating SDM by ameliorating oxidative stress responses. This therapeutic mechanism is postulated to operate via modulation of the PI3K/Akt/GSK-3β signaling pathway, coupled with the preservation of mitochondrial cristae morphology. Consequently, SQP attenuates the adverse effects of oxidative stress responses on pancreatic β-cell apoptosis induced by high-sugar and high-fat diets, thereby safeguarding pancreatic islet β cells.

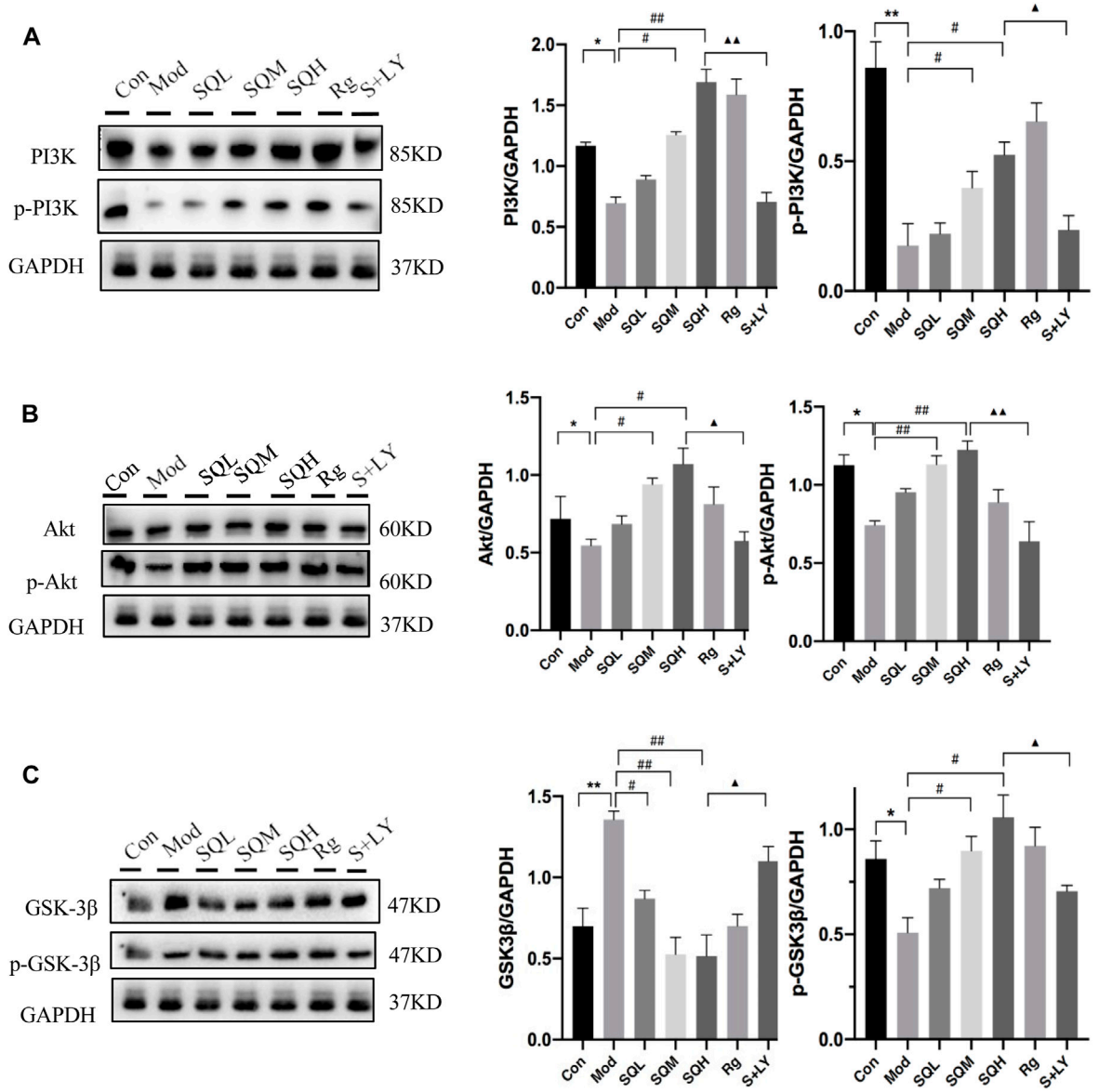

FIGURE 14
The expression of PI3K-Akt-GSK-3β signaling axis protein. **(A)** The expression of PI3K/p-PI3K **(B)** The expression of Akt/p-Akt **(C)** The expression of GSK-3β/p- GSK-3β [(Compared with Con, *$p < 0.05$, **$p < 0.01$; Compared with Mod, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; Compared with SQH, ▲$p < 0.05$, ▲▲$p < 0.01$)].

5 Conclusion

Improving the internal environment of oxidative stress can help protect pancreatic β-cells damage caused by type 2 diabetes. This study predicts the effective mechanism and possible targets of SQP in treating SDM. *In vivo* studies, SQP demonstrates efficacy in ameliorating the internal environment characterized by oxidative stress through the stabilization of blood sugar levels and regulation of blood lipids. Consequently, this intervention contributes to the preservation of mitochondrial morphology. SQP exhibits the potential to mitigate β-cell apoptosis by modulating the PI3K/Akt/GSK-3β signaling pathway. These results provide empirical evidence for the application of SQP in diabetes management. This study combines network analysis and *in vivo* experimental validation. This study aims to strengthen the scientific basis for SQP application and promote the widespread application of traditional Chinese medicine in clinical practice.

Data availability statement

The data supporting the findings of this study are available within the article/Supplementary Material. The data of the study are openly available in GeneCards (https://www.genecards.org/), OMIM (https://omim.org/), PharmGkb (https://www.pharmgkb.org/), BATMAN-TCM (http://bionet.ncpsb.org.cn/batman-tcm/),

Uniprot (https://www.uniprot.org/), MitoCarta3.0 (https://www.broadinstitute.org/mitocarta/mitocarta30-inventory-mammalian-mitochondrial-proteins-and-pathways) and GeneMANIA (http://genemania.org/) database.

Ethics statement

The animal study was approved by the Ethics Committee of Guang'anmen Hospital, China Academy of Chinese Medical Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YuZ: Writing–original draft. YaZ: Writing–original draft. ZW: Writing–original draft, Data curation. HW: Writing–original draft, Visualization. SZ: Writing–review and editing, Methodology. QN: Writing–review and editing, Funding acquisition, Conceptualization.

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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.2024.1339758/full#supplementary-material

References

Akhtar, A., and Sah, S. P. (2020). Insulin signaling pathway and related molecules: role in neurodegeneration and Alzheimer's disease. *Neurochem. Int.* 135, 104707. doi:10.1016/j.neuint.2020.104707

Andreas, E., Reid, M., Zhang, W., and Moley, K. H. (2019). The effect of maternal high-fat/high-sugar diet on offspring oocytes and early embryo development. *Mol. Hum. Reprod.* 25 (11), 717–728. doi:10.1093/molehr/gaz049

Andrieux, P., Chevillard, C., Cunha-Neto, E., and Nunes, J. P. S. (2021). Mitochondria as a cellular hub in infection and inflammation. *Int. J. Mol. Sci.* 22 (21), 11338. doi:10.3390/ijms222111338

Bansal, N., Dhaliwal, R., and Weinstock, R. S. (2015). Management of diabetes in the elderly. *Med. Clin. North Am.* 99 (2), 351–377. doi:10.1016/j.mcna.2014.11.008

Bhattamisra, S. K., Koh, H. M., Lim, S. Y., Choudhury, H., and Pandey, M. (2021). Molecular and biochemical pathways of catalpol in alleviating diabetes mellitus and its complications. *Biomolecules* 11 (2), 323. doi:10.3390/biom11020323

Bhattamisra, S. K., Yap, K. H., Rao, V., and Choudhury, H. (2019). Multiple biological effects of an iridoid glucoside, catalpol and its underlying molecular mechanisms. *Biomolecules* 10 (1), 32. doi:10.3390/biom10010032

Cao, F., Yang, K., Qiu, S., Li, J., Jiang, W., Tao, L., et al. (2023). Metformin reverses oxidative stress-induced mitochondrial dysfunction in pre-osteoblasts via the EGFR/GSK-3β/calcium pathway. *Int. J. Mol. Med.* 51 (4), 36. doi:10.3892/ijmm.2023.5239

Chen, Y., and He, X. (2020). Clinical research on improving insulin resistance in elderly diabetic patients with sarcopenia by therapy of invigorating spleen and tonifying kidney. *China Med. Pharm.* 10(19), 214–216. doi:10.3969/j.issn.2095-0616.2020.19.054

Chinese Elderly Type 2 Diabetes Prevention and Treatment of Clinical Guidelines Writing Group; Geriatric Endocrinology and Metabolism Branch of Chinese Geriatric Society (2022). Geriatric endocrinology and metabolism branch of chinese geriatric health care societygeriatric endocrinology and metabolism branch of chinese geriatric societygeriatric endocrinology and metabolism branch of chinese geriatric health care societygeriatric professional committee of beijing medical award foundation national clinical medical research center for geriatric diseases pla general hospital. Clinical guidelines for prevention and treatment of type 2 diabetes mellitus in the elderly in China. *Zhonghua Nei Ke Za Zhi* 61 (1), 12–50. doi:10.3760/cma.j.cn112138-20211027-00751

Cui, Z., Zhao, X., Amevor, F. K., Du, X., Wang, Y., Li, D., et al. (2022). Therapeutic application of quercetin in aging-related diseases: SIRT1 as a potential mechanism. *Front. Immunol.* 13, 943321. doi:10.3389/fimmu.2022.943321

Ferrucci, L., and Fabbri, E. (2018). Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat. Rev. Cardiol.* 15 (9), 505–522. doi:10.1038/s41569-018-0064-2

Finkel, T., and Oxidants, H. N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408 (6809), 239–247. doi:10.1038/35041687

Geidl-Flueck, B., Hochuli, M., Németh, Á., Eberl, A., Derron, N., Köfeler, H. C., et al. (2021). Fructose and sucrose but not glucose-sweetened beverages promote hepatic *de novo* lipogenesis: a randomized controlled trial. *J. Hepatol.* 75 (1), 46–54. doi:10.1016/j.jhep.2021.02.027

Han, Y. C., Tang, S. Q., Liu, Y. T., Li, A. M., Zhan, M., Yang, M., et al. (2021). AMPK agonist alleviate renal tubulointerstitial fibrosis via activating mitophagy in high fat and streptozotocin induced diabetic mice. *Cell. Death Dis.* 12 (10), 925. doi:10.1038/s41419-021-04184-8

Kim, J. Y., Lim, D. M., Moon, C. I., Jo, K. J., Lee, S. K., Baik, H. W., et al. (2010). Exendin-4 protects oxidative stress-induced β-cell apoptosis through reduced JNK and GSK3β activity. *J. Korean Med. Sci.* 25 (11), 1626–1632. doi:10.3346/jkms.2010.25.11.1626

Klionsky, D. J., Petroni, G., Amaravadi, R. K., Baehrecke, E. H., Ballabio, A., Boya, P., et al. (2021). Autophagy in major human diseases. *EMBO J.* 40 (19), e108863. doi:10.15252/embj.2021108863

Li, J. P., Yuan, Y., Zhang, W. Y., Jiang, Z., Hu, T. J., Feng, Y. T., et al. (2019). Effect of Radix isatidis polysaccharide on alleviating insulin resistance in type 2 diabetes mellitus cells and rats. *J. Pharm. Pharmacol.* 71 (2), 220–229. doi:10.1111/jphp.13023

Li, X., Li, C., Zhang, W., Wang, Y., Qian, P., and Huang, H. (2023). Inflammation and aging: signaling pathways and intervention therapies. *Signal Transduct. Target Ther.* 8 (1), 239. doi:10.1038/s41392-023-01502-8

Liang, D., Qi, Y., Liu, L., Chen, Z., Tang, S., Tang, J., et al. (2023). Jin-Gui-Shen-Qi Wan ameliorates diabetic retinopathy by inhibiting apoptosis of retinal ganglion cells through the Akt/HIF-1α pathway. *Chin. Med.* 18 (1), 130. doi:10.1186/s13020-023-00840-7

Lin, W., Chen, C., Guan, H., Du, X., and Li, J. (2016). Hospitalization of elderly diabetic patients: characteristics, reasons for admission, and gender differences. *BMC Geriatr.* 16, 160. doi:10.1186/s12877-016-0333-z

Lytrivi, M., Castell, A. L., Poitout, V., and Cnop, M. (2020a). Recent insights into mechanisms of β-cell lipo- and glucolipotoxicity in type 2 diabetes. *J. Mol. Biol.* 432 (5), 1514–1534. doi:10.1016/j.jmb.2019.09.016

Lytrivi, M., Castell, A. L., Poitout, V., and Cnop, M. (2020b). Recent insights into mechanisms of β-cell lipo- and glucolipotoxicity in type 2 diabetes. *J. Mol. Biol.* 432 (5), 1514–1534. doi:10.1016/j.jmb.2019.09.016

Maldonado, E., Morales-Pison, S., Urbina, F., and Solari, A. (2023). Aging hallmarks and the role of oxidative stress. *Antioxidants (Basel)* 12 (3), 651. doi:10.3390/antiox12030651

Nagao, Y., Amo-Shiinoki, K., Nakabayashi, H., Hatanaka, M., Kondo, M., Matsunaga, K., et al. (2022). Gsk-3-Mediated proteasomal degradation of ATF4 is a proapoptotic mechanism in mouse pancreatic β-cells. *Int. J. Mol. Sci.* 23 (21), 13586. doi:10.3390/ijms232113586

Nogales, C., Mamdouh, Z. M., List, M., Kiel, C., Casas, A. I., and Schmidt, H. H. H. W. (2022). Network pharmacology: curing causal mechanisms instead of treating symptoms. *Trends Pharmacol. Sci.* 43 (2), 136–150. doi:10.1016/j.tips.2021.11.004

Noor, F., Asif, M., Ashfaq, U. A., Qasim, M., and Tahir Ul Qamar, M. (2023). Machine learning for synergistic network pharmacology: a comprehensive overview. *Brief. Bioinform* 24 (3), bbad120. doi:10.1093/bib/bbad120

Pruett, J. E., Everman, S. J., Hoang, N. H., Salau, F., Taylor, L. C., Edwards, K. S., et al. (2022). Mitochondrial function and oxidative stress in white adipose tissue in a rat model of PCOS: effect of SGLT2 inhibition. *Biol. Sex. Differ.* 13 (1), 45. doi:10.1186/s13293-022-00455-x

Qian, B., Yang, Y., Tang, N., Wang, J., Sun, P., Yang, N., et al. (2021). M1 macrophage-derived exosomes impair beta cell insulin secretion via miR-212-5p by targeting SIRT2 and inhibiting Akt/GSK-3β/β-catenin pathway in mice. *Diabetologia* 64 (9), 2037–2051. doi:10.1007/s00125-021-05489-1

Rath, S., Sharma, R., Gupta, R., Ast, T., Chan, C., Durham, T. J., et al. (2021). MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. *Nucleic Acids Res.* 49 (D1), D1541–D1547. doi:10.1093/nar/gkaa1011

Sulkshane, P., Ram, J., Thakur, A., Reis, N., Kleifeld, O., and Glickman, M. H. (2021). Ubiquitination and receptor-mediated mitophagy converge to eliminate oxidation-damaged mitochondria during hypoxia. *Redox Biol.* 45, 102047. doi:10.1016/j.redox.2021.102047

Wang, D., Yang, Y., Zou, X., Zheng, Z., and Zhang, J. (2020). Curcumin ameliorates CKD-induced mitochondrial dysfunction and oxidative stress through inhibiting GSK-3β activity. *J. Nutr. Biochem.* 83, 108404. doi:10.1016/j.jnutbio.2020.108404

Wang, Q., Zhang, X., Fang, L., Guan, Q., Guan, L., and Li, Q. (2018). Prevalence, awareness, treatment and control of diabetes mellitus among middle-aged and elderly people in a rural Chinese population: a cross-sectional study. *PLoS One* 13 (6), e0198343. doi:10.1371/journal.pone.0198343

Xu, J., Lian, F., Zhao, L., Zhao, Y., Chen, X., Zhang, X., et al. (2015). Structural modulation of gut microbiota during alleviation of type 2 diabetes with a Chinese herbal formula. *ISME J.* 9 (3), 552–562. doi:10.1038/ismej.2014.177

Yin, X. M., and Ding, W. X. (2013). The reciprocal roles of PARK2 and mitofusins in mitophagy and mitochondrial spheroid formation. *Autophagy* 9 (11), 1687–1692. doi:10.4161/auto.24871

Yu, Y., Xie, K., Lou, Q., Xia, H., Wu, D., Dai, L., et al. (2020). The achievement of comprehensive control targets among type 2 diabetes mellitus patients of different ages. *Aging (Albany NY)* 12 (14), 14066–14079. doi:10.18632/aging.103358

Yu, Y., Xie, K., Lou, Q., Xia, H., Wu, D., Dai, L., et al. (2022). The clinical characteristics of Chinese elderly patients with different durations of type 2 diabetes mellitus. *Front. Endocrinol. (Lausanne)* 13, 904347. doi:10.3389/fendo.2022.904347

Yuan, H., Ma, Q., Cui, H., Liu, G., Zhao, X., Li, W., et al. (2017). How can synergism of traditional medicines benefit from network pharmacology? *Molecules* 22 (7), 1135. doi:10.3390/molecules22071135

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Association between the composite dietary antioxidant index and the prevalence and recurrence of kidney stones: results of a nationwide survey

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Aim: This study aims to evaluate the relationship between the Composite Dietary Antioxidant Index (CDAI) and the prevalence and recurrence of kidney stones.

Methods: Data from the National Health and Nutrition Examination Survey (NHANES) collected between 2007 and 2014 were used in this cross-sectional analysis. The CDAI was derived by standardizing the intake of dietary antioxidants from 24 h dietary recalls. The study assessed the prevalence and recurrence of kidney stones based on questionnaire responses. The association between the CDAI and both the prevalence and recurrence of kidney stones was investigated using multivariable logistic regression. Subgroup analyses and interaction tests further evaluated the robustness of this relationship.

Results: The study included 20,743 participants, and the reported incidence and recurrence rates of kidney stones were 9.09 and 2.90%, respectively. After stratifying the CDAI into tertiles, an inverse trend was observed in both kidney stones' prevalence and recurrence probabilities with increasing CDAI levels. Adjusting for confounding factors, individuals in the top tertile had a 23% lower prevalence of kidney stones (OR = 0.77, 95% CI: 0.66, 0.90, $p = 0.0011$) and a 39% lower recurrence rate (OR = 0.61, 95% CI: 0.47, 0.80, $p = 0.0003$) than those in the bottom tertile. In addition, interaction tests showed that age, gender, body mass index, hypertension, and diabetes did not significantly affect the relationship between CDAI levels and kidney stone prevalence and recurrence rates.

Conclusion: Our study suggests that increased levels of CDAI are associated with reduced incidence and recurrence rates of kidney stones. Therefore, increasing the intake of dietary antioxidants may be an effective strategy for preventing kidney stones and their recurrence.

KEYWORDS

composite dietary antioxidant index, NHANES, kidney stones, kidney stones recurrence, cross-sectional study

1 Introduction

Kidney stones, hard deposits of minerals and salts in urine, cause significant patient discomfort and impose a considerable economic burden on public health systems. In the United States, the annual expenditure on treating kidney stones is estimated to be approximately 10 billion dollars (1). The prevalence of kidney stones exhibits notable regional variations:

7–13% in North America, 5–9% in Europe, and is relatively lower in some areas of Asia (2). Absent preventive measures, it is estimated that approximately 50% of patients may experience a recurrence of kidney stones within 5 years (3). Recent studies have elucidated a close association between kidney stones and several health issues, such as hypertension, chronic kidney disease, and end-stage renal disease (4, 5). The increasing incidence of kidney stones, driven by global lifestyle and dietary habits shifts, underscores the urgency of implementing effective early prevention strategies (6, 7). Such strategies include maintaining a balanced fluid intake and adjusting dietary habits to mitigate the risk of kidney stone formation (8).

Antioxidants are substances that can prevent or slow down cell damage, including vitamin A, vitamin E, β-carotene, and various phytochemicals found in many foods (9). They neutralize free radicals in the body, reducing oxidative stress and releasing inflammatory mediators (10, 11). Through its metabolic processes, vitamin A can regulate the pH of the urine, keeping calcium oxalate crystals in a dispersed state and facilitating their excretion (12). Existing research suggests that the antioxidant selenium can reduce oxidative stress in the urine, inhibiting the tendency for calcium oxalate crystals to form and, consequently, reducing the incidence of kidney stones (13). Diets rich in antioxidants are associated with a lower incidence of kidney stones (14).

Recent research has started to explore the potential connection between antioxidants and kidney stone formation. However, the results have been inconsistent. For instance, an *in vitro* study using LLC-PK1 cell cultures found that vitamin C could reduce oxalate-induced oxidative renal damage and calcium oxalate crystal deposition (15). Conversely, an animal study involving male Wistar rats demonstrated that, despite being an effective antioxidant, vitamin C did not reduce oxidative stress-related damage associated with calcium oxalate (16). These findings suggest that the role of individual antioxidants may be limited, highlighting the need for a more comprehensive approach to antioxidant assessment. The Composite Dietary Antioxidant Index (CDAI) serves as an indicator for assessing the overall antioxidant capacity of the human body, encompassing vitamins A, C, and E, zinc, selenium, and carotenoids. It offers a thorough assessment of a diet's capacity to mitigate oxidative stress and neutralize free radicals (17). Previous research has demonstrated the ability of the CDAI to improve outcomes in conditions such as heart failure, hypertension, depression, and atherosclerotic cardiovascular disease, as well as reducing the risk of these conditions (18–21). However, the relationship between CDAI and both the occurrence and recurrence of kidney stones has not yet been investigated.

In this study, we analyzed data from the 2007–2014 National Health and Nutrition Examination Survey (NHANES) to investigate the association between CDAI and the prevalence and recurrence of kidney stones.

Abbreviations: CDAI, Composite Dietary Antioxidant Index; NHANES, National Health and Nutrition Examination Survey; PIR, Income-to-Poverty Ratio; BMI, Body mass index; NCHS, National Center for Health Statistics; ERB, Ethics Review Board; MEC, Mobile Examination Center; CAPI, Computer-Assisted Personal Interviewing; OGTT, Oral Glucose Tolerance Test; HbA1c, Hemoglobin A1c; CDC, Centers for Disease Control and Prevention; OBS, Oxidative Balance Score; ROS, Reactive Oxygen Species; OS, Oxidative stress.

2 Materials and methods

2.1 Study population

Data for this investigation were drawn exclusively from NHANES, a cross-sectional survey that conducts interviews and physical examinations on a randomly selected, nationally representative cohort to provide a comprehensive assessment of the health and nutritional status of the American population (22). The study's methodology received formal approval from the National Center for Health Statistics (NCHS). Written informed consent was obtained from all participants. The study initially enrolled 46,017 participants over four consecutive cycles from 2007 to 2014, with complete data on CDAI, kidney stones, and their recurrence only available within these cycles. After excluding people aged under 20 ($n = 23,482$), pregnant women ($n = 247$), participants with incomplete CDAI data ($n = 2,440$), and those without kidney stone data ($n = 52$), a total of 20,743 eligible participants were retained for the final analysis (Figure 1).

2.2 Assessment of composite dietary antioxidant index

The dietary recall interviews in NHANES were conducted face-to-face by trained dietary interviewers. These interviews follow a set of standardized guidelines for the nutritional assessment of each food item consumed. The first dietary recall interview takes place at the Mobile Examination Center (MEC), followed by a telephone interview occurring 3 to 10 days afterward. Throughout the data collection process, rigorous checks are made to address any omissions or inconsistencies in the reports and ensure the interview completeness (23). The calculation of the CDAI includes six major antioxidant components: vitamins A, C, E, zinc, selenium, and carotenoids. The sources of these dietary antioxidants are strictly limited to food intake,

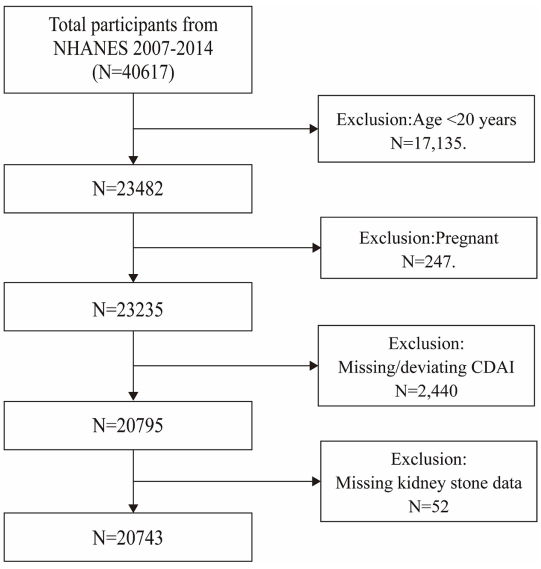

FIGURE 1
Flow chart of participants selection. NHANES, National Health and Nutrition Examination Survey.

explicitly excluding any contribution from nutritional supplements, pharmaceuticals, or antioxidants in drinking water. The CDAI score is derived by deducting the average intake of each antioxidant from the individual's intake, dividing this by the standard deviation, and subsequently aggregating the standardized scores for all antioxidant components.

$$CDAI = \sum_{i=1}^{6} \frac{\left(Antioxidant\ intake_i - mean_i\right)}{SD_i}$$

2.3 Diagnosis of kidney stones

In our selected cycles of NHANES, data regarding kidney stones and their expulsion were gathered at participants' residences through the Computer-Assisted Personal Interviewing (CAPI) system by extensively trained interviewers. The prevalence of kidney stones was determined by answering two key questions: "Have you ever had kidney stones?" and "How many times have you had kidney stones?" Participants who reported having had kidney stones in response to the first question were classified as having had kidney stones. Those who reported passing kidney stones twice or more in response to the second question were categorized as having recurrent kidney stones.

2.4 Covariables

The covariates in this study include demographic and health-related variables, such as age (measured in years); sex (male/female); race (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, and Other); educational attainment (less than high school, high school or GED equivalent, more than high school, and other); the poverty income ratio (PIR); the body mass index (BMI); serum levels of calcium, phosphorus, total cholesterol, and triglycerides; the presence of hypertension and diabetes; as well as intake of total energy, alcohol, and water. Hypertension was identified through either self-reported diagnosis, systolic blood pressure readings ≥140 mmHg, diastolic measurements ≥90 mmHg, or the use of antihypertensive medication (24). Diabetes mellitus was identified based on one or more of the following: clinical diagnosis, use of glucose-lowering medication, oral glucose tolerance test (OGTT) results ≥200 mg/dL, hemoglobin A1c (HbA1c) levels ≥6.5%, or fasting plasma glucose levels ≥126 mg/dL (25).

2.5 Statistical analysis

Statistical analyses in this study were performed using R (version 4.2.0) and EmpowerStats (version 4.2) with a statistical significance threshold of $p < 0.05$. In the descriptive analysis, continuous variables were analyzed using *t*-tests and presented with means and standard deviations, while categorical variables were evaluated with chi-squared tests and expressed as percentages. The CDAI was categorized into tertiles and evaluated using multivariable logistic regression to assess its association with the prevalence and recurrence of kidney stones across three distinct models. Model 1 served as the base model without any adjustments for covariates. Model 2, minimally adjusted, considered age, gender, and ethnicity. Model 3 included adjustments for a comprehensive set of covariates: age, gender, race, educational level, PIR, BMI, total energy intake, total alcohol intake, total water intake, serum creatinine levels, total cholesterol, triglycerides, serum calcium, serum phosphorus, hypertension, and diabetes mellitus status. Additionally, stratified analyses and interaction tests focused on age, gender, BMI, hypertension, and diabetes were conducted to explore further the relationship between CDAI and kidney stone prevalence and recurrence among various demographic groups.

3 Results

3.1 Baseline characteristics

Table 1 outlines the demographic characteristics of 20,743 individuals, detailing a mean age of 49.59 ± 17.66 years, with 49.33% males and 50.67% females. The CDAI was divided into tertiles (tertile 1: −8.29 to 1.55; tertile 2: −1.54 to 1.53; tertile 3: 1.54 to 48.66). In the highest tertile, participants were generally younger (48.06 ± 17.15 years), predominantly male (63.70%), chiefly non-Hispanic white (48.15%), and more often college-educated (60.25% with a degree). They exhibited a greater income-to-poverty ratio (2.76 ± 1.62) when compared with the lowest tertile. Additionally, the prevalence of hypertension and diabetes was less in T3 (38.60 and 15.09%, respectively) than in T1 (47.19 and 21.25%) (Table 1).

3.2 Association of CDAI with kidney stones

Table 2 illustrates how CDAI correlates with kidney stone cases across three progressively adjusted models. There's a marked negative link between CDAI levels and the prevalence of kidney stones. Complete adjustments reveal that a unit increase in CDAI correlates with a 3% lower prevalence of kidney stones (OR = 0.97; 95% CI: 0.95–0.98). Sensitivity analyses segmented CDAI into tertiles, showing in the fully adjusted scenario that individuals in the top CDAI tertile (T3) experienced a 23% lower chance of kidney stones compared to the bottom tertile (T1) (OR = 0.77; 95% CI: 0.66–0.90, $p = 0.0011$).

3.3 Association between CDAI and recurrence of kidney stones

A significant negative association with CDAI was observed for kidney stone recurrence. Within the fully adjusted model, each unit increase in CDAI correlated with a 7% decrease in the recurrence odds (OR = 0.93; 95% CI: 0.90–0.96). Dividing CDAI into tertiles, the analysis indicated that participants in the upper tertile had 39% lower odds of experiencing recurrent kidney stones compared to those in the lowest tertile (OR = 0.61; 95% CI: 0.47–0.80) (Table 2).

3.4 Subgroup analyses

To evaluate the consistency of the link between CDAI and kidney stone occurrence, subgroup analyses and interaction tests were conducted considering variables such as age, sex, BMI, hypertension,

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TABLE 1 Basic characteristics of participants by composite dietary antioxidant index among U.S. adults.

Variables	Composite dietary antioxidant index				*p*-value
	Total	T1 (−8.29–1.55)	T2 (−1.54–1.53)	T3 (1.54–48.66)	
N	20,743	6,912	6,911	6,920	
Age (years)	49.59 ± 17.66	51.01 ± 18.06	49.70 ± 17.65	48.06 ± 17.15	<0.001
Gender, (%)					<0.001
Male	10,232 (49.33%)	2,531 (36.62%)	3,293 (47.65%)	4,408 (63.70%)	
Female	10,511 (50.67%)	4,381 (63.38%)	3,618 (52.35%)	2,512 (36.30%)	
Races, (%)					<0.001
Mexican American	3,069 (14.80%)	1,076 (15.57%)	1,013 (14.66%)	980 (14.16%)	
Other Hispanic	2068 (9.97%)	756 (10.94%)	715 (10.35%)	597 (8.63%)	
Non-Hispanic White	9,317 (44.92%)	2,849 (41.22%)	3,136 (45.38%)	3,332 (48.15%)	
Non-Hispanic Black	4,378 (21.11%)	1,688 (24.42%)	1,389 (20.10%)	1,301 (18.80%)	
Other Races	1911 (9.21%)	543 (7.86%)	658 (9.52%)	710 (10.26%)	
Educational levels, (%)					<0.001
< high school	5,354 (25.81%)	2,383 (34.48%)	1,678 (24.28%)	1,293 (18.68%)	
High school or GED	4,742 (22.86%)	1719 (24.87%)	1,569 (22.70%)	1,454 (21.01%)	
> high school	10,624 (51.22%)	2,799 (40.49%)	3,656 (52.90%)	4,169 (60.25%)	
Others	23 (0.11%)	11 (0.16%)	8 (0.12%)	4 (0.06%)	
PIR	2.49 ± 1.57	2.14 ± 1.45	2.56 ± 1.58	2.76 ± 1.62	<0.001
BMI, (%)					<0.001
Normal weight	6,041 (29.12%)	1910 (27.63%)	1950 (28.22%)	2,181 (31.52%)	
Overweight	6,898 (33.25%)	2,232 (32.29%)	2,297 (33.24%)	2,369 (34.23%)	
Obesity	7,804 (37.62%)	2,770 (40.08%)	2,664 (38.55%)	2,370 (34.25%)	
Total energy (kcal)	2036.66 ± 867.46	1431.25 ± 497.12	1987.50 ± 553.24	2690.48 ± 952.47	<0.001
Total alcohol intake (gm)	8.62 ± 22.73	6.69 ± 20.99	8.49 ± 21.76	10.67 ± 25.07	<0.001
Total water drank (gm)	979.46 ± 963.57	848.11 ± 903.79	967.88 ± 903.94	1122.22 ± 1055.32	<0.001
Serum creatinine (mg/dL)	0.91 ± 0.45	0.92 ± 0.58	0.90 ± 0.42	0.91 ± 0.31	0.022
Serum calcium (mmol/L)	2.36 ± 0.09	2.35 ± 0.09	2.36 ± 0.09	2.36 ± 0.09	0.022
Serum phosphorus (mmol/L)	1.21 ± 0.18	1.21 ± 0.18	1.21 ± 0.18	1.21 ± 0.18	0.660
Triglyceride (mmol/L)	1.49 ± 1.01	1.49 ± 0.83	1.49 ± 1.16	1.48 ± 1.01	0.880
Total cholesterol (mmol/L)	5.01 ± 1.06	5.04 ± 1.07	5.02 ± 1.06	4.97 ± 1.04	<0.001
Hypertension, (%)	8,845 (42.64%)	3,262 (47.19%)	2,912 (42.14%)	2,671 (38.60%)	<0.001
Diabetes, (%)	3,756 (18.11%)	1,469 (21.25%)	1,243 (17.99%)	1,044 (15.09%)	<0.001
Kidney stone, (%)	1886 (9.09%)	658 (9.52%)	637 (9.22%)	591 (8.54%)	0.017
Kidney stone recurrence, (%)	601 (2.90%)	215 (3.11%)	214 (3.10%)	172 (2.49%)	0.010

Continuous variables were showed as mean ± SE, categorical variables were showed as frequency (percentage). PIR, poverty income ratio; BMI, body mass index; GED, general educational development.

and diabetes status. A consistent negative correlation between CDAI and kidney stone occurrence was observed without significant variations across different subgroups, as interaction effects were absent ($p > 0.05$) (Figure 2).

For kidney stone recurrence, similar subgroup analyses reaffirmed the uniform persistence of the inverse correlation with CDAI across stratified groups, with no significant interactions detected ($p > 0.05$), implying that the association between CDAI and stone recurrence was stable across the variables analyzed (Figure 2).

4 Discussion

In our cross-sectional analysis of 20,743 individuals, we observed that higher CDAI levels were inversely correlated with both the prevalence and recurrence of kidney stones. Further subgroup analyses and tests for interaction effects showed that this association did not change with variations in age, sex, BMI, hypertension, or diabetes status. This indicates an association between higher CDAI levels and a lower prevalence and recurrence of kidney stones. These findings highlight a potential approach for clinical practice: modifying dietary

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TABLE 2 Association between the composite dietary antioxidant index and kidney stones and kidney stone recurrence.

Variables	Model 1		Model 2		Model 3	
	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value
Kidney stone						
CDAI	0.99 (0.97, 1.00)	0.018	0.98 (0.97, 0.99)	0.002	0.97 (0.95, 0.98)	<0.001
CDAI tertile						
tertile 1	1		1		1	
tertile 2	0.96 (0.86, 1.08)	0.542	0.92 (0.82, 1.04)	0.189	0.89 (0.79, 1.01)	0.081
tertile 3	0.89 (0.79, 1.00)	0.045	0.83 (0.74, 0.94)	0.003	0.77 (0.66, 0.90)	0.001
P for trend		0.042		0.003		0.001
Kidney stone recurrence						
CDAI	0.97 (0.95, 0.99)	0.011	0.96 (0.94, 0.98)	<0.001	0.93 (0.90, 0.96)	<0.001
CDAI tertile						
tertile 1	1		1		1	
tertile 2	1.00 (0.82, 1.21)	0.962	0.91 (0.75, 1.10)	0.320	0.86 (0.70, 1.07)	0.174
tertile 3	0.79 (0.65, 0.97)	0.026	0.68 (0.55, 0.84)	<0.001	0.61 (0.47, 0.80)	<0.001
P for trend		0.022		<0.001		<0.001

Model 1: no covariates were adjusted.
Model 2: age, gender, and race were adjusted.
Model 3: age, gender, race, educational level, PIR, BMI, total energy intake, total alcohol intake, total water intake, serum creatinine levels, total cholesterol, triglycerides, serum calcium, serum phosphorus, hypertension, diabetes were adjusted.
PIR, poverty income ratio; BMI, body mass index; GED, general educational development.

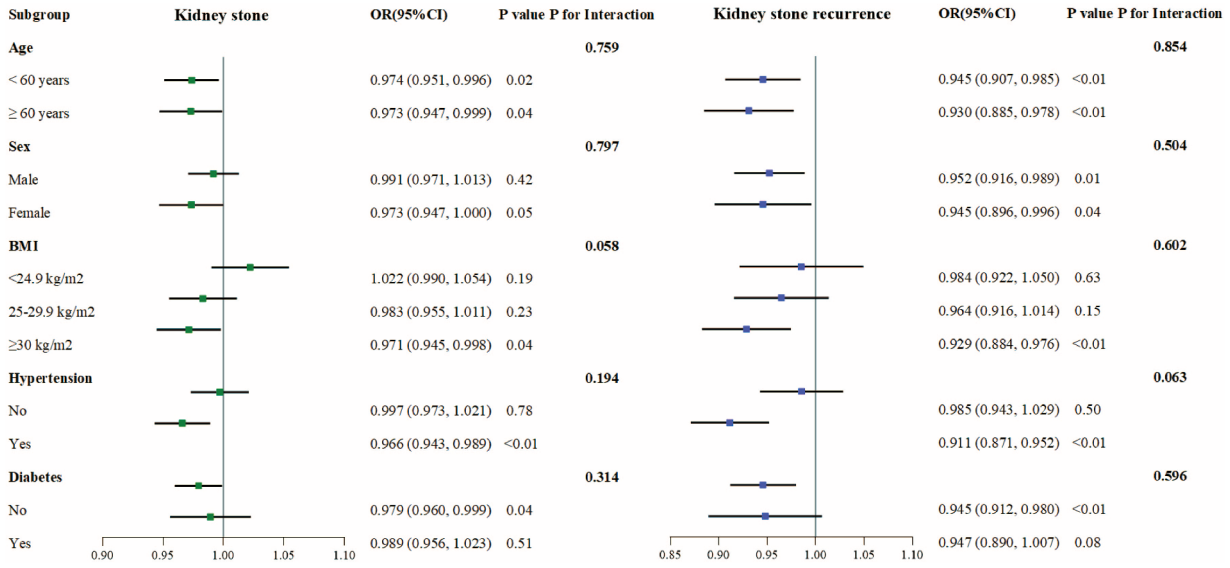

FIGURE 2
Subgroup analysis of the association between CDAI and prevalence and recurrence of kidney stones.

patterns to enhance antioxidant consumption could correlate with reduced prevalence and recurrence of kidney stones.

This research is the first to examine the association between the levels of CDAI and the prevalence and recurrence of kidney stones. Earlier studies have predominantly focused on the correlation between specific antioxidants and kidney stones. For example, in a case–control study with 75 kidney stone patients, Kato observed that the mean plasma concentrations of vitamins A and E in these patients (vitamin A: 13.18 ± 7.95 mg/d; vitamin E: 0.66 ± 0.23 mg/d) were notably lower compared to those in the healthy controls (vitamin A: 34.99 ± 11.40 mg/d; vitamin E: 1.10 ± 0.23 mg/d) (26). Similarly, in another case–control study involving 104 individuals with calcium oxalate stones, Atakan et al. found that urinary zinc and magnesium levels were significantly elevated in the healthy controls compared to the stone-forming group ($p < 0.0001$). This implies that zinc and magnesium may play a role in preventing the formation of calcium oxalate stones (27). Furthermore, recent research suggests that diets rich in antioxidants and certain dietary habits may be linked to a

reduced incidence of kidney stones. For instance, Ilbey et al. demonstrated that administering pomegranate juice to rats with ethylene glycol (EG)-induced hyperoxaluria lowered calcium oxalate stone formation by reducing the expression of ROS, NF-kB, and p38-MAPK, thereby inhibiting oxidative stress (28). In a longitudinal study spanning three large cohorts, Rodrigue found that participants with higher adherence to the Mediterranean diet had a 13–41% reduced risk of developing kidney stones compared to those with lower adherence (29). Nevertheless, findings on the relationship between antioxidants and kidney stones have been mixed; for instance, a prospective study across three sizable cohorts noted no link between vitamin B6 intake and kidney stone occurrence (30). Jian et al. found no association between dietary antioxidants and kidney stones in a cross-sectional study (31). In contrast, Lin's research using the NHANES database showed that the Oxidative Balance Score (OBS) could be a significant predictor for kidney stones (32). This discrepancy may stem from focusing solely on the effects of individual antioxidants on kidney stone formation, thereby neglecting potential interactions and synergistic effects among antioxidants. Moludi found in a cohort study that total dietary antioxidants had a positive effect on renal function but were not significantly associated with kidney stones (33). The limited age range and focus on specific ethnic groups in these studies require further investigation and verification. Given the controversial evidence mentioned above, our study is necessary and important.

Oxidative stress (OS), which arises from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defenses, significantly contributes to the formation of kidney stones (34). Under normal conditions, ROS perform essential functions such as signaling molecules, mediating cell growth, and immune responses. However, excessive production of ROS can damage biomolecules, triggering inflammatory responses and sustained renal tubular damage, ultimately facilitating the formation, growth, and aggregation of stone crystals (35). Hong et al. found that dietary polyphenols, a potent class of natural antioxidants, can modulate the expression and activity of endogenous antioxidant enzymes, influence OS-related signaling pathways, and maintain cellular morphology and functionality (36). Studies have shown that vitamins E and C can attenuate ROS production and protect renal epithelial cells from oxalate-induced oxidative damage, with synergistic effects observed when combined (15). Including foods rich in antioxidants in the daily diet may, therefore, help to reduce the prevalence and recurrence of kidney stones.

Similarly, changes in urine pH are identified as another critical factor in the formation of kidney stones. Uric acid tends to crystallize

TABLE 3 Animal experimental and clinical evidence of the impact of antioxidants on kidney stone formation.

Antioxidants	Study type	Study design	Possible mechanisms	Ref.
Vitamin A	Animal Model	Male Wistar rats induced with hyperoxaluria (oral 0.5% ethylene glycol)	Urine pH ↑ citrate excretion ↑	(12)
	Clinical Participants	Vitamin A-deficient boys administered 24,000 IU of retinyl palmitate daily	Inhibitory activity on calcium oxalate crystal growth ↑ excretion of calcium and oxalate ↓	(47)
Vitamin C	*In Vitro* Experiment	Evaluation of the effects of physiological urinary oxalate concentrations on normal and antioxidant-deficient LLC-PK1 cell cultures	ROS production ↓ oxalate-induced tubular oxidative damage ↓	(15)
Vitamin E	Animal Model	Male Sprague–Dawley rats induced with hyperoxaluria (oral 150 mg ethylene glycol)	Oxidative stress damage ↓ calcium oxalate crystal deposition ↓	(48)
	Animal Model	Male Wistar rats induced with hyperoxaluria (oral 0.75% ethylene glycol)	Tubular epithelial cell death ↓ THP defensive functions ↑ crystal deposition and stone formation ↓	(49)
	Clinical Participants	Patients with calcium oxalate stones (daily oral 400 mg Vitamin E)	THP function restored to normal inhibitory activity on calcium oxalate crystallization ↑	(50)
Zinc	*In Vitro* Experiment	Crystallization of calcium oxalate monohydrate was investigated in the presence of various organic molecules in combination with zinc ions	Chelation with oxalate ions, oxalate ion activity ↓ nucleation rate ↓	(51)
	Animal Model	Male Sprague–Dawley rats induced with calcium oxalate nephrolithiasis (oral 1% ethylene glycol solution)	Inflammatory marker expression ↓ oxalate degradation activity ↑	(52)
Selenium	Animal Model	Male Wistar rats induced with hyperoxaluria (intraperitoneal injection of 0.7% ethylene glycol)	Crystal formation, growth, and aggregation ↓	(53)
	Animal Model	Male dogs induced with calcium oxalate nephrolithiasis (oral 1% ethylene glycol solution)	OPN expression ↓ formation of calcium oxalate stones ↓	(13)
Carotenoids	Animal Model	Male Sprague–Dawley rats induced with calcium oxalate nephrolithiasis (oral 1% ethylene glycol solution)	Oxidative stress damage ↓ inflammation response ↓ deposition of calcium oxalate crystals ↓	(54)

↑ demonstrates increasing trend; ↓ demonstrates decreasing trend. THP (Tamm-Horsfall Protein): A regulatory agent in the nucleation, growth, and aggregation of calcium oxide crystals. OPN (Osteopontin): One of the most crucial components in the matrix of urinary calcium oxalate stones, contributing to the formation of stones. Citrate: Exhibits high affinity for calcium, thereby inhibiting the deposition of calcium crystals in urine. ROS, reactive oxygen species; THP, Tamm-Horsfall protein; OPN, Osteopontin.

and form stones when the urine pH drops below 5.5, increasing the likelihood of uric acid stones (37–39). Certain antioxidants can alter the chemical properties of urine; for example, vitamin A can elevate urinary pH and enhance citrate excretion, thereby decreasing the potential for stone formation (12, 40). In addition, antioxidants can directly or indirectly affect the nucleation, growth, and aggregation of crystals in the urine, reducing the risk of stone formation by reducing crystal formation or promoting crystal dissolution (41). Animal studies have shown that antioxidants such as quercetin, vitamin E, and taurine can reduce crystal deposition in rat models of hyperoxaluria-induced kidney stones, consistent with the above findings (42).

Thirdly, inflammatory immune responses are closely linked to the formation of kidney stones (43). The excessive production of free radicals activates several inflammatory cells and cytokines, increasing cytokines such as TNFα, IL-1β, IL-8, and IL-10 (44). This, in turn, damages renal tubular epithelial cells and causes calcium oxalate crystals to adhere, facilitating stone formation in the papillary and medullary regions of the kidney (45). Antioxidants can reduce inflammatory responses by neutralizing free radicals. In addition, antioxidants may inhibit kidney stone formation by suppressing inflammatory mediator production by inhibiting NF-kB and MAPKs pathways (46). Oxidative stress and inflammatory responses are mutually reinforcing in kidney stone formation, and antioxidants control the occurrence of kidney stones by inhibiting these two processes. For more details on the possible mechanisms by which antioxidants may counteract kidney stone formation, please refer to Table 3.

There are several strengths to our study. First, it is based on data from NHANES. This database uses a complex sampling design and follows rigorous quality control and standardization procedures to ensure the accuracy and national representativeness of the data. Second, we adjusted for confounding covariates to increase the reliability of our results. In addition, subgroup analyses and sensitivity analyses have demonstrated the robustness of our findings. However, our study is subject to several limitations. First, the cross-sectional nature of our design prevents us from determining causal relationships between CDAI levels and the prevalence and recurrence of kidney stones. Second, antioxidant intake was derived from two 24 h recall interviews, subject to recall bias. Third, due to limitations in the NHANES database, we lack data on the composition of kidney stones and cannot precisely record and analyze the types of water intake and specific foods consumed by the subjects. This restricts our analysis of the relationship between CDAI levels and various types of kidney stones and may affect our comprehensive assessment of the factors associated with kidney stone formation. Fourth, although we controlled for several conventional variables, the influence of all potential confounders could not be completely excluded. Finally, our study population consisted of American adults, which may affect the generalizability of our results.

5 Conclusion

Our findings suggest an association between increased intake of specific dietary antioxidants and reduced prevalence and recurrence of kidney stones, highlighting the potential role of these nutrients in reducing such occurrences. These insights could inform prevention and treatment strategies for kidney stones. However, given the variability in individual antioxidant responses and the complexity of dietary factors, additional prospective studies and foundational research are required to confirm these associations and elucidate the underlying mechanisms.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: detailed information about this study can be found at the NHANES online website: https://www.cdc.gov/nchs/nhanes/index.htm.

Ethics statement

The studies involving humans were approved by NCHS Research Ethics Review Board, which is affiliated with the National Center for Health Statistics (NCHS). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

HZ: Writing – original draft, Conceptualization, Data curation, Formal analysis. YC: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. YF: Conceptualization, Writing – review & editing. HC: Conceptualization, Data curation, Formal analysis, Writing – original draft.

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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

1. Scales CD Jr, Tasian GE, Schwaderer AL, Goldfarb DS, Star RA, Kirkali Z. Urinary stone disease: advancing knowledge, patient care, and population health. *Clin J Am Soc Nephrol*. (2016) 11:1305–12. doi: 10.2215/cjn.13251215

2. Sorokin I, Mamoulakis C, Miyazawa K, Rodgers A, Talati J, Lotan Y. Epidemiology of stone disease across the world. *World J Urol*. (2017) 35:1301–20. doi: 10.1007/s00345-017-2008-6

3. D'Costa MR, Haley WE, Mara KC, Enders FT, Vrtiska TJ, Pais VM, et al. Symptomatic and radiographic manifestations of kidney stone recurrence and their prediction by risk factors: a prospective cohort study. *J Am Soc Nephrol*. (2019) 30:1251–60. doi: 10.1681/asn.2018121241

4. Khan SR, Pearle MS, Robertson WG, Gambaro G, Canales BK, Doizi S, et al. Kidney stones. *Nat Rev Dis Prim*. (2016) 2:16008. doi: 10.1038/nrdp.2016.8

5. Rule AD, Krambeck AE, Lieske JC. Chronic kidney disease in kidney stone formers. *Clin J Am Soc Nephrol*. (2011) 6:2069–75. doi: 10.2215/cjn.10651110

6. Shabani E, Khorshidi A, Sayehmiri K, Moradi K, Nabi AE. The effect of nutritional factors on urolithiasis: a case-control study. *J Med Life*. (2023) 16:1062–9. doi: 10.25122/jml-2022-0321

7. Abufaraj M, Xu T, Cao C, Waldhoer T, Seitz C, D'Andrea D, et al. Prevalence and trends in kidney stone among adults in the USA: analyses of National Health and nutrition examination survey 2007–2018 data. *Eur Urol Focus*. (2021) 7:1468–75. doi: 10.1016/j.euf.2020.08.011

8. Prieto RM, Rodriguez A, Sanchis P, Morey M, Fiol M, Grases F, et al. Association of Adherence to the Mediterranean diet with urinary factors favoring renal Lithiasis: cross-sectional study of overweight individuals with metabolic syndrome. *Nutrients*. (2019) 11:1708. doi: 10.3390/nu11081708

9. Rafieian-Kopaei M, Baradaran A, Rafieian M. Plants antioxidants: from laboratory to clinic. *J Nephropathol*. (2013) 2:152–3. doi: 10.5812/nephropathol.12116

10. Conner EM, Grisham MB. Inflammation, free radicals, and antioxidants. *Nutrition*. (1996) 12:274–7. doi: 10.1016/s0899-9007(96)00000-8

11. Nirumand MC, Hajialyani M, Rahimi R, Farzaei MH, Zingue S, Nabavi SM, et al. Dietary plants for the prevention and Management of Kidney Stones: preclinical and clinical evidence and molecular mechanisms. *Int J Mol Sci*. (2018) 19:765. doi: 10.3390/ijms19030765

12. Bardaoui M, Sakly R, Neffati F, Najjar MF, El Hani A. Effect of vitamin a supplemented diet on calcium oxalate renal stone formation in rats. *Exp Toxicol Pathol*. (2010) 62:573–6. doi: 10.1016/j.etp.2009.08.005

13. Liu Y, Xu H, Zhong W, Shen Q, Zhuang T, Huang K. Organic selenium alleviated the formation of ethylene glycol-induced calcium oxalate renal calculi by improving Osteopontin expression and antioxidant capability in dogs. *Biol Trace Elem Res*. (2015) 168:392–400. doi: 10.1007/s12011-015-0373-9

14. Avila-Nava A, Medina-Vera I, Rodríguez-Hernández P, Guevara-Cruz M, Heredia GCPK, Tovar AR, et al. Oxalate content and antioxidant activity of different ethnic foods. *J Ren Nutr*. (2021) 31:73–9. doi: 10.1053/j.jrn.2020.04.006

15. Thamilselvan V, Menon M, Thamilselvan S. Oxalate at physiological urine concentrations induces oxidative injury in renal epithelial cells: effect of A-tocopherol and ascorbic acid. *BJU Int*. (2014) 114:140–50. doi: 10.1111/bju.12642

16. Fishman AI, Green D, Lynch A, Choudhury M, Eshghi M, Konno S. Preventive effect of specific antioxidant on oxidative renal cell injury associated with renal crystal formation. *Urology*. (2013) 82:489.e1–8. doi: 10.1016/j.urology.2013.03.065

17. Wright ME, Mayne ST, Stolzenberg-Solomon RZ, Li Z, Pietinen P, Taylor PR, et al. Development of a comprehensive dietary antioxidant index and application to lung Cancer risk in a cohort of male smokers. *Am J Epidemiol*. (2004) 160:68–76. doi: 10.1093/aje/kwh173

18. Liu C, Lai W, Zhao M, Zhang Y, Hu Y. Association between the composite dietary antioxidant index and atherosclerotic cardiovascular disease in postmenopausal women: a cross-sectional study of Nhanes data, 2013–2018. *Antioxidants (Basel, Switzerland)*. (2023) 12. doi: 10.3390/antiox12091740

19. Ma Y, Liu J, Sun J, Cui Y, Wu P, Wei F, et al. Composite dietary antioxidant index and the risk of heart failure: a cross-sectional study from Nhanes. *Clin Cardiol*. (2023) 46:1538–43. doi: 10.1002/clc.24144

20. Wu M, Si J, Liu Y, Kang L, Xu B. Association between composite dietary antioxidant index and hypertension: insights from NHANES. *Clin Exp Hypertens*. (2023) 45:2233712. doi: 10.1080/10641963.2023.2233712

21. Zhao L, Sun Y, Cao R, Wu X, Huang T, Peng W. Non-linear association between composite dietary antioxidant index and depression. *Front Public Health*. (2022) 10:988727. doi: 10.3389/fpubh.2022.988727

22. Johnson CL, Dohrmann SM, Burt VL, Mohadjer LK. National Health and nutrition examination survey: sample design, 2011–2014. *Vital Health Stat*. (2014) 162:1–33.

23. Zhang HR, Yang Y, Tian W, Sun YJ. Dietary Fiber and all-cause and cardiovascular mortality in older adults with hypertension: a cohort study of Nhanes. *J Nutr Health Aging*. (2022) 26:407–14. doi: 10.1007/s12603-022-1770-3

24. Fryar CD, Ostchega Y, Hales CM, Zhang G, Kruszon-Moran D. Hypertension prevalence and control among adults: United States, 2015–2016. *NCHS Data Brief*. (2017) 289:1–8.

25. Menke A, Casagrande S, Geiss L, Cowie CC. Prevalence of and trends in diabetes among adults in the United States, 1988–2012. *JAMA*. (2015) 314:1021–9. doi: 10.1001/jama.2015.10029

26. Kato J, Ruram AA, Singh SS, Devi SB, Devi TI, Singh WG. Lipid peroxidation and antioxidant vitamins in urolithiasis. *Indian J Clin Biochem*. (2007) 22:128–30. doi: 10.1007/bf02912895

27. Atakan IH, Kaplan M, Seren G, Aktoz T, Gül H, Inci O. Serum, urinary and stone zinc, Iron, magnesium and copper levels in idiopathic calcium oxalate stone patients. *Int Urol Nephrol*. (2007) 39:351–6. doi: 10.1007/s11255-006-9050-4

28. Ilbey YO, Ozbek E, Simsek A, Cekmen M, Somay A, Tasci AI. Effects of pomegranate juice on Hyperoxaluria-induced oxidative stress in the rat kidneys. *Ren Fail*. (2009) 31:522–31. doi: 10.1080/08860220902963871

29. Rodriguez A, Curhan GC, Gambaro G, Taylor EN, Ferraro PM. Mediterranean diet adherence and risk of incident kidney stones. *Am J Clin Nutr*. (2020) 111:1100–6. doi: 10.1093/ajcn/nqaa066

30. Ferraro PM, Taylor EN, Gambaro G, Curhan GC. Vitamin B6 intake and the risk of incident kidney stones. *Urolithiasis*. (2018) 46:265–70. doi: 10.1007/s00240-017-0999-5

31. Jian Z, Wang M, Jin X, Li H, Wang K. Diet-derived antioxidants and risk of kidney stone disease: results from the NHANES 2007–2018 and Mendelian randomization study. *Front Nutr*. (2021) 8:738302. doi: 10.3389/fnut.2021.738302

32. Lin G, Zhan F, Zhu J, Xue L, Wei W. Relationship between oxidative balance score and kidney stone prevalence in us adults. *Int Urol Nephrol*. (2024) 56:877–85. doi: 10.1007/s11255-023-03866-w

33. Moludi J, Tandorost A, Kamari N, Abdollahzad H, Pakzad R, Najafi F, et al. Dietary Total antioxidant capacity and its association with renal function and kidney stones: results of a Rancd cohort study. *Food Sci Nutr*. (2022) 10:1442–50. doi: 10.1002/fsn3.2753

34. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox Signalling. *Biochem J*. (2012) 441:523–40. doi: 10.1042/bj20111451

35. Khan SR. Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis. *Transl Androl Urol*. (2014) 3:256–76. doi: 10.3978/j.issn.2223-4683.2014.06.04

36. Hong SY, Qin BL. The protective role of dietary polyphenols in urolithiasis: insights into antioxidant effects and mechanisms of action. *Nutrients*. (2023) 15:3753. doi: 10.3390/nu15173753

37. Abou-Elela A. Epidemiology, pathophysiology, and Management of Uric Acid Urolithiasis: a narrative review. *J Adv Res*. (2017) 8:513–27. doi: 10.1016/j.jare.2017.04.005

38. Ngo TC, Assimos DG. Uric acid nephrolithiasis: recent progress and future directions. *Rev Urol*. (2007) 9:17–27.

39. Sakhaee K. Uric acid metabolism and uric acid stones In: NP Rao, GM Preminger and JP Kavanagh, editors. *Urinary tract stone disease*. London: Springer London (2011). 185–93.

40. Munday JS, McKinnon H, Aberdein D, Collett MG, Parton K, Thompson KG. Cystitis, pyelonephritis, and urolithiasis in rats accidentally fed a diet deficient in vitamin a. *J Am Assoc Lab Anim Sci*. (2009) 48:790–4.

41. Khan SR. Reactive oxygen species as the molecular modulators of calcium oxalate kidney stone formation: evidence from clinical and experimental investigations. *J Urol*. (2013) 189:803–11. doi: 10.1016/j.juro.2012.05.078

42. Yang X, Ding H, Qin Z, Zhang C, Qi S, Zhang H, et al. Metformin prevents renal stone formation through an antioxidant mechanism in vitro and in vivo. *Oxidative Med Cell Longev*. (2016) 2016:4156075. doi: 10.1155/2016/4156075

43. Sakhaee K. Exploring the role of inflammation toward the pathogenesis of calcium nephrolithiasis. *Clin J Am Soc Nephrol*. (2022) 17:338–9. doi: 10.2215/cjn.00510122

44. Khan SR, Canales BK, Dominguez-Gutierrez PR. Randall's plaque and calcium oxalate stone formation: role for immunity and inflammation. *Nat Rev Nephrol*. (2021) 17:417–33. doi: 10.1038/s41581-020-00392-1

45. Dominguez-Gutierrez PR, Kusmartsev S, Canales BK, Khan SR. Calcium oxalate differentiates human monocytes into inflammatory M1 macrophages. *Front Immunol*. (2018) 9:1863. doi: 10.3389/fimmu.2018.01863

46. Arulselvan P, Fard MT, Tan WS, Gothai S, Fakurazi S, Norhaizan ME, et al. Role of antioxidants and natural products in inflammation. *Oxidative Med Cell Longev*. (2016) 2016:5276130–15. doi: 10.1155/2016/5276130

47. Kancha RK, Anasuya A. Contribution of vitamin a deficiency to Calculogenic risk factors of urine: studies in children. *Biochem Med Metab Biol*. (1992) 47:1–9. doi: 10.1016/0885-4505(92)90002-g

48. Thamilselvan S, Menon M. Vitamin E therapy prevents Hyperoxaluria-induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status. *BJU Int*. (2005) 96:117–26. doi: 10.1111/j.1464-410X.2005.05579.x

49. Huang HS, Chen J, Chen CF, Ma MC. Vitamin E attenuates crystal formation in rat kidneys: roles of renal tubular cell death and crystallization inhibitors. *Kidney Int*. (2006) 70:699–710. doi: 10.1038/sj.ki.5001651

50. Sumitra K, Pragasam V, Sakthivel R, Kalaiselvi P, Varalakshmi P. Beneficial effect of vitamin E supplementation on the biochemical and kinetic properties of Tamm-Horsfall glycoprotein in hypertensive and Hyperoxaluric patients. *Nephrol Dial Transplant*. (2005) 20:1407–15. doi: 10.1093/ndt/gfh794

51. Barker T, Boon M, Jones F. The role of zinc ions in calcium oxalate monohydrate crystallization. *J Cryst Growth*. (2020) 546:125777. doi: 10.1016/j.jcrysgro.2020.125777

52. Wu F, Cheng Y, Zhou J, Liu X, Lin R, Xiang S, et al. Zn(2+) regulates human oxalate metabolism by manipulating oxalate decarboxylase to treat calcium oxalate stones. *Int J Biol Macromol*. (2023) 234:123320. doi: 10.1016/j.ijbiomac.2023.123320

53. Sakly R, Chaouch A, el Hani A, Najjar MF. Effects of intraperitoneally administered vitamin E and selenium on calcium oxalate renal stone formation: experimental study in rat. *Ann Urol*. (2003) 37:47–50. doi: 10.1016/s0003-4401(03)00007-x

54. Gao X, Lin B, Chen C, Fang Z, Yang J, Wu S, et al. Lycopene from tomatoes and tomato products exerts Renoprotective effects by ameliorating oxidative stress, apoptosis, Pyroptosis, fibrosis, and inflammatory injury in calcium oxalate nephrolithiasis: the underlying mechanisms. *Food Funct*. (2024) 15:4021–36. doi: 10.1039/d4fo00042k

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Ophiopogonin D: review of pharmacological activity

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Background: Ophiopogon D is an important natural organic compound in *Ophiopogon japonicus*, which often has significant biological activity.

Purpose: The purpose of this review is to systemically summarize and discuss the pharmacological activity and underlying mechanisms of OP-D in recent years.

Method: PubMed and Web of Science were searched with the keywords: "*Ophiopogon japonicus*", "Ophiopogon D" "pharmacology", and "pharmacokinetics". There was no restriction on the publication year, and the last search was conducted on 1 Jan 2024.

Results: Emerging evidence suggests that OP-D possess numerous pharmacological activities, including bone protection, cardiovascular protection, immune regulation, anti-cancer, anti-atherosclerosis, anti-inflammatory and anti-NAFLD.

Conclusion: OP-D has a potential value in the prevention and treatment of many diseases. We hope that this review will contribute to therapeutic development and future studies of OP-D.

KEYWORDS

***Ophiopogon japonicus*, Ophiopogon D, pharmacology, pharmacokinetics, drug**

1 Introduction

Since the 21st century, researchers have increasingly turned their attention to traditional Chinese herbs, recognizing their advantage of reduced side effects (Zeng et al., 2019). *Ophiopogon japonicus*, a well-known Chinese herb, has long been considered a health-promoting substance (Fang et al., 2018). At the same time, *Ophiopogon japonicus* is also a popular ornamental plant in East Asia (Yuan et al., 2019). Literature investigation has shown that *Ophiopogon japonicus* contains many active compounds, such as Dwarf Lilyturf Tuber-13 (DT-13), Ophiopogon-B(OP-B), Ophiopogon-D (OP-D), Liriopesides-B (LP-B), Ruscogenin (RUS), and Ophiopogon-D′ (OP-D′) (Ren-ping et al., 2014; Dong et al., 2021;

Abbreviations: AD, Atopic dermatitis; Ang II, Angiotensin II; DMSO, Dimethyl sulfoxide; DT-13, Dwarf lilyturf tuber-13; ERS, Endoplasmic reticulum stress; ESI-MS, Electrospray ionization mass spectrometry; HUVECs, Human umbilical vein endothelial cells; LC, Liquid chromatography; LP-B, Liriopesides B; MCF-7, Human breast carcinoma-7; MI/R, Myocardial Ischemia-Reperfusion; MMP-9, Matrix metalloproteinase; NSCLC, Non-small cell lung carcinoma; NAFLD, Nonalcoholic fatty liver disease; OATPs, Organic anion-transporting polypeptides; OP-B, Ophiopogon-B; OP-D, Ophiopogon-D; RUS, Ruscogenin; OP-D′, Ophiopogon-D′; PECAM1, Platelet and endothelial cell adhesion molecule 1; ROS, Reactive oxygen species; STAT3, Signal transducer and activator of transcription 3; SM-I, Shenmai injection; SLE, Systemic lupus erythematosus; TNBC, Triple-negative breast cancer.

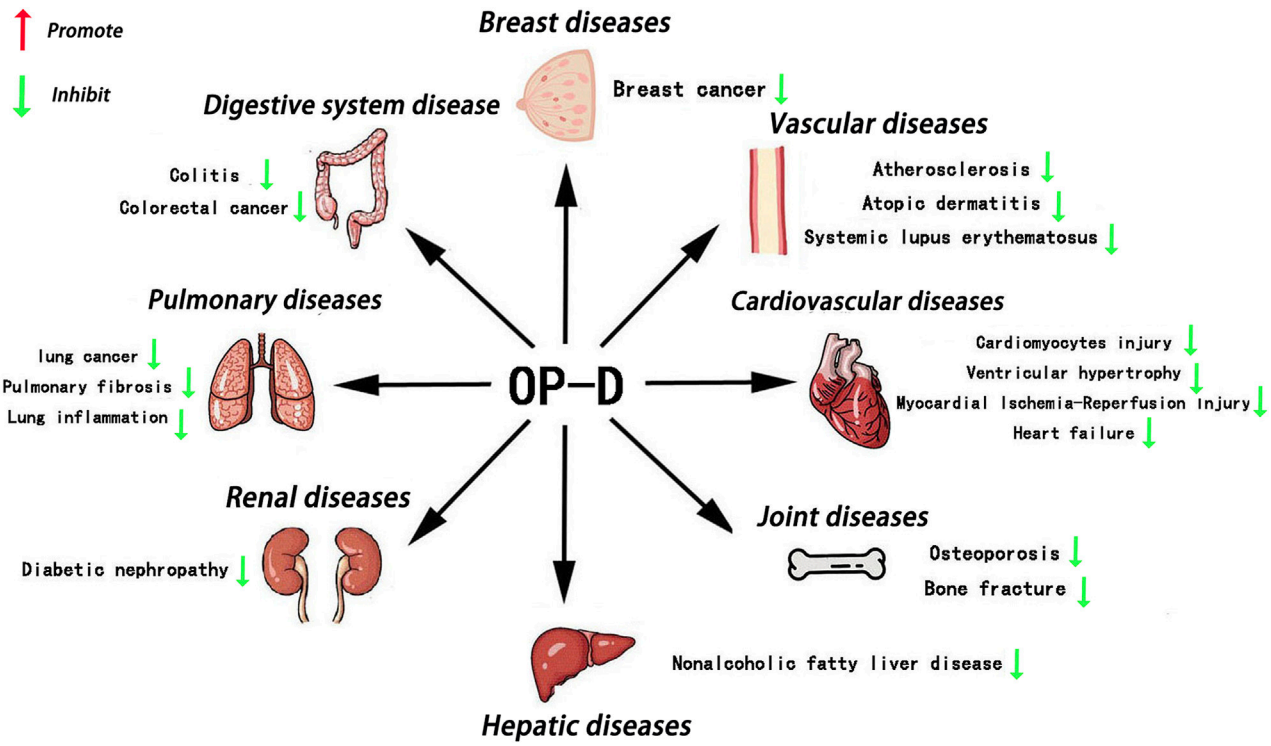

FIGURE 1
Main roles of OP-D in various tissues OP-D plays an important role in various organs, such as the bone, blood vessel, heart, breast, kidney, liver, lungs, colon and so on.

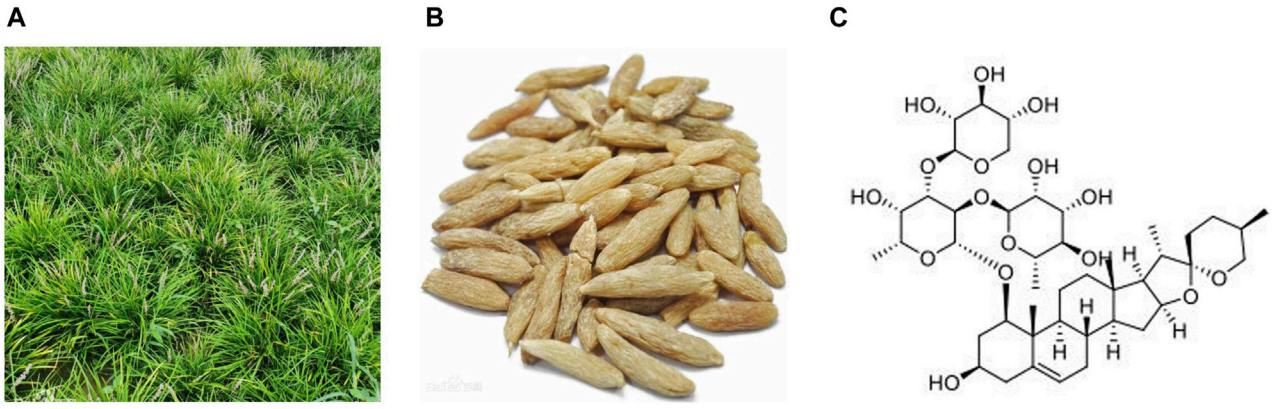

FIGURE 2
The whole plant **(A)**, roots **(B)** and structures **(C)** of OP-D.

Liu et al., 2023). OP-D is a rare C27 steroid glycoside isolated from the tuber of *Ophiopogon japonicus*. Over the past few years, extensive research on OP-D across animal and human models has demonstrated its multifaceted benefits, including anti-inflammatory, anti-cancer, anti-atherosclerosis, anti-NAFLD, immunomodulatory, osteoprotective, and cardioprotective effects (Figure 1). However, there is still a lack of comprehensive and critical review of OP-D pharmacological activity. We hope that this review will contribute to therapeutic development and future studies of OP-D.

2 Chemical properties and plant sources of OP-D

Ophiopogon japonicus is a perennial bushy herb characterized by its bushy growth and small, oval or spindle-shaped roots, typically found in the middle or near the ends of the root system. The small tuberous roots are light brownish-yellow and very short (Figure 2). The leaf base is clumped. The seeds are spherical, and the flowers are solitary or in pairs. The flowering period is from May to August, and the fruiting period lasts from August to September (Lei et al., 2021).

OP-D is identified as a white crystalline powder with a molecular formula of $C_{44}H_{70}O_{16}$, and a molecular weight of 855.07. It is soluble in methanol, ethanol, and dimethyl sulfoxide (DMSO), and features eight OH groups and six CH3 groups (Chen et al., 2016) (Figure 2).

3 Pharmacokinetics

Liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS) are two high-performance physical separation techniques, particularly for the detection of OP-D and its pharmacokinetics in rats. These two methods exhibited a good correlation coefficient over the investigated concentration range ($r > 0.997$, LLOD < 2.0 ng/mL, RSD < 7.5% and accuracies were 97.5%–107.0%) (Xia C. et al., 2008). The two-compartment pharmacokinetic model describes the evolution of drug levels in the organism by depicting the body as two pharmacokinetic compartments (the central and the peripheral compartments, also commonly referred to as compartment 1 and compartment 2, in that order). After intravenous dosing (77.0 μg/kg), the plasma concentration-time profile for OP-D was best fitted to an open two-compartment model (Cl = 0.024 ± 0.010 L/min/kg, $T_{1/2}$ = 17.29 ± 1.70 min). As a component of 'SHENMAI' injection, the pharmacokinetics of OP-D revealed a significantly smaller clearance compared with a pure OP-D compound (Xia CH. et al., 2008). OP-D also influences on the pharmacokinetics and transport of other drugs. As a natural quinone compound, cryptotanshinone was found to have anti-inflammatory activities, anti-cancer activities, anti-metabolic disorders, cardiovascular protection and other functions. OP-D significantly increased the C_{max} and $T_{1/2}$ of cryptotanshinone, while decreasing the clearance rate of cryptotanshinone. *In vitro*, OP-D improved metabolic stability by lowering intrinsic clearance and dramatically inhibited the transport of cryptotanshinone through a reduced efflux rate. The combination of cryptotanshinone and OP-D could inhibit the transport of cryptotanshinone and reduce the bioavailability of cryptotanshinone (Wang et al., 2023).

4 Anti-inflammatory activities

In colitis mouse model, OP-D ameliorates the colitis by inhibiting epithelial NF-κB signaling pathway (Wang et al., 2022). In streptozotocin-induced diabetic nephropathy rats, OP-D ameliorates renal function by inhibiting inflammatory response (Qiao et al., 2020). Related studies have shown that particulate matter with a diameter of less than 2.5 μm (PM2.5) can cause lung inflammation. In mouse pulmonary epithelial cells, OP-D significantly ameliorates PM2.5-induced inflammation by inhibiting the AMPK/NF-κB signaling pathway (Wang Y. et al., 2020). Atopic dermatitis (AD) is a prevalent condition globally, marked by symptoms such as itching and eczema. In atopic dermatitis mouse models and inflamed HaCaT cells, OP-D can treat inflammatory skin diseasessuch as AD (An et al., 2020). In addition, related studies have observed the anti-inflammatory effects of OP-D in the spleen. The level of cytokine expression in the blood of OP-D-treated mice is significantly decreased (An et al., 2020).

5 Anti-cancer activities

Tumours pose a significant threat to human health. In a study by Zhang Y et al., the potential of OP-D to inhibit melanoma was explored. The research revealed that OP-D effectively suppressed both the invasion and proliferation of MDA-MB-435 melanoma cells. Additionally, OP-D was found to inhibit the adhesion of these cells to fibronectin. Mechanically, OP-D suppressed the phosphorylation of p38 and the expression of matrix metalloproteinase-9 (MMP-9) (Zhang Y. et al., 2015). Exploring the anti-metastasis effect of OP-D in triple-negative breast cancer (TNBC) cells and its mechanism should be endowed with paramount importance. OP-D inhibited the migration, invasion and proliferation of MDA-MB-231 cells. Mechanically, OP-D upregulated the nuclear β-catenin and reduced the phosphorylation of FAK/Src/AKT by abolishing ITGB1 expression (Zhu et al., 2020). In addition, the number of viable cells and colony formation significantly decreased when the cells were exposed to OP-D. OP-D inhibits the proliferation of MCF-7 cells and causes cell cycle arrest at the G (2)/M phase. Mechanically, cyclin B1 downregulation was linked to OP-D-induced G (2)/M cell cycle arrest. Moreover, OP-D-induced apoptosis included the activation of caspase-8 and caspase-9 (Zang et al., 2016). OP-D may be a promising natural anti-cancer agent for the treatment of colorectal cancer and throat cancer. In human laryngocarcinoma cells, OP-D boosted caspase-3/9 activity, induced apoptosis, promoted cytotoxicity, and decreased cell growth. OP-D markedly elevated p-p38 MAPK protein expression while dramatically downregulating the expression of cyclin B1 and matrix metalloproteinase-9 (MMP-9) proteins (Yan et al., 2019). Ko HM et al. aimed to investigate the anti-colorectal cancer effect of OP-D. They found that OP-D (20–40 uM) significantly inhibits cell viability and possesses anti-proliferative properties. By preventing IPO7 and XPO1 from being produced, OP-D (40 uM) caused nucleolar stress and suppressed the expression of Ki67 (a marker for cell proliferation). Furthermore, OP-D controlled CDK4 and cyclin D1. Furthermore, in a dose-dependent manner, OP-D consistently suppressed the phosphorylation of AKT expression. The $T_{1/2}$ of c-Myc was shortened by OP-D in a time-dependent way (Ko et al., 2022). In human lung cancer cells, OP-D suppresses the proliferation of cells and reduces the expression of several carcinogenic gene products by inhibiting the NF-κB, PI3K/AKT, and AP-1 pathways (Lee et al., 2018b). Another study suggests that OP-D can induce apoptosis and exert anti-tumor effects by inhibiting of signal transducer and activator of transcription 3 (STAT3) signaling pathways in non-small cell lung carcinoma (NSCLC) cells (Lee et al., 2018a) (The potential anti-cancer effects and mechanisms of OP-D are shown in Figure 3).

6 Cardiovascular protection activities

OP-D is recognized as the principal bioactive constituent of traditional Chinese medicine formulations such as Shenmai san, Shenmai injection (SM-I), and Radix *Ophiopogon japonicus* (Jiang et al., 2014). The study demonstrated that OP-D attenuated doxorubicin-induced cardiomyocyte injury by suppressing

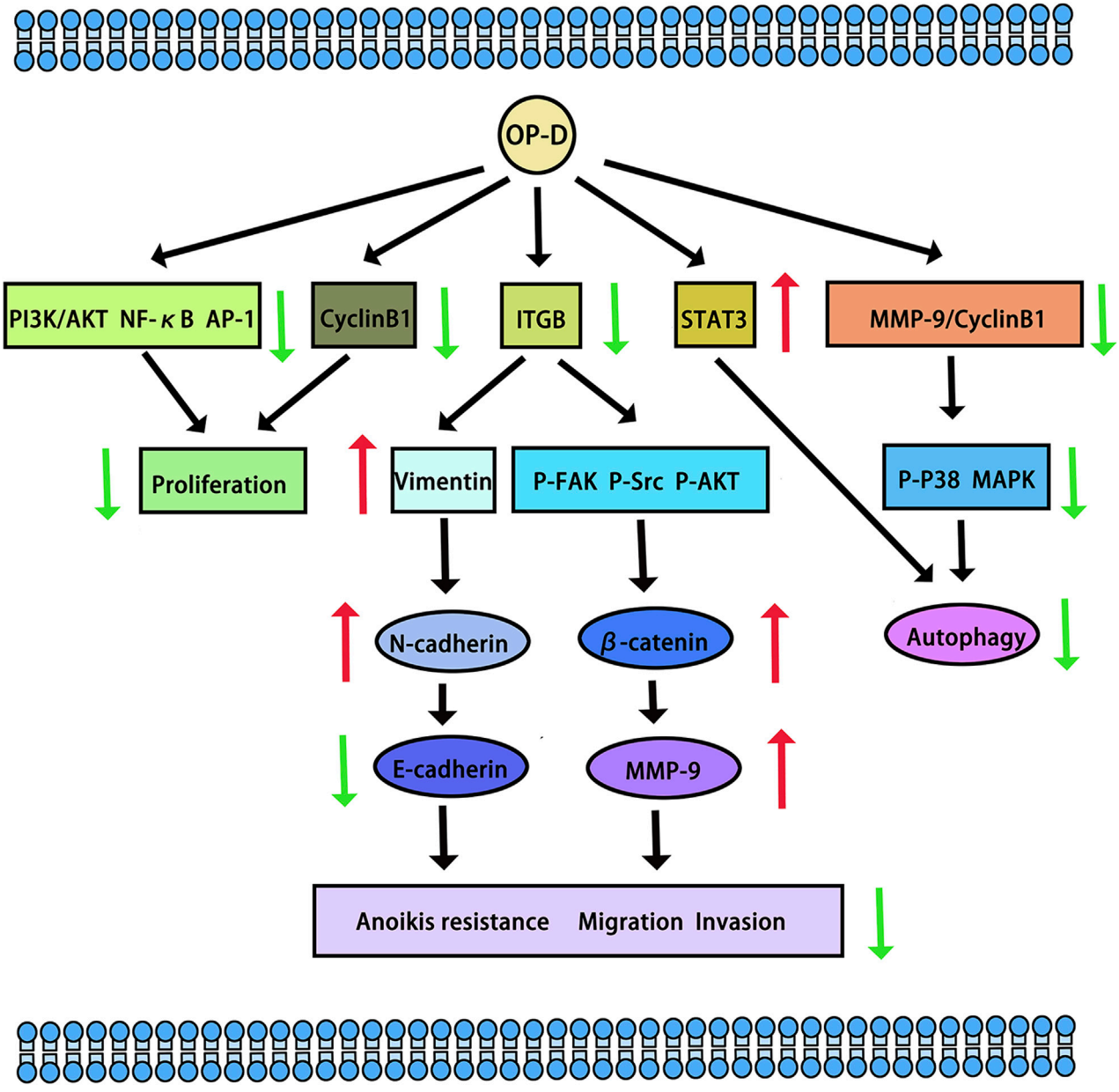

FIGURE 3
The potential anti-cancer effects and mechanisms of OP-D. The red arrow means up-regulation, the green arrow means down-regulation.

endoplasmic reticulum stress (ERS) and relieving mitochondrial damage (Meng et al., 2014). Mechanically, OP-D mitigated autophagy activity by diminishing the production of reactive oxygen species (ROS) (Zhang YY. et al., 2015). Another study pointed out that OP-D reduced diabetic myocardial injuries by regulating the dynamics of mitochondria. In type 2 diabetes mice, OP-D lowered blood lipid levels and alleviated mitochondrial dysfunction. In myocardial lipotoxicity models, OP-D inhibited the mitochondrial dysfunction and promoted the cell survival rate (Li et al., 2021). Furthermore, OP-D also prevented H_2O_2-induced injury in human umbilical vein endothelial cells (HUVECs), where OP-D dose-dependently reduced the mRNA levels of anti-oxidant, pro-inflammatory, and apoptotic genes. Pretreatment with OP-D reduced H_2O_2-induced lipid peroxidation and protein carbonylation. In addition, in cells treated with OP-D, mitochondrial ROS production and cell death were diminished. Furthermore, OP-D prevented the release of inflammatory cytokines and restored the entire antioxidative capacity of the cell (Qian et al., 2010). Zhang GC found that OP-D protected isoproterenol-induced cardiomyocyte injury by regulating multiple signaling pathways of target proteins (Zhang et al., 2022). Ophiopogon-D′ (OP-D′) and OP-D are the two main active components in *Ophiopogon japonicus*, and these factors have the same molecular formula and similar structures. Interestingly, OP-D′ induced cardiomyocyte mitophagy and mitochondrial damage (Lei et al., 2022). OP-D protected against OP-D′-induced cardiomyocyte injury through the inhibition of ERS. The rate of apoptosis was significantly increased by OP-D′ (6 uM) and genes

related to ERS had increased expression. The endoplasmic reticulum's morphology was altered, and myocardial cell damage caused by OP-D′ could be partially mitigated at varying concentrations of OP-D (Wang et al., 2019).

CYP2J2, CYP4F3, CYP4A11, CYP4A22, CYP4F2, and CYP4F3 were common fatty acid metabolic enzymes in cardiomyocytes. The research revealed that low concentrations of OP-D did not impact the viability of cardiomyocytes. Conversely, concentrations of OP-D exceeding 20 μM could potentially enhance cell viability. At concentrations below 100 μM, OP-D did not significantly alter the morphology or quantity of cardiomyocytes. At 5 μM, OP-D mildly upregulated CYP2J2 and CYP4F3 mRNA expression, whereas high concentrations of OP-D substantially enhanced these expressions in a dose-dependent manner. On the mRNA expressions of CYP4A11, CYP4A22, and CYP4F2, the same concentration of OP-D had a minor impact. In a dose-dependent manner, 20 uM OP-D might considerably increase the expression of CYP4F3 in the protein (Tang et al., 2021). CYP2J2 was highly expressed in cardiovascular tissue. Huang X et al. found that OP-D has an endothelial protective effect via activating the CYP2J2-PPARα pathway in HUVECs. By upregulating CYP2J2/EETs and PPARα in HUVECs, OP-D dramatically reduced Ang II-induced NF-κB nuclear translocation, IκBα downregulation, and activation of pro-inflammatory cytokines (TNF-α, IL-6, and VCAM-1) (Huang et al., 2017). Another study demonstrated OP-D's ability to inhibit Angiotensin II (Ang II)-induced vascular endothelial cell death by upregulating CYP2J2 (Huang XY. et al., 2018). Meanwhile, OP-D was closely related to CYP2J3. By inhibiting inflammation *in vivo* and upregulating CYP2J3 *in vitro*, OP-D reduced ventricular hypertrophy in rats. Ang II treatment was administered to H9c2 cells. In response to Ang II-induced hypertrophy, specific hypertrophy genes and NF-κB signaling molecules were expressed at higher levels. Nevertheless, OP-D combined with Ang II negated these inductive effects (Wang et al., 2018). By upregulating CYP2J3, OP-D also reduced Myocardial Ischemia-Reperfusion (MI/R) Injury in rats. OP-D provided a range of preventive measures against MI/R injury. These included improving the healing of damaged myocardial structures, reducing the synthesis of creatine kinase and lactate dehydrogenase, attenuating the size of myocardial infarcts, and regulating heart function. There was potential in developing OP-D as a unique medication for the treatment of MI/R damage (Huang X. et al., 2018). Another study demonstrated that OP-D upregulated CYP2J3 and suppressed ER stress in rat cardiomyocytes to preserve Ca^{2+} homeostasis *in vitro* (You et al., 2016). Besides, Wang, J et al. found that OP-D could increase SERCA2a interaction with phospholamban by inducing the increase of CYP2J3 in rat cardiomyocytes. Through increasing SERCA2a activity and phosphorylating PLB, the occurrence of heart failure was reduced. In a heart failure model, the reduction of CYP2J3 eliminated these positive effects of OP-D on heart failure (Wang J. et al., 2020) (The cardiovascular protective mechanisms of OP-D are shown in Figure 4).

7 Bone protection activities

OP-D is also a new herbal agent against osteoporosis. The research showed that OP-D markedly increased the proliferation of MC3T3-E1 cells. Furthermore, in RAW264.7 cells, both TRAP activity and the mRNA expressions of osteoclastic genes were decreased. One of the main factors contributing to the development of osteoporosis was an excess of ROS. In MC3T3-E1 cells and RAW264.7 cells, OP-D inhibited the generation of ROS. Serum bone degradation indicators, such as TRAP and CTX-1, showed decreased activity following OP-D treatment. Subsequent investigations revealed that OP-D exhibited anti-osteoporosis properties by lowering ROS via the FoxO3a-β-catenin signaling pathway (Huang et al., 2015). Clinical evidence has indicated a high failure rate of titanium implants in diabetic patients. Under diabetic conditions, excessive oxidative stress at the bone-implant interface plays an important role in the impaired osteointegration. OP-D ameliorated the osteointegration of titanium alloy implants by preventing ROS overproduction through the Wnt/β-catenin signaling pathway (Ma et al., 2018). PECAM1 (platelet and endothelial cell adhesion molecule 1) holds considerable significance for angiogenesis and osteogenesis and is involved in bone regeneration. Endothelial-specific KLF3 knockout mice showed increased PECAM1 and accelerated bone formation in the bone regeneration area. As a KLF3 inhibitor, OP-D stimulated the formation of vessels both *in vivo* and *in vitro*. When OP-D was administered, PECAM1 abundance rose and bone healing accelerated. These findings offered a novel therapeutic target for the treatment of bone fractures and the enhancement of bone regeneration (Yang et al., 2020).

8 Other activities

Atherosclerosis is a common cardiovascular disease. OP-D plays an important role in atherosclerosis. When compared to the model group, OP-D dramatically reduced the amount of serum lipid and the formation of plaque. Furthermore, OP-D decreased hepatocyte steatosis while enhancing insulin resistance and oral glucose tolerance. Subsequent investigation showed that OP-D might reduce atherosclerosis by blocking mTOR phosphorylation and targeting lipid metabolism pathways regulated by SREBP1 and SCD1, as observed in both vivo and vitro. The gut microbiota was confirmed to be crucial in atherosclerosis pathogenesis. In HFD-fed mice, OP-D treatment resulted in notable structural alterations in the gut microbiota and faecal metabolites. Additionally, it decreased the relative abundance of Erysipelotrichaceae genera linked to the metabolism of cholesterol (Zhang et al., 2021).

Gut dysbiosis also plays a critical role in the pathogenesis of obesity. In HFD-fed mice, OP-D ameliorated body weight, hyperglycemia, hyperlipidemia, and insulin resistance. Specifically, OP-D reversed HFD-induced gut dysbiosis (Chen et al., 2018).

Nonalcoholic fatty liver disease (NAFLD), a clinicopathologic syndrome characterized by excessive fat deposition in the liver cells, not caused by alcohol or other liver-specific toxins. In HFD-fed obese mice, OP-D reduced NAFLD by enhancing oxidative stress, lipid metabolism, and inflammatory response. *In vitro*, OP-D treatment lowered the levels of inflammation and lipogenesis.

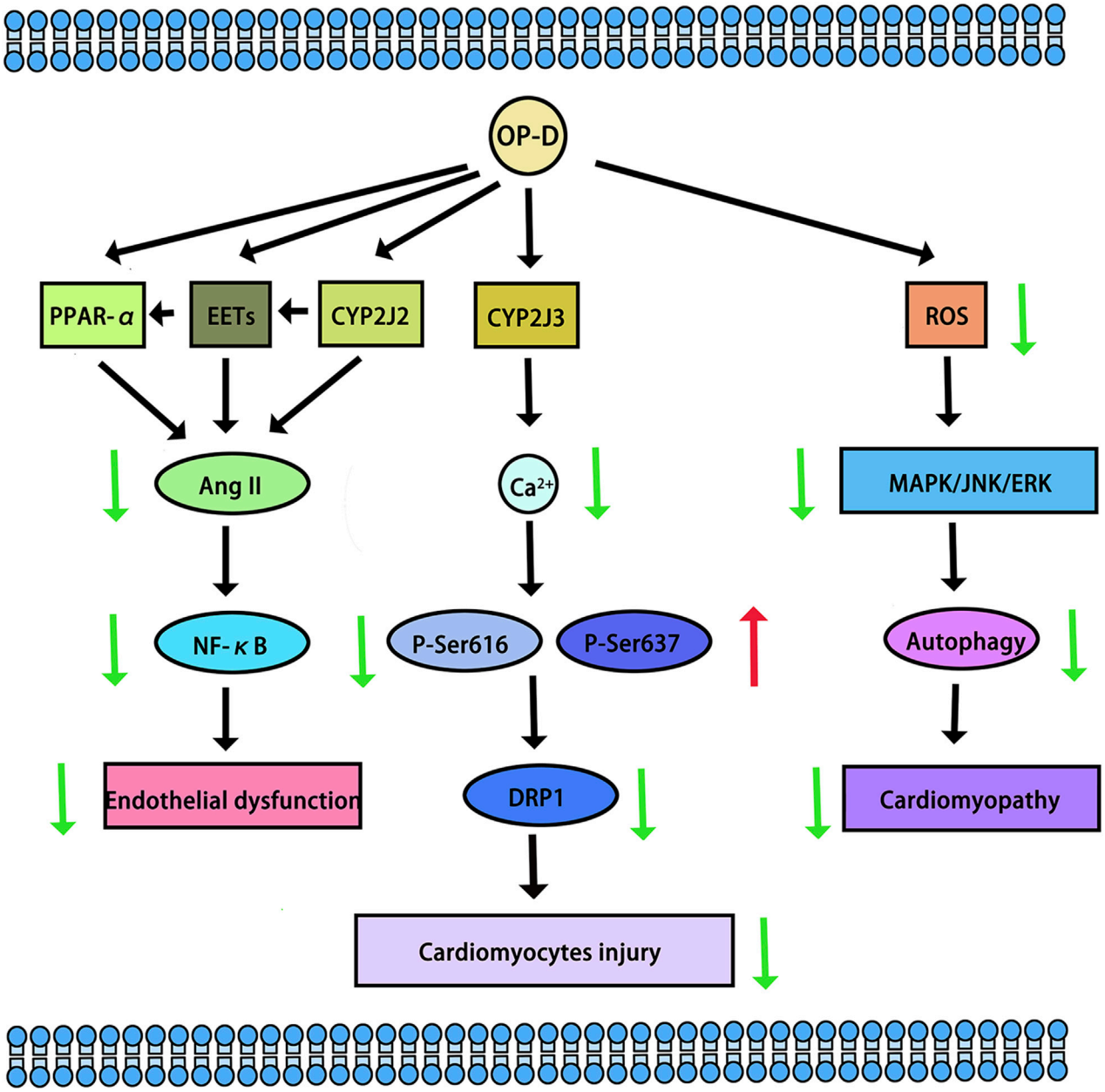

FIGURE 4
The cardiovascular protective mechanisms of OP-D. The red arrow means up-regulation, the green arrow means down-regulation.

One possible explanation for OP-D's beneficial effects on NAFLD was the NF-κB signaling pathway (Huang et al., 2023).

Systemic lupus erythematosus (SLE) is an autoimmune disease. The activation of autoreactive B cell differentiation will promote the development of SLE. In MRL/lpr mice, the treatment of OP-D decreased the serum levels of IgG, IgM, and anti-dsDNA autoantibodies. Meanwhile, OP-D improved the progression of SLE by decreasing the number of B cells (Nie et al., 2023).

OP-D is a potential anti-pulmonary fibrosis drug. *In vivo* and *in vitro* models, OP-D inhibited epithelial-mesenchymal transition and excessive extracellular matrix deposition, accelerated lung fibroblast apoptosis, and prevented lung fibroblasts from differentiating into myofibroblasts. According to multi-omics techniques and bioinformatics analysis, the AKT/GSK3β pathway was inhibited by OP-D. OP-D combined with PI3K/AKT inhibitors could effectively alleviate pulmonary fibrosis (Bao et al., 2023).

OP-D can reduce the excitability of airway parasympathetic ganglion neurons. The hyperpolarizing effect of OP-D on paratracheal neurones by activating the potassium conductance might explain the mechanisms of the antitussive effect (Ishibashi et al., 2001). By directly affecting airway epithelial cells, OP-D can also enhance mucin secretion and production (Park et al., 2014).

TABLE 1 Pharmacological activities of OP-D.

Subjects	Pharmacologic action	Function	Ref.
Male C57BL/6 J mice IEC-6 cells	Anti-colitis	GSH↑ SOD↑ TNF-α↓ IL-1β↓ IL-6↓	Wang et al. (2022)
Male Sprague Dawley rats	Anti-diabetic nephropathy	GSH↑ SOD↑ CAT↑ TNF-α↓ TNF-α↓	Qiao et al. (2020)
MLE-12 cells	Anti-lung inflammation	TNF-α↓ IL-1β↓ IL-6↓ IL-8↓	Wang J. et al. (2020)
Female BALB/c mice HaCaT cells	Anti-atopic dermatitis	TNF-α↓ IL-1β↓ IL-6↓	An et al. (2020)
MDA-MB-435 cells	Anti-breast cancer	Cells proliferation↓ invasion↓ migration↓	Zhang et al. (2015a)
MDA-MB-231 cells	Anti-breast cancer	Cells proliferation↓ invasion↓ migration↓	Zhu et al. (2020)
MCF-7 cells	Anti-breast cancer	apoptosis↑	Zang et al. (2016)
AMC-HN-8 cells	Anti-laryngocarcinoma	Cells proliferation↓ apoptosis↑	Yan et al. (2019)
HCT116 cells	Anti-colorectal cancer	Cells proliferation↓ apoptosis↑	Ko et al. (2022)
H1299 cells A549 cells	Anti-lung cancer	Cells proliferation↓ apoptosis↑	Lee et al. (2018a)
H1299 cells A549 cells H460 cells	Anti-lung cancer	Cells proliferation↓ apoptosis↑	Lee et al. (2018b)
H9C2 cells	Cardiovascular protection	ROS↓ ATF6α↓ GRP78↓ CHOP↓	Meng et al. (2014)
C57BL/6 J mice H9C2 cells	Cardiovascular protection	Autophagy↓ ROS↓ EF↑ LVFS↑	Zhang et al. (2015b)
Male db/db mice Male C57BL/6 J mice H9C2 cells	Cardiovascular protection	ALT↓ MFN1↑ MFN2↑ OPA1↑	Li et al. (2021)
Human umbilical vein endothelial cells	Cardiovascular protection	ROS↓ HO-1↓ PGC-1α↓	Qian et al. (2010)
Human umbilical vein endothelial cells	Cardiovascular protection	TNF-α↓ IL-6↓ VCAM-1↓	Huang et al. (2017)
Male Sprague-Dawley rats H9C2 cells	Cardiovascular protection	ANP↓ BNP↓ β-MHC↓	Wang et al. (2018)
Male Sprague-Dawley rats	Cardiovascular protection	LDH↓ SOD2↓ CK↓	Huang X. et al. (2018)
H9C2 cells	Cardiovascular protection	SERCA2a↑ PLB↑ RyR2↑ FKBP12.6↑	You et al. (2016)
H9C2 cells	Cardiovascular protection	SERCA2a↑ PLB↑	Wang Y. et al. (2020)
Female BALB/c mice MC3T3-E1 cells RAW264.7 cells	Bone protection	ROS↓ Calciumdeposition↑ ALP ↑ NFATc1↑ TRAP↑ CTX-1↑	Huang et al. (2015)
Male New Zealand rabbits Rabbit osteoblasts	Bone protection	ALP↑ Runx2↑ Osterix↑ Col 1↑ OPN↑ SOD↓ ROS↓	Ma et al. (2018)
Cdh5-Cre transgenic mice Human microvascular endothelial cells	Bone protection	CD31↑ EMCN↑ JUNB↑ VEGFA↑ VEGFB↑ PDGFA↑ PDGFB↑	Yang et al. (2020)
Male ApoE$^{-/-}$ mice Male C57BL/6 mice Human LO2 cells	Anti-atherosclerosis	TG↓ TC↓ LDL-C↓ MDA↓ LDH↓ ALT↓ AST↓	Zhang et al. (2021)
Male C57BL/6 J mice	Anti-obesity	TG↓ TC↓ LDL-C↓ TNF-a↓ MCP-1↓ ALT↓ AST↓	Chen et al. (2018)
Male C57BL/6 J mice Primary hepatocytes	Anti-NAFLD	TG↓ TC↓ FFA↓ LDL-C↓ SOD↓ TNF-α↓ IL-1β↓ IL-6↓	Huang et al. (2023)
Female MRL/lpr mice	Anti-systemic lupus erythematosus	Proteinuria levels↓ Serum creatinine levels↓	Nie et al. (2023)
Male C57BL/6 J mice	Anti-pulmonary fibrosis	GSH-PX↑ MDA↓ HIF-1α↓ IL-6↓ TNF-α↓ α-SMA↓	Bao et al. (2023)
Wistar rats	Antitussive effect	Excitability of airway parasympathetic ganglion neurons ↓	Ishibashi et al. (2001)
NCI-H292 cells	Antitussive effect	Mucin production and secretion↑	Park et al. (2014)

9 Discussion

As mentioned above, OP-D has a wide range of pharmacological effects, including anti-cancer, anti-inflammatory, bone protection and cardiovascular protection. OP-D has a potential value in the prevention and treatment of many diseases (Table 1). In addition, OP-D also plays an important role in other aspects. Ginsenosides Rb1, Ginsenosides Rd, rosuvastatin, and glycyrrhizic acid significantly reduce the uptake of OP-D in liver (Zhang et al., 2017; Liu et al., 2018). As an ingredient of vaccines, adjuvants can directly stimulate or promote the immune responses. It has been discovered that OP-D works well as a vaccine adjuvant. The problems of the low solubility and toxicity of OP-D can be effectively overcome by using a low-energy emulsification method to prepare nanoemulsion OP-D (Tong et al., 2018; Luo et al., 2022). Interestingly, OP-D and OP-D′ act as isomers of each other. *In vitro* studies showed that only OP-D′ induced a hemolysis reaction, whereas *in vivo*, both OP-D and OP-D′ were found to cause hemolysis. The hemolytic effects of OP-D and OP-D′ were thought to be closely associated with disruptions in phospholipid metabolism (Xu et al., 2021). In some difficult problems, such as the osteointegration of titanium alloy implants, the studies have also confirmed the application value of OP-D. Nevertheless, there are still many questions of OP-D that need to be discussed. On the one hand, the toxicity testing of OP-D in animals is currently insufficient. Yu J et al. assess the long-term toxicokinetic profiles of the OP-D in SM-I. They found that OP-D exhibited an extremely low exposure level and a rapid elimination rate after injection (Yu et al., 2014). As the main component of *Ophiopogon japonicus*, we speculate that OP-D may have side effects such as gastrointestinal reactions and allergic reactions like *Ophiopogon japonicus*. On the other hand, the anti-cancer effects and mechanisms of OP-D require further investigation. Future research is anticipated to ensure OP-D's safety and efficacy in protecting human health.

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K-qC: Writing–original draft. S-zW: Writing–review and editing. H-bL: Writing–review and editing. XL: Writing–review and editing.

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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

An, E. J., Kim, Y., Lee, S. H., Choi, S. H., Chung, W. S., and Jang, H. J. (2020). Ophiopogonin D ameliorates DNCB-induced atopic dermatitis-like lesions in BALB/c mice and TNF-α- inflamed HaCaT cell. *Biochem. Biophys. Res. Commun.* 522 (1), 40–46. doi:10.1016/j.bbrc.2019.10.190

Bao, S., Chen, T., Chen, J., Zhang, J., Zhang, G., Hui, Y., et al. (2023). Multi-omics analysis reveals the mechanism of action of ophiopogonin D against pulmonary fibrosis. *Phytomedicine* 121, 155078. doi:10.1016/j.phymed.2023.155078

Chen, M. H., Chen, X. J., Wang, M., Lin, L. G., and Wang, Y. T. (2016). *Ophiopogon japonicus*--A phytochemical, ethnomedicinal and pharmacological review. *J. Ethnopharmacol.* 181, 193–213. doi:10.1016/j.jep.2016.01.037

Chen, S., Li, X., Liu, L., Liu, C., and Han, X. (2018). Ophiopogonin D alleviates high-fat diet-induced metabolic syndrome and changes the structure of gut microbiota in mice. *Faseb J.* 32 (3), 1139–1153. doi:10.1096/fj.201700741RR

Dong, W., Dong, Q., and Ding, H. (2021). Ophiopogonin B induces reactive oxygen species-dependent apoptosis through the Hippo pathway in nasopharyngeal carcinoma. *Mol. Med. Rep.* 24 (1), 534. doi:10.3892/mmr.2021.12173

Fang, J., Wang, X., Lu, M., He, X., and Yang, X. (2018). Recent advances in polysaccharides from *Ophiopogon japonicus* and Liriope spicata var. prolifera. *Int. J. Biol. Macromol.* 114, 1257–1266. doi:10.1016/j.ijbiomac.2018.04.022

Huang, X. Y., Wang, Y. G., Wang, Y., and Gao, Y. (2018). Effect of ophiopogonin D in resisting vascular endothelial cell apoptosis induced by AngⅡthrough up-regulating CYP2J2/EETs. *Zhongguo Zhong Yao Za Zhi* 43 (2), 377–384. doi:10.19540/j.cnki.cjcmm.20171027.019

Huang, Q., Gao, B., Wang, L., Zhang, H. Y., Li, X. J., Shi, J., et al. (2015). Ophiopogonin D: a new herbal agent against osteoporosis. *Bone* 74, 18–28. doi:10.1016/j.bone.2015.01.002

Huang, X., Ji, Q., She, C. Y., Cheng, Y., Zhou, J. R., and Wu, Q. M. (2023). Ophiopogonin D ameliorates non-alcoholic fatty liver disease in high-fat diet-induced obese mice by improving lipid metabolism, oxidative stress and inflammatory response. *Exp. Ther. Med.* 26 (3), 418. doi:10.3892/etm.2023.12116

Huang, X., Wang, Y., Wang, Y., Yang, L., Wang, J., and Gao, Y. (2018). Ophiopogonin D reduces myocardial ischemia-reperfusion injury via upregulating CYP2J3/EETs in rats. *Cell. Physiol. Biochem.* 49 (4), 1646–1658. doi:10.1159/000493500

Huang, X., Wang, Y., Zhang, Z., and Chen, X. (2017). Ophiopogonin D and EETs ameliorate Ang II-induced inflammatory responses via activating PPARα in HUVECs. *Biochem. Biophys. Res. Commun.* 490 (2), 123–133. doi:10.1016/j.bbrc.2017.06.007

Ishibashi, H., Mochidome, T., Okai, J., Ichiki, H., Shimada, H., and Takahama, K. (2001). Activation of potassium conductance by ophiopogonin-D in acutely dissociated rat paratracheal neurones. *Br. J. Pharmacol.* 132 (2), 461–466. doi:10.1038/sj.bjp.0703818

Jiang, M., Kang, L., Wang, Y., Zhao, X., Liu, X., Xu, L., et al. (2014). A metabonomic study of cardioprotection of ginsenosides, schizandrin, and ophiopogonin D against acute myocardial infarction in rats. *BMC Complement. Altern. Med.* 14, 350. doi:10.1186/1472-6882-14-350

Ko, H. M., Jee, W., Lee, D., Jang, H. J., and Jung, J. H. (2022). Ophiopogonin D increase apoptosis by activating p53 via ribosomal protein L5 and L11 and inhibiting the expression of c-Myc via CNOT2. *Front. Pharmacol.* 13, 974468. doi:10.3389/fphar.2022.974468

Lee, J. H., Kim, C., Lee, S. G., Sethi, G., and Ahn, K. S. (2018a). Ophiopogonin D, a steroidal glycoside abrogates STAT3 signaling cascade and exhibits anti-cancer activity by causing GSH/GSSG imbalance in lung carcinoma. *Cancers (Basel)* 10 (11), 427. doi:10.3390/cancers10110427

Lee, J. H., Kim, C., Lee, S. G., Yang, W. M., Um, J. Y., Sethi, G., et al. (2018b). Ophiopogonin D modulates multiple oncogenic signaling pathways, leading to suppression of proliferation and chemosensitization of human lung cancer cells. *Phytomedicine* 40, 165–175. doi:10.1016/j.phymed.2018.01.002

Lei, F., Weckerle, C. S., and Heinrich, M. (2021). Liriopogons (genera ophiopogon and liriope, asparagaceae): a critical review of the phytochemical and pharmacological research. *Front. Pharmacol.* 12, 769929. doi:10.3389/fphar.2021.769929

Lei, S., Feng, Y., Huang, P., Chen, B., Bao, K., Wu, Q., et al. (2022). Ophiopogonin D'-induced mitophagy and mitochondrial damage are associated with dysregulation of the PINK1/Parkin signaling pathway in AC16 cells. *Toxicology* 477, 153275. doi:10.1016/j.tox.2022.153275

Liu, J. C., Zhao, Q. F., Zhang, L., Yu, B. Y., Li, F., and Kou, J. P. (2023). Ruscogenin alleviates myocardial ischemia via myosin IIA-dependent mitochondrial fusion and fission balance. *Am. J. Chin. Med.* 51 (7), 1879–1904. doi:10.1142/S0192415X23500830

Liu, X., Chen, L., Liu, M., Zhang, H., Huang, S., Xiong, Y., et al. (2018). Ginsenoside Rb1 and Rd remarkably inhibited the hepatic uptake of ophiopogonin D in Shenmai injection mediated by OATPs/oatps. *Front. Pharmacol.* 9, 957. doi:10.3389/fphar.2018.00957

Li, W., Ji, L., Tian, J., Tang, W., Shan, X., Zhao, P., et al. (2021). Ophiopogonin D alleviates diabetic myocardial injuries by regulating mitochondrial dynamics. *J. Ethnopharmacol.* 271, 113853. doi:10.1016/j.jep.2021.113853

Luo, X., Tong, Y., Zeng, X., Ye, Y., Yang, Y., Song, Z., et al. (2022). *In vitro* cellular activity evaluation of the nanoemulsion vaccine adjuvant ophiopogonin D. *J. Vis. Exp.* 190. doi:10.3791/64291

Ma, X. Y., Wen, X. X., Yang, X. J., Zhou, D. P., Wu, Q., Feng, Y. F., et al. (2018). Ophiopogonin D improves osteointegration of titanium alloy implants under diabetic conditions by inhibition of ROS overproduction via Wnt/β-catenin signaling pathway. *Biochimie* 152, 31–42. doi:10.1016/j.biochi.2018.04.022

Meng, C., Yuan, C. H., Zhang, C. C., Wen, M. D., Gao, Y. H., Ding, X. Y., et al. (2014). Ophiopogonin D protects cardiomyocytes against doxorubicin-induced injury through suppressing endoplasmic reticulum stress. *Yao Xue Xue Bao* 49 (8), 1117–1123.

Nie, Y., Li, C., and Sun, N. (2023). Ophiopogonin D attenuates the progression of murine systemic lupus erythematosus by reducing B cell numbers. *J. Biochem. Mol. Toxicol.* 37 (7), e23361. doi:10.1002/jbt.23361

Park, S. H., Lee, H. J., Ryu, J., Son, K. H., and Kwon, S. Y. (2014). Effects of ophiopogonin D and spicatoside A derived from Liriope Tuber on secretion and production of mucin from airway epithelial cells. *Phytomedicine* 21 (2), 172–176. doi:10.1016/j.phymed.2013.08.013

Qian, J., Jiang, F., Wang, B., Yu, Y., Zhang, X., Yin, Z., et al. (2010). Ophiopogonin D prevents H2O2-induced injury in primary human umbilical vein endothelial cells. *J. Ethnopharmacol.* 128 (2), 438–445. doi:10.1016/j.jep.2010.01.031

Qiao, Y., Jiao, H., Wang, F., and Niu, H. (2020). Ophiopogonin D of *Ophiopogon japonicus* ameliorates renal function by suppressing oxidative stress and inflammatory response in streptozotocin-induced diabetic nephropathy rats. *Braz J. Med. Biol. Res.* 53 (7), e9628. doi:10.1590/1414-431X20209628

Ren-Ping, Z., Sen-Sen, L., Yuan, S. T., Yu, B. Y., Bai, X. S., Sun, L., et al. (2014). DT-13, a saponin of dwarf lilyturf tuber, exhibits anti-cancer activity by down-regulating C-C chemokine receptor type 5 and vascular endothelial growth factor in MDA-MB-435 cells. *Chin. J. Nat. Med.* 12 (1), 24–29. doi:10.1016/S1875-5364(14)60005-4

Tang, X. L., Lin, Y., Wang, Y. G., and Gao, Y. (2021). Effects of ophiopogonin D on fatty acid metabolic enzymes in cardiomyocytes. *Zhongguo Zhong Yao Za Zhi* 46 (14), 3672–3677. doi:10.19540/j.cnki.cjcmm.20210311.401

Tong, Y. N., Yang, L. Y., Yang, Y., Song, Z., Peng, L. S., Gao, J. N., et al. (2018). An immunopotentiator, ophiopogonin D, encapsulated in a nanoemulsion as a robust adjuvant to improve vaccine efficacy. *Acta Biomater.* 77, 255–267. doi:10.1016/j.actbio.2018.07.034

Wang, J., Wang, N. N., Ge, Y. X., Tan, H. L., Ma, Z. C., Wang, Y. G., et al. (2019). Ophiopogonin D protects cardiomyocytes against ophiopogonin D'-induced injury through suppressing endoplasmic reticulum stress. *Zhongguo Zhong Yao Za Zhi* 44 (9), 1876–1881. doi:10.19540/j.cnki.cjcmm.20190102.003

Wang, J., You, W., Wang, N., Zhou, W., Ge, Y., Ma, Z., et al. (2020). Ophiopogonin D increases SERCA2a interaction with phospholamban by promoting CYP2J3 upregulation. *Oxid. Med. Cell. Longev.* 2020, 8857906. doi:10.1155/2020/8857906

Wang, L., Gu, H., and Chen, K. (2023). Effect of ophiopogonin D on the pharmacokinetics and transport of cryptotanshinone during their co-administration and the potential mechanism. *Chem. Biol. Drug Des.* 102 (3), 557–563. doi:10.1111/cbdd.14269

Wang, L., Yang, H., Qiao, L., Liu, J., Liao, X., Huang, H., et al. (2022). Ophiopogonin D inhibiting epithelial NF-κB signaling pathway protects against experimental colitis in mice. *Inflammation* 45 (4), 1720–1731. doi:10.1007/s10753-022-01655-8

Wang, Y., Huang, X., Ma, Z., Chen, X., and Gao, Y. (2018). Ophiopogonin D alleviates cardiac hypertrophy in rat by upregulating CYP2J3 *in vitro* and suppressing inflammation *in vivo*. *Biochem. Biophys. Res. Commun.* 503 (2), 1011–1019. doi:10.1016/j.bbrc.2018.06.110

Wang, Y., Li, D., Song, L., and Ding, H. (2020). Ophiopogonin D attenuates PM2.5-induced inflammation via suppressing the AMPK/NF-κB pathway in mouse pulmonary epithelial cells. *Exp. Ther. Med.* 20 (6), 139. doi:10.3892/etm.2020.9268

Xia, C. H., Sun, J. G., Hao, H. P., Wang, G. J., Yan, B., Gu, S. H., et al. (2008). Quantitative determination of ophiopogonin d by liquid chromatography/electrospray ionization mass spectrometry and its pharmacokinetics in rat. *Planta Med.* 74 (15), 1832–1836. doi:10.1055/s-0028-1088339

Xia, C., Wang, G., Sun, J., Hao, H., Xiong, Y., Gu, S., et al. (2008). Simultaneous determination of ginsenoside Rg1, Re, Rd, Rb1 and ophiopogonin D in rat plasma by liquid chromatography/electrospray ionization mass spectrometric method and its application to pharmacokinetic study of 'SHENMAI' injection. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 862 (1-2), 72–78. doi:10.1016/j.jchromb.2007.11.020

Xu, H. H., Jiang, Z. H., Huang, C. S., Sun, Y. T., Xu, L. L., Tang, X. L., et al. (2021). Global metabolomic and lipidomic analysis reveals the potential mechanisms of hemolysis effect of Ophiopogonin D and Ophiopogonin D' *in vivo*. *Chin. Med.* 16 (1), 3. doi:10.1186/s13020-020-00412-z

Yang, M., Li, C. J., Xiao, Y., Guo, Q., Huang, Y., Su, T., et al. (2020). Ophiopogonin D promotes bone regeneration by stimulating CD31(hi) EMCN(hi) vessel formation. *Cell. Prolif.* 53 (3), e12784. doi:10.1111/cpr.12784

Yan, Z., Liu, G., Liang, M., and Xu, Y. (2019). Ophiopogonin D inhibits cell proliferation and induces apoptosis of human laryngocarcinoma through downregulation of cyclin B1 and MMP-9 and upregulation of p38-MAPK signaling. *Oncol. Lett.* 17 (2), 1877–1882. doi:10.3892/ol.2018.9788

You, W. T., Zhou, T., Ma, Z. C., Liang, Q., Xiao, C., Tang, X., et al. (2016). Ophiopogonin D maintains Ca2+ homeostasis in rat cardiomyocytes *in vitro* by upregulating CYP2J3/EETs and suppressing ER stress. *Acta Pharmacol. Sin.* 37 (3), 368–381. doi:10.1038/aps.2015.146

Yuan, C., Peng, F., Tao, S., Sha, X. F., Xiong, M., Chen, Y. Y., et al. (2019). The complete chloroplast genome of Ophiopogon japonicus, an ornamental and medicinal plant. *Mitochondrial DNA B Resour.* 4 (2), 2843–2844. doi:10.1080/23802359.2019.1659110

Yu, J., Xin, Y. F., Gu, L. Q., Gao, H. Y., Xia, L. J., You, Z. Q., et al. (2014). One-month toxicokinetic study of SHENMAI injection in rats. *J. Ethnopharmacol.* 154 (2), 391–399. doi:10.1016/j.jep.2014.04.014

Zang, Q. Q., Zhang, L., Gao, N., and Huang, C. (2016). Ophiopogonin D inhibits cell proliferation, causes cell cycle arrest at G2/M, and induces apoptosis in human breast carcinoma MCF-7 cells. *J. Integr. Med.* 14 (1), 51–59. doi:10.1016/S2095-4964(16)60238-8

Zeng, P., Li, J., Chen, Y., and Zhang, L. (2019). The structures and biological functions of polysaccharides from traditional Chinese herbs. *Prog. Mol. Biol. Transl. Sci.* 163, 423–444. doi:10.1016/bs.pmbts.2019.03.003

Zhang, G. C., Wang, Y. H., Ruan, P. P., Zhang, Z. Q., Shen, N. N., Liu, Y. F., et al. (2022). Protective effect of ophiopogonin D against isoproterenol-induced cardiomyocyte injury and targets. *Zhongguo Zhong Yao Za Zhi* 47 (10), 2721–2728. doi:10.19540/j.cnki.cjcmm.20211216.702

Zhang, Y. X., Qu, S. S., Zhang, L. H., Gu, Y. Y., Chen, Y. H., Huang, Z. Y., et al. (2021). The role of ophiopogonin D in atherosclerosis: impact on lipid metabolism and gut microbiota. *Am. J. Chin. Med.* 49 (6), 1449–1471. doi:10.1142/S0192415X21500683

Zhang, Y. Y., Meng, C., Zhang, X. M., Yuan, C. H., Wen, M. D., Chen, Z., et al. (2015b). Ophiopogonin D attenuates doxorubicin-induced autophagic cell death by relieving mitochondrial damage *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.* 352 (1), 166–174. doi:10.1124/jpet.114.219261

Zhang, W., Xiong, X., Chen, L., Liu, M., Xiong, Y., Zhang, H., et al. (2017). Hepatic uptake mechanism of ophiopogonin D mediated by organic anion transporting polypeptides. *Eur. J. drug metabolism Pharmacokinet.* 42 (4), 669–676. doi:10.1007/s13318-016-0384-8

Zhang, Y., Han, Y., Zhai, K., Sun, M., Liu, J., Yu, B., et al. (2015a). Ophiopogonin-D suppresses MDA-MB-435 cell adhesion and invasion by inhibiting matrix metalloproteinase-9. *Mol. Med. Rep.* 12 (1), 1493–1498. doi:10.3892/mmr.2015.3541

Zhu, X., Wang, K., and Chen, Y. (2020). Ophiopogonin D suppresses TGF-β1-mediated metastatic behavior of MDA-MB-231 breast carcinoma cells via regulating ITGB1/FAK/Src/AKT/β-catenin/MMP-9 signaling axis. *Toxicol Vitro* 69, 104973. doi:10.1016/j.tiv.2020.104973

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Nutraceutical formulation based on a synergic combination of melatonin and palmitoylethanolamide for the management of allergic events

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The management of allergic events is a growing global health issue, especially in industrialized countries. This disease is an immune-mediated process, regulated by the interaction of IgE with an allergen, resulting in mast cell activation, which concerns the release of several immune-inflammatory modulators, i.e., histamine, β-hexosaminidase, COX-2, IL-6, and TNF-α, responsible for the main allergic-reaction associated symptoms. The aim of the present study was the efficacy evaluation of an alternative remedy, an innovative nutraceutical formulation (NF) based on the synergic combination of melatonin (MEL) and palmitoylethanolamide (PEA) for the prevention and treatment of immune disease. At first, the intestinal bioaccessibility of PEA and MEL in NF was assessed at 1.6 and 36%, respectively. Then the MEL and PEA ability to modulate the release of immune-inflammatory modulators in the human mast cell line (HMC-1.2) at their bioaccessible concentration was investigated. Our results underline that NF treatment was able to reduce COX-2 mRNA transcription levels (−30% vs. STIM, $p < 0.0001$) in stimulated HMC-1.2 and to contract COX-2 enzymatic activity directly (IC_{50}: 152 μg/mL). Additionally, NF showed valuable ability in reducing histamine and β-hexosaminidase release in stimulated HMC-1.2, as well as in decreasing TNF-α and IL-6 mRNA transcription levels and protein production.

KEYWORDS

melatonin, palmitoylethanolamide, synergic combination, nutraceutical formulation, immune response, human mast cell

1 Introduction

Allergic diseases represent a prevalent global health issue that has seen a significant rise in incidence, particularly with the onset of industrialization (1). It is estimated that more than 30% of the global population is affected by one or more allergic-related disorders, especially in Western countries (1). The most common and diffused allergic pathology worldwide include allergies to pollen, house dust mites, animal dander, and obviously food allergens (2). Allergy

is an immune-mediated disease, classified as one of the four types of hypersensitivity reactions, formally known as type I (or immediate) hypersensitivity reaction. From the molecular point of view, it is mainly mediated by the interaction of IgE with allergens, leading to a consequent release of mast cell and basophil-derived mediators into the blood system. The allergic reaction could be divided into two different phases, an early step starts from the moment in which the allergen binds to the mast cell with its specific IgE receptors, causing mast cell degranulation, which in turn releases the main molecule mediators responsible for the symptoms typical of immune responses, such as histamines, proteases, proteoglycans, and tumor necrosis factor-alpha (TNF-α) (3). The late phase of the allergic event usually begins within 4–6 h after the allergen exposure and involves the further release of additional inflammatory modulators, including IL-6, IL-1β, and IL-13.

Even though immune events can be accurately diagnosed and effectively treated with conventional drug-based therapy, concerns persist regarding the potential side effects of immunomodulatory drugs and the increasing costs associated with medical treatment (4). Therefore, the identification of innovative and alternative natural sources of bioactive compounds useful for the management and prevention of allergic and allergic-related inflammatory diseases is a worldwide challenge. In this context, several natural molecules have proven a remarkable anti-allergic and immunomodulatory potential. For example, several food-derived compounds such as curcumin (5), resveratrol (6), and quercetin (7) have shown valuable immunoregulatory effects both *in vitro* and in clinical models.

In this sense, other natural compounds, palmitoylethanolamide (PEA) and melatonin (MEL) that are both endogenously produced and could be integrated by external supplementation have also shown a valuable potential in the management of allergic events.

Specifically, PEA is an important endogenous lipid mediator belonging to the fatty acid ethanolamine class (8). It was well reported that PEA could effectively contrast the immune response by downregulating mast-cell degranulation (9, 10). Interestingly, PEA is not only endogenously produced, but it could be introduced also with diet. Specifically, PEA is contained in a wide type of food matrices, including the lipid fraction of egg yolk, peanut oil, some varieties of legumes, such as peas and beans (11), as well as in vegetables including tomatoes and potatoes (12). In this regard, several studies underlined the PEA-based treatment as an alternative remedy for the care of immune-related disorders, including allergic dermatitis (13, 14), attenuating airway allergic symptoms in rats (15), and reducing the symptoms of allergic asthma in humans (16).

MEL (N-acetyl-5-methoxy-tryptamine) is a natural substance largely diffused in the plant world (17). It plays a key role in the regulation of several plant physiological functions, such as plant growth, photosynthesis (18), seed germination (19), and protection against abiotic/biotic stress agents (18). In humans, MEL is a hormone produced mainly in the pineal gland, although its valuable amount is also released from other human body districts, such as the gastrointestinal system, epithelial hair follicles, skin, retina, salivary glands, platelets, and lymphocytes (20, 21). In mammals, MEL was commonly known for its, extensively studied, pivotal role in circadian cycle regulation (22), for which it was used as a functional ingredient in nutraceutical formulation for the management of sleep disorders. Additionally, MEL has proven a general anti-inflammatory potential, reducing the expression levels of the most relevant inflammatory mediators, i.e., metallo-proteinase-9 and -2, TNF-α, cyclooxygenase-2 (Cox-2), nitric oxide synthase, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), interleukin 4 (IL-4), and C-reactive protein, in different *in vitro* and pre-clinical inflammation models (22). This evidence was clinically corroborated by the results of a recent systematic review summarizing the effects of MEL-based treatment on the main inflammatory markers plasma levels recorded by 31 different clinical trials conducted on patients affected by a different type of chronic inflammatory disease (23). They described that the IL-6, IL-8, and TNF-α plasma concentrations were statistically decreased after MEL-based supplementation, without significant side effects showed (23). Directly connected with MEL anti-inflammatory activity, several studies have additionally reported the potential MEL activity for the management of immune reactions. Specifically, Liu and colleagues have described that MEL treatment in mice reduced the inflammation state in the allergic airway mice model (24), these results were in line with those described by others, who reported that MEL treatment reduced total nasal symptom scores and serum ovalbumin-specific IgE, in ovalbumin-induced allergic rhinitis model in rats (25). Moreover, additional studies described the positive effects of MEL supplementation on dermatologic allergic diseases, including atopic dermatitis and chronic spontaneous urticaria (26). As described for PEA, MEL could also be introduced with diet. The main MEL food sources include tomato (32 pg./g), red pepper (93 ng/g), strawberries (12 pg./g), mango (699 pg./g), apples (48 pg./g), oranges (150 pg./g), bananas (378 pg./g), and particularly rice (1,006 pg./g) (27, 28).

Based on these considerations, the present study aimed to evaluate the immunomodulatory activity of an innovative nutraceutical formulation (NF) based on the MEL and PEA combination. At first, to assess the effective NF health-promoting effects, the NF intestinal bioaccessibility was calculated through the application of an *in vitro* digestion protocol. After the evaluation of the bioaccessibility of NF, PEA, and MEL, the NF formulation and the pure compounds MEL and PEA were tested on human mast cells (HMC-1.2). To evaluate NF treatment immune-modulatory and allergic-related inflammation effects on HMC, the histamine and β-hexosaminidase release was evaluated, as well as the expression and release of TNF-α and IL-6. Finally, the ability of NF to reduce at the transcriptional level the expression of COX-2, and their capacity to modulate COX-2 enzymatic activity was further explored.

2 Materials and methods

2.1 Nutraceutical formulation

The studied NF was based on an innovative combination of Ph. Eur. PEA and MEL (ratio 6,000:1). PEA and MEL doses were 300 mg of PEA and 50 μg of MEL. Ph. Eur. PEA powder was a synthetic compound made up of raw materials of vegetable origin and was purchased from Farmalabor SRL (Canosa di Puglia, Italy). MEL powder was a synthetic compound purchased from Sigma-Aldrich (Milan, Italy). The powders were mixed in the selected ratio to obtain a homogeneous mixture, which was the formulation NF.

2.2 Simulated gastro-intestinal digestion of melatonin/PEA formulation

A dose of NF formulation was subjected to successive oral, gastric, and intestinal *in vitro* digestion, following a harmonized procedure

reported by the COST action INFOGEST network. Simulated digestion fluids, namely gastric fluid (SGF), salivary fluid (SSF), and intestinal fluid (SIF) were prepared according to our previously published protocol (27). Briefly, 1 g of the formulation was mixed with 3.5 mL of SSF at the temperature of 37°C. Next, 0.5 mL of α-amylase solution (75 U/mL), 25 μL of 0.3 M calcium chloride, and 975 μL of water were added and mixed. A solution of 1 M hydrochloride acid (HCl) was added to reduce the pH of the solution to 7, and the mixture was incubated at 37°C for 2 min in an orbital shaker bath at 200 rpm. Then, for simulating gastric conditions, 7.5 mL SGF, 1.6 mL pepsin solution (2000 U/mL), 5 μL 0.3 M calcium chloride, and 695 μL of water were added and thoroughly mixed. Next, a solution of 1 M HCl was used to modify the pH of the solution to 3, and the mixture was incubated for 120 min at 37°C in an orbital shaker bath at 200 rpm. Afterward, to recreate the intestinal stage, 11 mL SIF, 2.5 mL bile salt solution (65 mg/mL), 5 mL pancreatin solution (100 U/mL of trypsin activity), 1.3 mL of water, and 40 μL of 0.3 M calcium chloride were added. After that, the solution was mixed, and 1 M NaOH was added to modify the pH of the mixture to 7. The solution was incubated at 37°C for 120 min in an orbital shaker bath at 200 rpm. At the end of the incubation, the samples were centrifuged for 10 min at 37°C at 9000 rpm. Finally, the supernatant was collected and diluted until 30% of acetonitrile to block the enzymatic activity (29). Then, the samples were freeze-dried and the powder obtained was subjected to a hydroalcoholic extraction to remove salts and enzymes added during the simulated gastrointestinal digestion before the analysis. Specifically, to the solid residue, a volume of 10 mL methanol was added. The mixture was vortexed for 1 min and placed in an ultrasonic bath (Branson Fisher Scientific 150 E Sonic Dismembrator) for 10 min. Samples were then shaken on an orbital shaker (Sko-DXL, Argolab, Carpi, Italy) at 600 rpm for 10 min and centrifuged at 9000 rpm for 10 min. The supernatants were collected and stored at 4°C protected from the light. The obtained pellets were re-extracted with 10 mL methanol using the same procedure. Finally, the extracted obtained were evaporated to dryness under a light stream of nitrogen, reconstituted in an aqueous solution at 50% methanol at a concentration of 10 mg/mL, and stored at −20°C until analysis. A blank sample of gastro-intestinal digestion was prepared following the same procedure previously described, in the absence of a matrix to digest.

2.3 PEA and melatonin quantitative analysis by HPLC-DAD

A Jasco Extrema LC-4000 HPLC system (Jasco Inc., Easton, MD, United States), coupled with an autosampler, a binary solvent pump, a diode-array detector (DAD), and a fluorescence detector (FLD), was used for the analysis. The chromatographic analysis was performed according to the following conditions: elution was performed on a Kinetex® C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, United States). The mobile phases were an ammonium acetate buffer 5 mM containing 0.1% formic acid (A) and acetonitrile (B). The elution gradient was performed under the following conditions: 0–5 min, isocratic on 30% phase B; 5–8 min, linear gradient from 30 to 85% B; 8–10 min, linear gradient from 85 to 95% B; 10–15 min, isocratic with 95% B; 15–20 min, isocratic with 30% B for column reconditioning. The separation parameters were as follows: column temperature was set at 30°C, injection volume was 20 μL, and flow rate was set at 1 mL/min. The quantification of PEA was performed at 205 nm with DAD detection, while the quantification of MEL was performed with an excitation wavelength of 280 nm and an emission wavelength of 310 nm.

2.4 HPLC-DAD method validation

2.4.1 Linearity and sensitivity of PEA and MEL HPLC-DAD-FLD analysis

Analytical standards of PEA and MEL were used to develop and validate the HPLC-DAD-FLD method used to evaluate the compound's concentration in the simulated gastrointestinal products. A mixing stock solution of the two standards was prepared at a concentration of 1,000 ppm using HPLC-grade methanol as solvent. A mixing sub-stock solution at a concentration of 500 ppm for PEA and 50 ppm for MEL was prepared by diluting the stock solution with water to obtain a final composition of water/methanol 50:50 (v/v). Three quality control (QC) working solutions (PEA: 500 ppm, 100 ppm, 50 ppm; MEL: 25 ppm, 5 ppm, 1 ppm), eight different calibration working solutions (0.2, 0.4, 0.8, 1.5, 3.0, 6.0, 12.0, and 25 ppm) for MEL analysis and five calibration working solutions (12.5, 25.0, 50.0, 100.0, and 500 ppm) for PEA were prepared by diluting the mixing working solution (PEA at 500 pm and MEL at 50 ppm) with an aqueous solution at 50% methanol. The calibration working solutions were analyzed by HPLC in triplicate. The calibration curves were constructed by plotting the peak area against the standard concentration to evaluate the linearity of the method. Limits of detection (LODs) and limits of quantification (LOQs) were determined to evaluate the sensitivity of the method. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and previously described LODs establishing the minimum concentration at which the analyte can be reliably detected as is defined as the lowest detectable concentration of analyst that the analytical system can reliably distinguish from the background level (S (signal of compound)/N (signal of noise)) = 3, while LOQ is defined as the lowest quantifiable concentration of analyst that can be measured with a standard level of confidence, and it is typically calculated using (S/N) = 10. Potential interferences at the retention time of the analytes were demonstrated in the gastro-intestinal blank solution at a concentration of 20 mg/mL and the mixture of two analytes at the lower limit of quantification (LLOQ). The absence of peaks at the target retention times in the blank was verified to evaluate the selectivity.

2.4.2 Accuracy and precision of MEL and PEA HPLC-DAD-FLD analysis

As recommended by the ICH guidelines (30), to validate an analytical method, it is essential to determine the accuracy (estimated by calculating the % bias) and precision (estimated by calculating the % CV, coefficient of variation %) of the developed method (27). Accuracy (% bias) was calculated by intraday and inter-day analysis of calibration standards. Three different concentrations of the two compounds were injected 3 times per day (intra-day) and once for 3 consecutive days (inter-day). Precision (% CV, coefficient of variation %) was determined by an intraday and inter-day analysis of the two compounds at 3 different concentrations. Each analyte was injected 3 times per day (intra-day) and once for 3 consecutive days (inter-day).

2.4.3 Matrix effect of PEA and MEL extraction

In order to evaluate the efficacy of the extraction process from the lyophilized bioaccessible fraction the matrix effect was investigated. Specifically, it was investigated by calculating the ratio of the peak area in the presence of matrix (matrix spiked with PEA and MEL post extraction) to the peak area in the absence of matrix (PEA and MEL in acetonitrile). The matrix was spiked with the analyte in triplicate with 1 µg (low), 5 µg (medium), and 10 µg (high) for MEL, and with 50 µg (low), 250 µg (medium), and 500 µg (high) for PEA. The ratio was calculated as follows (31):

$$\text{Matrix effect } \% = \frac{\text{Peak area in presence of matrix}}{\text{Peak area in solvent}} \times 100$$

2.4.4 Recovery of PEA and melatonin extraction

The matrix effect was investigated by calculating the ratio of the peak area in the pre-extraction spiked samples (matrix spiked with PEA and MEL pre-extraction) to the peak area in the post-extraction spiked samples (matrix spiked with PEA and MEL post-extraction). The matrix was spiked with the analyte in triplicate with 1 µg (low), 5 µg (medium), and 10 µg (high) for MEL, and with 50 µg (low), 250 µg (medium) and 500 µg (high) for PEA either before (pre-extraction spiked) or after (post-extraction spiked) extraction. The ratio was calculated as follows:

$$\text{Recovery}\% = \frac{\text{Peak area}_{\text{pre-extraction spiked sample}}}{\text{Peak area}_{\text{post-extraction spiked sample}}} \times 100$$

2.5 Cyclooxygenase 2 *in vitro* inhibitory activity assay

The cyclooxygenase 2 (COX-2) inhibitory activity assays were performed using a Cayman Chemical COX Colorimetric Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, United States). The method is able to evaluate the peroxidase activity of COXs by calorimetrically monitoring the appearance of oxidized N, N,N′, N′-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. Samples were divided into a positive control (100% of COX activity), containing 150 µL of 0.1 M Tris–HCl buffer (pH 8.0), 10 µL of heme, and 10 µL of the enzyme, and 10 µL of sample solution at different concentrations. Samples were incubated at 25°C for 5 min, and then 20 µL of arachidonic acid (AA) solution and 20 µL of a colorimetric substrate solution (TMPD) were added. After 2 min of incubation at 25°C, the absorbance at 590 nm was read (32). The COX-2 inhibitory activities were calculated as follows:

$$\%\text{inhibition} = \left(\frac{\text{Activity of COX2} - \text{Activity of COX2}}{\text{Activity of COX2}} \right) \times 100$$

Results were expressed as IC_{50} (inhibitory concentration), which is the concentration of inhibitor required to inhibit COX activity by 50%.

2.6 Cell culture

The human mast cells HMC-1.2 cell line was purchased from Sigma-Aldrich (Milan, Italy) and cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamin, 100 µg/mL streptomycin, 100 U/mL of penicillin, and 0.08% 1-Thioglycerol (all from Gibco; New York, NY, United States) at 37°C in a humidified incubator under 5% CO_2.

2.7 MTT assay

HMC-1.2 cells were seeded in a 96-multiwell plate (5×10^4/well) and treated with increasing concentrations of PEA (2–32 µM), MEL (10–160 nM), and their combination. After 24 h, 25 µL of MTT (Sigma, Milan, Italy) (5 mg/mL in saline) were added and the plate was incubated for 3 h at 37°C in a humidified incubator under 5% CO_2. After that, the plate was centrifuged at 300 RCF for 3 min and the supernatants were discarded. The dark blue crystals were lysed with 100 µL of a solution containing 50% (v/v) N, N-dimethylformamide, and 20% (w/v) sodium dodecylsulfate with an adjusted pH of 4.5. The optical density of each well was measured at 620 nm using a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

2.8 Measurement of β-hexosaminidase

HMC-1.2 cells were plated in a 24-well plate (2×10^5/well) and pretreated with PEA 16 µM, MEL 80 nM, and NF 16 µM + 80 nM and, 1 h later, stimulated with phorbol myristate acetate (PMA) 50 nM and ionophore 1 µM. After 120 min, 50 µL supernatant was transferred into a 96-well plate and β-hexosamine 1 mM (in citrate buffer 0.05 M, pH 4.5) was added. Similarly, once the 24-well plate was centrifuged, the cell pellet was resuspended with the same solution. Following a 37°C incubation of 90 min, the reaction was stopped by adding 0.1 mM Na_2CO_3/$NaHCO_3$. The absorbance was measured at 405 nm using the Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

2.9 Measurement of histamine release

For histamine release quantification, supernatants were assayed by a histamine ELISA kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions.

2.10 RNA extraction and quantitative real-time PCR

HMC-1.2 mast cells (5×10^4/well) were pretreated with PEA, MEL, and NF at the same concentrations as the previous experiment for 1 h and then stimulated with PMA 50 nM and ionophore 1 µM. Cells were incubated for 6 h, and then total RNA was extracted using TRI-Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions. Subsequently, the retro-transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad) and qPCR was carried out in the Bio-Rad CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with the following primers:

IL-6: 5′-CGGAGAGGAGACTTCACAGAG-3′; 5′-ATTTCCA CGATTTCCCAGAG-3′.

TNF-A: 5′-CAGTAGACAGAAGAGCGTGGT-3′; 5′-AGGCAC TCCCCCA AAAGA-3′.

PTGS2: 5′-CTGGCGCTCAGCCATACAG-3′; 5′-CGCACTTAT ACTGGTCAAATCCC-3′.

Relative gene expression was obtained by normalizing the Ct values against the housekeeping gene ribosomal protein S16 transcript level, using the $2^{-\Delta Ct}$ formula.

2.11 Cytokine quantification

IL-6 and TNF-α supernatant levels were measured by BioLegend's LEGENDplex™ bead-based immunoassays HU Th Cytokine Panel (12-plex). The analyses were performed according to the manufacturer's instructions.

2.12 Statistics

Statistical analysis was performed using GraphPad Prism software version 9 (San Diego, CA, United States). For the comparison of two groups, a *t*-test was used, and for the comparison of multiple groups, an ANOVA test was used. The data were shown as mean ± SEM. A *p*-value <0.05 was considered statistically significant and was labeled with *; *p*-values <0.01, 0.001, or 0.0001 were labeled with **, ***, or ****, respectively.

3 Results

3.1 NF bioaccessibility

In order to establish the effectiveness of the proposed formulation, a bioaccessibility study was conducted. Specifically, the NF was subjected to the *in vitro* gastrointestinal digestion protocol, which provides several consecutive steps including, the salivary, gastric, and duodenal phases. The results of the quantitative analysis of the NF duodenal phase are reported in Table 1. Our results indicate that MEL was moderately resistant to the gastrointestinal digestion process, with a calculated duodenal bioaccessibility of 36% vs. undigested matrix. Conversely, PEA was particularly sensible to the gastrointestinal process, leading to a calculated bioaccessible fraction of only 1.59% (Table 1).

3.2 HPLC-DAD-FLD method validation

In order to identify and quantify simultaneously PEA and MEL in the bioaccessible fraction, a single HPLC-DAD-FLD method was developed. These compounds showed clear chromatographic separation, allowing PEA and MEL simultaneous analysis (Figure 1). The method validation was conducted according to the ICH guidelines (27, 30). Specifically, the linearity studies were conducted by preparing calibration curves on a wide range of calibration points (0.1–1,000 ppm). All determinations were acquired in triplicate, and each analytical standard concentration was plotted versus each peak area, resulting in a linear relation described by a correlation factor R^2 of 0.99. The sensitivity of the analytical method was assessed by determining the LOQ and LOD values for both the studied molecules (Table 2). The obtained data

TABLE 1 Assessment of duodenal bioaccessibility of MEL and PEA after *in vitro* digestion of an NF day dose.

Compound	Duodenal bioaccessibility
Melatonin	36%
PEA	1.59%

indicate the developed method was 100 times more sensitive in MEL (LOD: 0.1 ppm; LOQ 0.3 ppm) than PEA detection (LOD: 10 ppm; LOQ: 30 ppm). Since both PEA and MEL LOD and LOQ values were largely below the lowest concentrations detected and quantified in all samples analyzed, this analytical method may be considered a reliable protocol for simultaneous MEL and PEA detection and quantification.

Furthermore, intra-day and inter-day accuracy (% bias), and precision (% CV, coefficient of variation %) (33) were also calculated at four different MEL (25, 5, 1, and 0.3 ppm) and PEA (500, 100, 50, and 30 ppm) concentration levels (Table 3). As expected, the higher % CV values were obtained at the lowest concentrations analyzed for both compounds. Specifically, PEA at the lowest concentration tested (30 ppm) showed an intraday and interday % CV of 4.4 and 8.8, respectively. The same trend was also followed by MEL, where the highest % CV values were acquired at 0.3 ppm MEL concentration level (Table 3). In addition, the % bias ranged from 0.1% to −1.8% for the estimation of intra-day PEA accuracy and from −0.6 to −1.7% for the determination of PEA inter-day accuracy. Higher accuracy was found for MEL detection, described by low % bias values, both intraday and inter-day determinations at all the concentration levels tested (Table 3). Both precision and accuracy displayed values lower than 15%, which is considered a limit criterion normally accepted for analytical method validation (27, 28).

3.3 MEL and PEA recovery and matrix effects in NF duodenal bioaccessible fraction

Due to the complex composition of NF bioaccessible fractions, the assessment of extraction protocol efficacy is highly required. Thus, the recovery (%) and the matrix effect (%) were determined at PEA and MEL different spiked concentrations, 50, 250, and 500 μg for PEA and 1, 5, and 10 μg for MEL (Table 4) in NF bioaccessible fraction. As expected, the higher % matrix effect was calculated at the lowest PEA spiked concentration (50 μg), with a calculated % matrix effect of 5.5%. The same trend was followed also by MEL analysis that led to the most relevant matrix effect of −5.4% at the lowest MEL spiked concentration (1 μg). Regarding the recovery % evaluation, PEA and MEL follow a similar trend. While for PEA detection the highest error was calculated at the maximum PEA spiked concentration (500 μg), the MEL recovery highest error was detected at the minimum melatonin spiked concentration (1 μg) (Table 4). Generally, considering the low values obtained for the % matrix effect and % recovery values close to 100%, the present extraction and analysis methods could be considered as a reproducible and reliable protocol for the simultaneous PEA and MEL quantification by HPLC-DAD-FLD analysis in a complex sample. In particular, % recovery values ranged between 80 and 110%, which are provided by FDA guidelines as values for a good recovery assay (34).

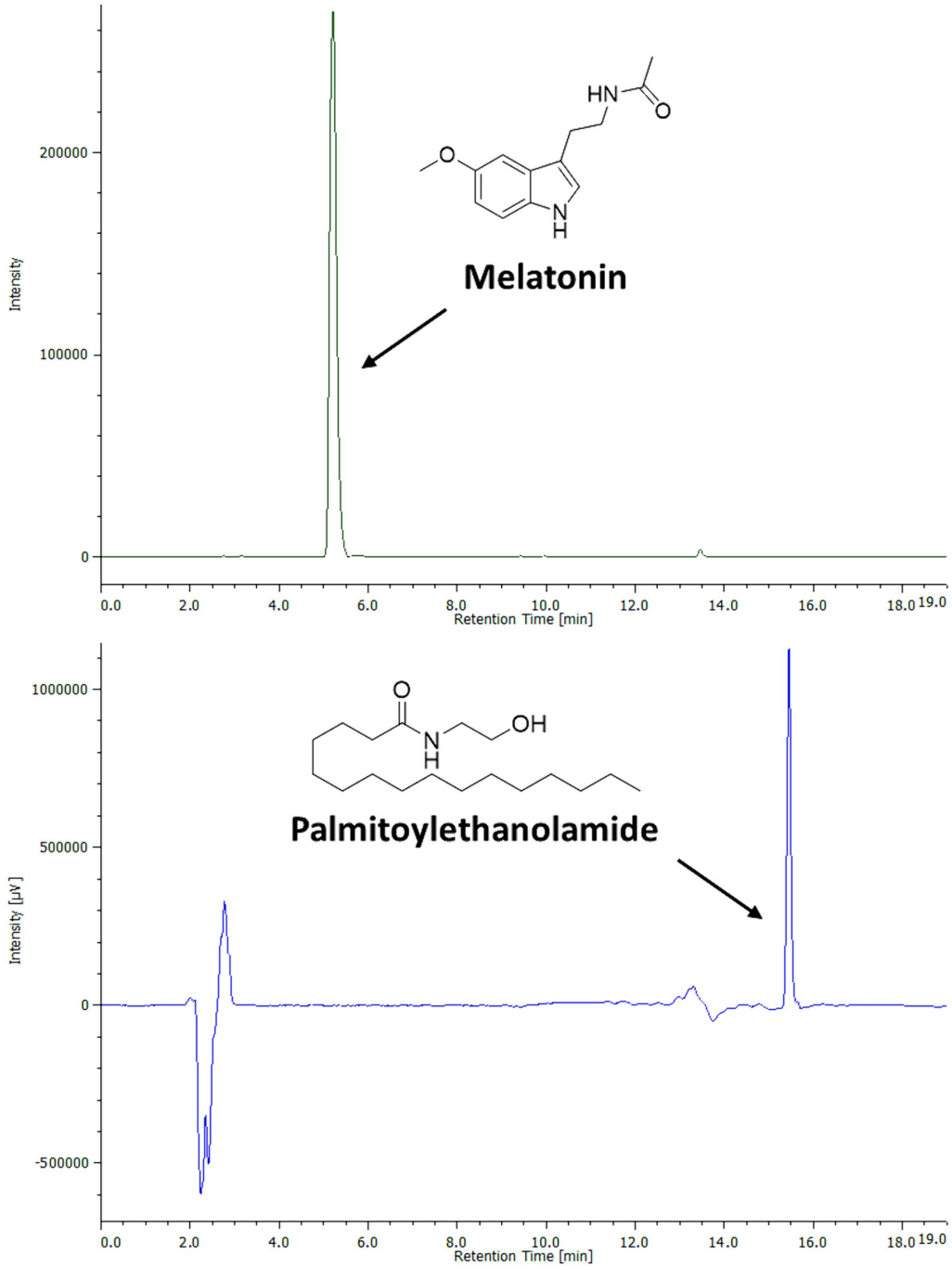

FIGURE 1
Chromatographic profile and chemical structures of PEA and MEL standards by HPLC-DAD-FLD analysis. PEA (500 ppm) was analyzed by HPLC-DAD at the wavelength of 205 nm. MEL (25 ppm) was analyzed by HPLC-FLD.

3.4 Cytotoxic effect of PEA, MEL, and NF on HMC human mast cell lines

To evaluate the ability of NF to modulate the activity of mast cells, we used the human mast cell line HMC-1.2 (referred to as HMC). First, we evaluated whether NF, MEL, and PEA single components may exert a cytotoxic effect on HMC cells. Cells were treated with increased concentrations of PEA (2–32 µM), of MEL (10–160 nM), and of NF (mix of PEA with MEL, tested exactly at the same concentration evaluated for every single component) for 72 h and cell viability was evaluated by performing an MTT assay. As shown in Figure 2A, we found that all the PEA concentrations tested were safe and did not impair HMC cell viability. Similarly, MEL (Figure 2B) and NF (Figure 2C) did not affect in any way the cell viability at the same concentrations tested. Thus, according to the results obtained in our bioaccessibility experiments, for the next experiments we used the concentrations of 16 µM and 80 nM for PEA and MEL, respectively.

3.5 Pretreatment with NF reduced histamine and β-hexosaminidase release in HMC cells

To characterize the effect of NF on mast cell activation, we first evaluated the release of histamine and β-hexosaminidase that occur in response to allergens or inflammatory agents, acting as important players in the inflammatory response. Thus, we pretreated HMC cells with NF or PEA and MEL alone for 1 h before stimulation with PMA and Ionophore for 2 h, the most used triggers for mast cell degranulation (referred to as STIM). As shown in Figure 3A, the stimulation with PMA and Ionophore significantly increased the release of histamine. In contrast, pretreatment with NF as well as the single compounds significantly reduced the release of histamine. In line with this result, stimulation with PMA Ionophore induced b-hexosaminidase release by HMC1, which was significantly reduced by NF (Figure 3B). These results highlight the potential synergistic effects of NF in inhibiting the degranulation of mast cells.

3.6 NF reduced the mRNA expression and release of pro-inflammatory cytokines in HMC cells

Once activated, mast cells are able to release different cytokines that sustain the allergic-related inflammatory response. Therefore, we evaluated by qPCR analysis the RNA expression levels of both TNF-α and IL-6 in NF-treated HMC cells. As expected, the stimulation with PMA and Ionomycin significantly increased the RNA expression levels of the two cytokines tested. Conversely, as shown in Figures 4A,B, the pretreatment with NF, PEA, and MEL significantly reduced the expression of both TNF-α and IL-6. Interestingly, this effect was more pronounced in HMC cells treated with the NF compared to the single compounds. These results were confirmed by the quantification of cytokines in cell culture supernatants of HMC cells, pretreated or not with NF, PEA, and MEL (Figure 4C). In line with the qPCR analysis, we observed a significant reduction of both cytokines in the cultured medium of HMC. However, the pretreatment with NF showed a major reduction compared to the single compounds.

3.7 NF reduced the expression and inhibited the activity of the COX-2 enzyme

It is well known that type-2 cyclooxygenase enzyme (COX-2) is the inducible form of COX enzyme that sustains the inflammatory process by modulating the function of different immune cells including mast cells (35). Thus, we evaluated the effect of PEA, MEL, and NF on the expression of COX-2 enzyme at transcriptional levels by qPCR analysis. As shown in Figure 5A, stimulation with PMA/Ionophore increased the expression of COX2 whereas pretreatment with PEA, MEL, and NF resulted in a valuable reduction in COX-2 expression vs. STIM. Specifically, the NF was shown more relevant effects on COX-2 transcription rate than its single components, PEA and MEL. Moreover, we also tested the ability of our compounds to directly inhibit the enzymatic activity of COX2. Our results indicate that both MEL and PEA tested separately at concentration levels in the range of their previously described bioaccessible concentrations show a valuable COX-2 inhibitory activity with a calculated IC_{50} of 47.47 μg/mL and of 0.152 μg/mL (Figures 5B,C), for MEL and PEA, respectively. The inhibitory activity of the NF was described by a calculated IC_{50} of 0.135 μg/mL (Figure 5D), with a non-significant difference from the

TABLE 2 Linearity and sensitivity of PEA and MEL detection.

Compound	Calibration line	R^2	LOD (ppm)	LOQ (ppm)
Palmitoylethanolamide	$y = 3 \times 10^6 x + 32{,}333$	0.99	10	30
Melatonin	$y = 5 \times 10^7 x + 28{,}200$	0.99	0.1	0.3

LOQ, limit of quantification; LOD, limit of detection.

TABLE 4 Recovery (%) and matrix effect (%) of PEA and MEL extraction process.

Compound	Spike (μg)	Recovery (%)	Matrix effect (%)
Palmitoylethanolamide	50	102.3 ± 6.2	5.5 ± 2.1
	250	102.3 ± 3.7	−1.9 ± 2.0
	500	104.8 ± 5.6	0.4 ± 1.0
Melatonin	1	104.2 ± 6.0	−5.4 ± 4.1
	5	98.2 ± 9.1	1.1 ± 1.2
	10	102.1 ± 3.3	3.8 ± 2.3

TABLE 3 Intra-day and inter-day precision and accuracy of melatonin and PEA detection.

Compound	Concentration (ppm)	Precision (% CV)		Accuracy (% bias)	
		Intraday	Interday	Intraday	Interday
Palmitoylethanolamide	500	1.9	3.3	0.1	−0.6
	100	2.2	3.2	2.4	2.2
	50	4.0	7.1	1.0	0.4
	30	4.4	8.8	−1.8	−1.7
Melatonin	25	0.7	0.2	−0.1	−0.1
	5	0.3	0.3	0.2	−0.2
	1	2.6	1.3	0.3	0.1
	0.3	4.7	4.0	−0.1	−0.4

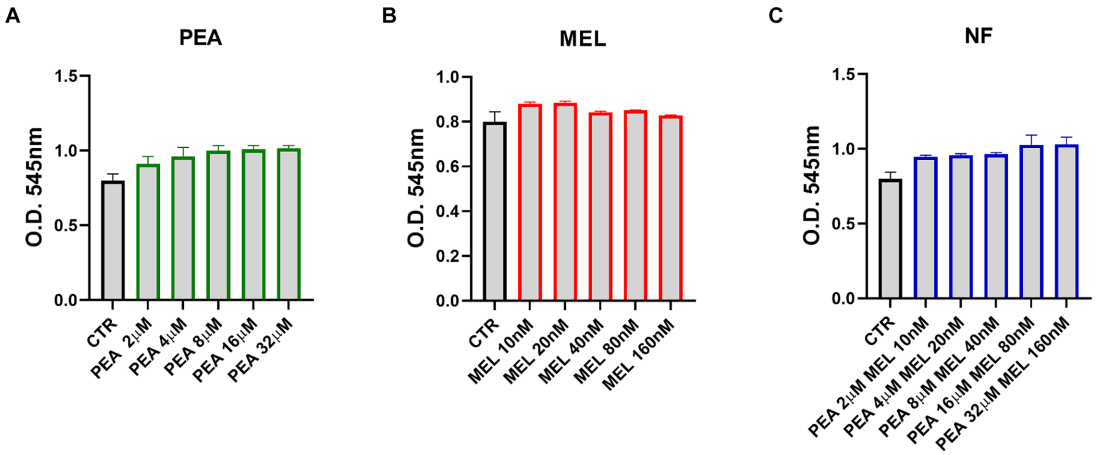

FIGURE 2
Cytotoxic effect of PEA **(A)**, melatonin **(B)**, and NF **(C)** on HMC-1.2 cells after 72 h evaluated by the MTT assay. Values are expressed as mean ± SEM from three independent experiments.

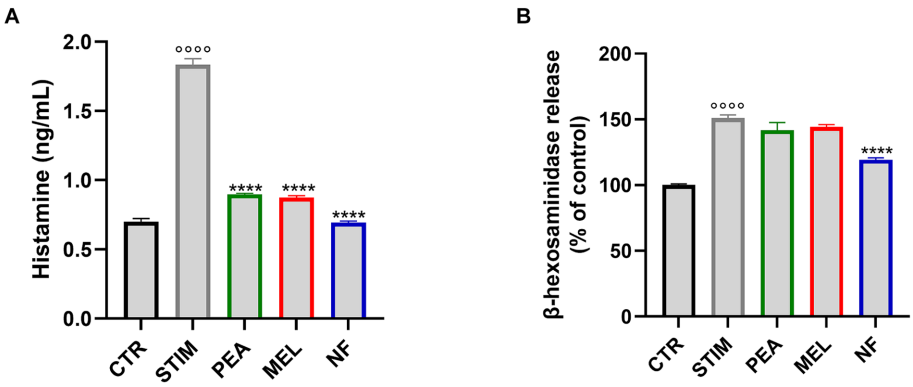

FIGURE 3
Histamine **(A)** and β-hexosaminidase **(B)** release in HMC-1.2 cells following pretreatment with PEA, MEL, and NF and stimulation with PMA 50 nM and ionophore 1 μM. Values are expressed as mean ± SEM from three independent experiments. °°°°$p < 0.0001$ indicates a significant effect of PMA/Ionophore compared to unstimulated cells (CTR); ****$p < 0.0001$ indicates significant effect of PEA, MEL, and NF compared to stimulated cells.

PEA anti-COX2 activity described. Regarding MEL, despite showing a valuable enzymatic inhibitory activity, its IC_{50} of 47.47 μg/mL (204.3 nM) is higher than its calculated bioaccessibility, thus this result has weak health significance. Collectively, these results demonstrated that NF, on the one hand, was able to modulate the expression of COX-2 enzyme at mRNA levels in stimulated HMC-1.2, and, on the other hand, was able to directly contract its enzymatic activity with a calculated IC_{50} of 0.208 μg/mL, a concentration largely below its intestinal bioaccessibility.

4 Discussion

Allergic diseases represent a prevalent global health issue with an increasing incidence, particularly in Western countries. Allergic events are characterized by an immune-mediated inflammatory reaction to typically harmless environmental allergens, leading to the proliferation and activation of mast cells, heightened production, and release of IgE in the bloodstream (36). From the molecular point of view, this process was mediated by multistep complex molecular mechanisms that modulate the activity of different immune cells both from the innate and adaptive arms.

Thus, the inhibition or the expression-regulation of such molecular modulators could be considered a useful tool for the management of immune response. In this context, the NF exhibited a valuable anti-allergic activity, acting in both the early and late phases of immune events. Nevertheless, concerning the assessment of nutraceutical formulation's human health effectiveness, the evaluation of its intestinal bioaccessibility plays a key role. Thus, in the current study, at first, the intestinal bioaccessibility of PEA and MEL was calculated, after the *in vitro* gastrointestinal digestion protocol. Our results indicate that the digestion process drastically reduces the effective active concentration of PEA, with a bioaccessible concentration of only 1.59% concerning the initial concentration,

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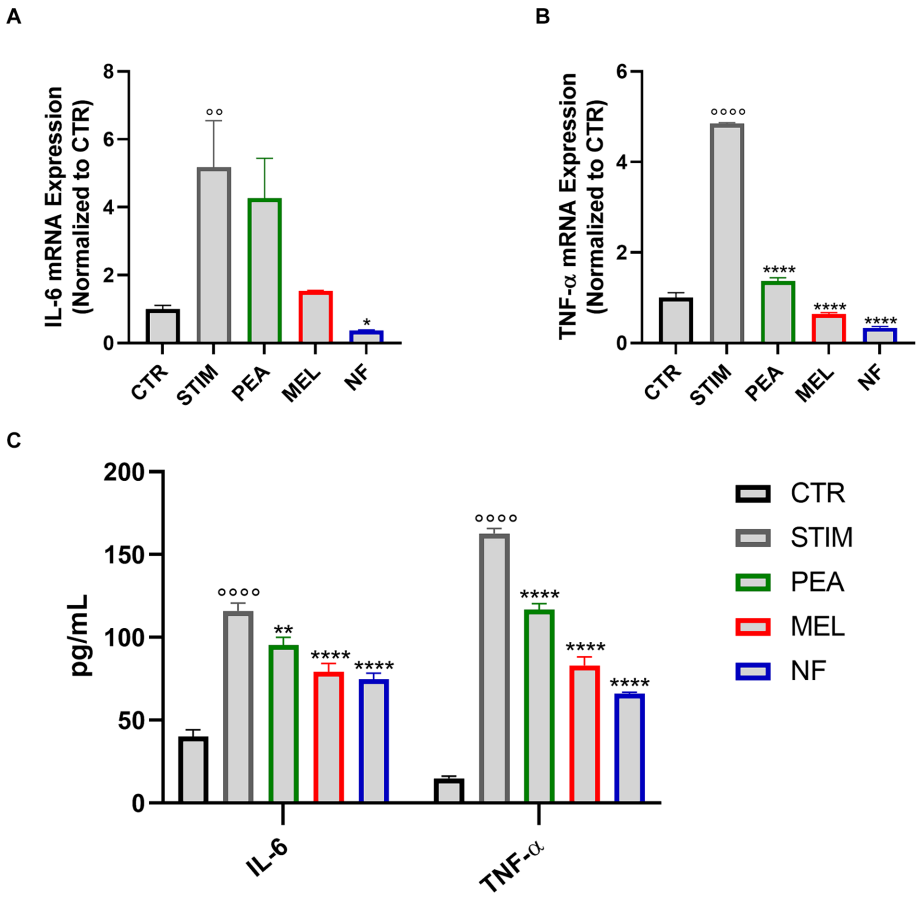

FIGURE 4
Expression of IL-6 **(A)** and TNF-α **(B)** assessed with qPCR analysis in HMC-1.2 cells pretreated for 1 h with PEA 16 μM, melatonin 80 nM and NF 16 μM + 80 nM and stimulated with PMA 50 nM and ionophore 1 μM for 6 h. **(C)** Levels of IL-6 and TNF-α in supernatants assessed by multiplex assay. Values are expressed as mean ± SEM from three independent experiments. °°°°$p < 0.0001$ indicates a significant effect of PMA/Ionophore compared to unstimulated cells (CTR); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicate a significant effect of PEA, MEL or NF compared to stimulated cells.

while for MEL a valuable resistance during the digestion process was detected, with a calculated bioaccessibility of 36%. These results, describe a first attempt to define the intestinal bioaccessibility of MEL and PEA in biocomponent nutraceutical formulation. Concerning MEL, scant literature evaluates its bioaccessibility, particularly in its pure form. A single study estimated 80% of MEL bioaccessibility encapsulated in a formulation based on glycosylated egg proteins (37), while another investigation reported that MEL contained in pistachios seeds has shown intestinal bioaccessibility of 21% (38). Interestingly, despite the widespread use of PEA in nutraceutical formulations and its numerous biological activities, there is a surprising lack of scientific evidence addressing this specific topic.

From an analytical point of view, to produce reliable and accurate data about the PEA and MEL intestinal bioaccessibility, these parameters were calculated using an opportunely validated HPLC-DAD-FLD method, which allows their simultaneous accurate and sensitive determination in bioaccessible fraction. According to the analytical validation process conducted, the current method could be considered, accurate, sensitive, reliable, and reproducible, and the extraction process used for the recovery of PEA and MEL from bioaccessible fraction was exhaustive and efficient.

In this context, once the bioaccessible fractions of PEA (15.95 mM) and MEL (215 nM) for a single dose treatment were assessed, the biological assays performed were conducted in a concentration range below such levels, in order to ensure the plausibility of the health-promoting effects investigated. Thus, at first, we evaluated the HMC-1.2 tolerability through an MTT test. Our results indicate that both PEA, MEL, and NF are particularly well-tolerated in the range tested, not showing any toxic effects. Thus to explore the immunomodulatory effects, their activity on the histamine and β-hexosaminidase release from HMC-1.2 stimulated cells was explored. Specifically, these two mediators play a pivotal role in the progression of the initial phase of the allergic reaction, where histamine is responsible for the main symptoms associated with the allergic and immune reaction inducing smooth muscle contraction, heightened vascular permeability, and vasodilation, while β-hexosaminidase is responsible for the progression of inflammatory response. In this sense, β-hexosaminidase contributes to the modification of the extracellular matrix, catalyzing the removal of N-acetylglucosamine (GlcNAc) groups from glycosaminoglycans structural components of the extracellular matrix, facilitating the dispersion and

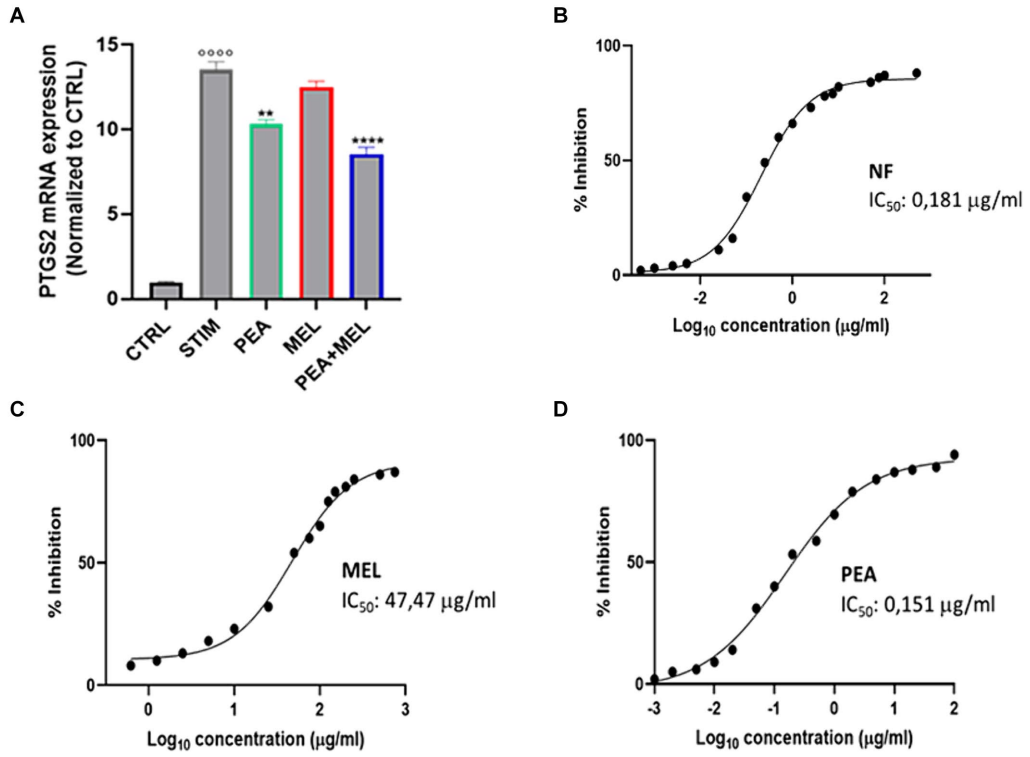

FIGURE 5
(A) Expression of PTGS2 assessed with qPCR analysis in HMC-1.2 cells treated with PEA 16 µM, melatonin 80 nM, and NF 16 µM + 80 nM for 1 h following stimulation with PMA 50 nM and ionophore 1 µM and treatment for 6 h. Inhibition of COX-2 activity (expressed in %) of PEA **(B)** MEL **(C)** and NF **(D)**. Values are expressed as mean ± SEM from three independent experiments. °°°°$p < 0.0001$ indicates a significant effect of PMA/Ionophore compared to unstimulated cells (CTR); **$p < 0.01$ and ****$p < 0.0001$ indicate a significant effect of PEA, MEL or NF compared to stimulated cells.

mobilization of inflammatory cells and mediators, enhancing the progression of the allergic-inflammatory response. However, the stronger effect of NF on histamine compared to β-hexosaminidase could be related to the slow release of the latter compared to histamine whose release is faster during mast cell degranulation (39).

Our results highlight that the treatment with both MEL (80 nM) and PEA (16 µM) was able to decrease the level of histamine release rate by 55% vs. STIM ($p < 0.0001$), while the treatment with NF led to a more evident reduction of histamine release (65% vs. STIM, $p < 0.0001$) than the single component treatment. Conversely, the release of β-hexosaminidase follows a completely different trend, while the single MEL and PEA treatment led to weak effects (with no significative difference vs. STIM), but their combination shows a valuable reduction in terms of β-hexosaminidase release. In this regard, the ability of MEL to reduce β-hexosaminidase release in a dose-dependent manner (over the concentration of 0.2–1 mM) in rat basophilic leukemia (RBL-2H3) cells has already been reported (40). On the same cellular model was additionally studied the PEA ability to reduce the histamine and β-hexosaminidase in dose dose-dependent manner. Particularly, Petrosino and colleagues stated that PEA treatment at the concentration range of 0.1–10 mM strongly reduced the release of β-hexosaminidase and histamine in stimulated RBL-2H3 cell lines (13). Additionally, other authors have described the same PEA treatment effects in canine mast cells isolated from skin biopsies, at the concentration of 30 mM could simultaneously reduce the histamine and TNF-α release by 60 and 40%, respectively (41).

Molecularly, these effects could be explained considering the two different molecular pathways separately activated by PEA and MEL. It was well reported that PEA modulates the activity of mast cells by a specific interaction with the cannabinoid receptor, the CB2 receptor (9). Regarding the MEL activity on mast cells, it was well described that MEL could exert a direct effect on HMC cells through the interaction with MEL receptors 1 and 2 (MT1 and MT2), two G-protein–coupled receptors, expressed in human mast cells. Specifically, MEL through their activation could lead to the inhibition of NF-κB activation, which in turn could downregulate MC degranulation, proliferation, and differentiation, resulting potentially in a reduction of histamine release (42). Thus, in light of the difference, the results obtained could be explained as considered a synergic activation of both molecular via described. Interestingly, considering the role of mast cell activation in the pathogenesis of coeliac disease and inflammatory bowel diseases (IBD) (43, 44), the PEA and MEL ability to modulate the mast cell degranulation could be considered as a potential alternative treatment for intestinal-immune-based disorders.

Next, to investigate the NF potential immunomodulatory activity in the tardive phase of the immune response process, its ability to regulate both at the transcriptional level and the production of IL-6 and TNF-a was investigated. The NF treatment

leads to a valuable reduction in mRNA expression levels for both IL-6 and TNF-α, and reasonably leads to decreased synthesis of these cytokines and interleukins. These data were supported by previously published findings, reporting that PEA through interaction with PPARγ in RBL-2H3 and rat mast cells, leads to a reduction of cytokine release, including TNF-α in both models (9, 45). Regarding MEL, other evidence described that the pre-treatment with MEL (100 nM) in stimulated mast cells, significantly reduced the levels of TNF-α and IL-6 via inhibition of IKK/NF-kB (46). Based on such consideration, it could be hypothesized the NF's greater effect in reducing IL-6 and TNF-α than its single components, could be related to a synergic combination of both the mechanism of action mentioned above, the PEA component acting via PPARγ while the MEL component via IKK/NF-kB.

Finally, we moved to investigate the NF's ability to transcriptionally modulate the expression of COX-2 in stimulated HMC-1.2 cell lines. Our results indicate that NF, has a powerful activity in reducing at the transcriptional level the COX-2 expression (−30% vs. STIM, $p < 0.0001$), while no statistically significant results were obtained after the monocomponent treatment, suggesting a potential synergic effect. These results were in line with other published data where PEA demonstrated to downregulate the COX-2 mRNA expression level (47).

Once established the NF efficacy in reducing the COX-2 transcription rate, we investigated whether NF could also exert a direct inhibitory effect on the COX-2 enzymatic activity. Our results did not confirm the same trend shown for the data obtained at the transcription level. Above all, NF has shown a comparable COX-2 inhibitory activity to PEA single-component treatment, with a similar calculated IC_{50} value (0.151 μg/mL for PEA and 0.198 μg/mL for NF). Not surprisingly, these could be related principally to the fact that PEA has shown a higher inhibitory potential toward COX-2 enzymatic activity than MEL (IC_{50} 47.47 μg/mL), and, in addition, represents the main functional ingredient in NF. These data agreed with literature data, which reported that PEA has shown a valuable direct inhibitory COX-2 enzymatic activity, with a reported IC_{50} value of 0.57 mM (48), as well as for MEL was confirmed the calculated inhibitory activity by the results of a previously published study where was reported an IC_{50} of 60 μg/mL (49).

5 Conclusion

This study represents the first attempt to evaluate a potential synergic immunomodulatory activity of innovative nutraceutical formulation based on the combination of PEA and MEL. Our results underline that both these components, at concentration levels lower than their intestinal bioaccessible calculated fractions, can positively modulate the mast cell degranulation, and consequently reduce the liberation of the main immune-inflammatory molecular modulators, i.e., TNF-α, IL-6, and COX-2 in treated HMC-1.2. In conclusion, this formulation could be considered as a potential alternative remedy for the treatment of allergic or immune-inflammatory diseases at different body districts, including at the intestinal level. Nevertheless, further studies are required to establish their bioavailability and to clarify the specific molecular pathways involved in the collected results.

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

MM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. VP: Data curation, Investigation, Software, Writing – review & editing. AM: Data curation, Investigation, Methodology, Writing – review & editing. DM: Formal analysis, Investigation, Methodology, Writing – original draft. BR: Data curation, Formal analysis, Investigation, Writing – review & editing. VS: Supervision, Visualization, Writing – review & editing. GT: Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Investigation, Project administration, Resources. GE: Supervision, Visualization, Writing – review & editing, Data curation, Formal analysis, Methodology, Validation, Writing – original draft. AI: Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Kaag S, Lorentz A. Effects of dietary components on mast cells: possible use as nutraceuticals for allergies? *Cells*. (2023) 12:22602. doi: 10.3390/cells12222602

2. Orban NT, Jacobson MR, Nouri-Aria KT, Durham SR, Eifan AO. Repetitive nasal allergen challenge in allergic rhinitis: priming and Th2-type inflammation but no evidence of remodelling. *Clin Exp Allergy*. (2021) 51:329–38. doi: 10.1111/cea.13775

3. Jang MH, Piao XL, Kim JM, Kwon SW, Park JH. Inhibition of cholinesterase and amyloid-& bgr; aggregation by resveratrol oligomers from *Vitis amurensis*. *Phytother Res*. (2008) 22:544–9. doi: 10.1002/ptr.2406

4. Li QS, Wang YQ, Liang YR, Lu JL. The anti-allergic potential of tea: a review of its components, mechanisms and risks. *Food Funct*. (2021) 12:57–69. doi: 10.1039/d0fo02091e

5. Kong ZL, Sudirman S, Lin HJ, Chen WN. In vitro anti-inflammatory effects of curcumin on mast cell-mediated allergic responses via inhibiting Fcε RI protein expression and protein kinase C delta translocation. *Cytotechnology*. (2020) 72:81–95. doi: 10.1007/s10616-019-00359-6

6. Shirley D, McHale C, Gomez G. Resveratrol preferentially inhibits IgE-dependent PGD2 biosynthesis but enhances TNF production from human skin mast cells. *Biochim Biophys Acta Gen Subj*. (2016) 1860:678–85. doi: 10.1016/J.BBAGEN.2016.01.006

7. Yamada S, Shirai M, Inaba Y, Takara T. Effects of repeated oral intake of a quercetin-containing supplement on allergic reaction: a randomized, placebo-controlled, double-blind parallel-group study. *Eur Rev Med Pharmacol Sci*. (2022) 26:4331–45. doi: 10.26355/eurrev_202206_29072

8. Mattace Raso G, Russo R, Calignano A, Meli R. Palmitoylethanolamide in CNS health and disease. *Pharmacol Res*. (2014) 86:32–41. doi: 10.1016/J.PHRS.2014.05.006

9. Facci L, Dal Toso R, Romanello S, Buriani A, Skaper SD, Leon A. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc Natl Acad Sci USA*. (1995) 92:3376–80. doi: 10.1073/pnas.92.8.3376

10. Berdyshev EV, Schmid PC, Dong Z, Schmid HHO. Stress-induced generation of N-acylethanolamines in mouse epidermal JB6 P+ cells. *PT Cells*. (2000) 346:369–74. doi: 10.1042/bj3460369

11. Venables BJ, Waggoner CA, Chapman KD. N-acylethanolamines in seeds of selected legumes. *Phytochemistry*. (2005) 66:1913–8. doi: 10.1016/J.PHYTOCHEM.2005.06.014

12. Rawyler AJ, Braendle RA. N-acylphosphatidylethanolamine accumulation in potato cells upon energy shortage caused by anoxia or respiratory inhibitors. *Plant Physiol*. (2001) 127:240–51. doi: 10.1104/pp.127.1.240

13. Petrosino S, Schiano Moriello A, Verde R, Allarà M, Imperatore R, Ligresti A, et al. Palmitoylethanolamide counteracts substance P-induced mast cell activation in vitro by stimulating diacylglycerol lipase activity. *J Neuroinflammation*. (2019) 16:274–16. doi: 10.1186/s12974-019-1671-5

14. Vaia M, Petrosino S, De Filippis D, Negro L, Guarino A, Carnuccio R, et al. Palmitoylethanolamide reduces inflammation and itch in a mouse model of contact allergic dermatitis. *Eur J Pharmacol*. (2016) 791:669–74. doi: 10.1016/J.EJPHAR.2016.10.005

15. Roviezzo F, Rossi A, Caiazzo E, Orlando P, Riemma MA, Iacono VM, et al. Palmitoylethanolamide supplementation during sensitization prevents airway allergic symptoms in the mouse. *Front Pharmacol*. (2017) 8:1–11. doi: 10.3389/fphar.2017.00857

16. Briskey D, Ebelt P, Rao A. The effect of Levagen+ (Palmitoylethanolamide) supplementation on symptoms of allergic rhinitis—a double-blind placebo-controlled trial. *Nutrients*. (2023) 15:34940. doi: 10.3390/nu15234940

17. Favero G, Franceschetti L, Bonomini F, Rodella LF, Rezzani R. Melatonin as an anti-inflammatory agent modulating inflammasome activation. *Int J Endocrinol*. (2017) 2017:1–13. doi: 10.1155/2017/1835195

18. Arnao MB, Hernández-Ruiz J. Functions of melatonin in plants: a review. *J Pineal Res*. (2015) 59:133–50. doi: 10.1111/jpi.12253

19. Janas KM, Posmyk MM. Melatonin, an underestimated natural substance with great potential for agricultural application. *Acta Physiol Plant*. (2013) 35:3285–92. doi: 10.1007/s11738-013-1372-0

20. Zhang HM, Zhang Y. Melatonin: a well-documented antioxidant with conditional pro-oxidant actions. *J Pineal Res*. (2014) 57:131–46. doi: 10.1111/jpi.12162

21. Zagajewski J, Drozdowicz D, Brzozowska I, Hubalewska-Mazgaj M, Stelmaszynska T, Laidler PM, et al. Conversion L-tryptophan to melatonin in the gastrointestinal tract: the new high performance liquid chromatography method enabling simultaneous determination of six metabolites of L-tryptophan by native fluorescence and UV-VIS detection. *J Physiol Pharmacol*. (2012) 63:613–21. doi: 10.26402/jpp.2021.6.15

22. Nabavi SM, Nabavi SF, Sureda A, Xiao J, Dehpour AR, Shirooie S, et al. Anti-inflammatory effects of melatonin: a mechanistic review. *Crit Rev Food Sci Nutr*. (2019) 59:S4–S16. doi: 10.1080/10408398.2018.1487927

23. Cho JH, Bhutani S, Kim CH, Irwin MR. Anti-inflammatory effects of melatonin: a systematic review and meta-analysis of clinical trials. *Brain Behav Immun*. (2021) 93:245–53. doi: 10.1016/j.bbi.2021.01.034

24. Liu X, Zhang Y, Ren Y, Li J. Melatonin prevents allergic airway inflammation in epicutaneously sensitized mice. *Biosci Rep*. (2021) 41:1–12. doi: 10.1042/BSR20210398

25. Cakir Cetin A, Ecevit MC, Gumustekin M, Pekcetin C, Ozbal S, Efe H, et al. Therapeutic effects of melatonin on an ovalbumin-induced allergic rhinitis model in rats. *Auris Nasus Larynx*. (2021) 48:1109–19. doi: 10.1016/j.anl.2021.04.010

26. Bešlić I, Lugović-Mihić L, Vrtarić A, Bešlić A, Škrinjar I, Hanžek M, et al. Melatonin in dermatologic allergic diseases and other skin conditions: current trends and reports. *Int J Mol Sci*. (2023) 24:44039. doi: 10.3390/ijms24044039

27. Maisto M, Schiano E, Novellino E, Piccolo V, Iannuzzo F, Salviati E, et al. Application of a rapid and simple technological process to increase levels and bioccessibility of free phenolic compounds in Annurca apple nutraceutical product. *Food Secur*. (2022) 11:1453. doi: 10.3390/foods11101453

28. Candeias SX, Gallardo E, Matos AC. Caffeine content of retail market coffee in Portugal. *Food Anal Methods*. (2009) 2:251–6. doi: 10.1007/s12161-008-9058-8

29. Fiorillo B, Roselli R, Finamore C, Biagioli M, di Giorgio C, Bordoni M, et al. Discovery of a novel class of dual GPBAR1 agonists-RORγt inverse agonists for the treatment of IL-17-mediated disorders. *ACS Omega*. (2023) 8:5983–94. doi: 10.1021/acsomega.2c07907

30. Ohno Y. ICH guidelines—implementation of the 3Rs (refinement, reduction, and replacement): incorporating best scientific practices into the regulatory process. *ILAR J*. (2002) 43:S95–8. doi: 10.1093/ilar.43.suppl_1.s95

31. Maisto M, Piccolo V, Novellino E, Schiano E, Iannuzzo F, Ciampaglia R, et al. Optimization of Ursolic acid extraction in oil from Annurca apple to obtain Oleolytes with potential cosmeceutical application. *Antioxidants*. (2023) 12:224. doi: 10.3390/antiox12020224

32. Iannuzzo F, Piccolo V, Novellino E, Schiano E, Salviati E, Summa V, et al. A food-grade method for enhancing the levels of low molecular weight Proanthocyanidins with potentially high intestinal bioavailability, vol. 23 (2022).

33. Annunziata G, Maisto M, Schisano C, Ciampaglia R, Narciso V, Hassan STS, et al. Effect of grape pomace polyphenols with or without pectin on TMAO serum levels assessed by LC/MS-based assay: a preliminary clinical study on overweight/obese subjects. *Front Pharmacol*. (2019) 10:575. doi: 10.3389/fphar.2019.00575

34. Piccolo V, Maisto M, Cerrato LM, Esposito E, Panza E, Sorrentino R, et al. Characterization and quantification of intact glucosinolates in Catozza rapeseeds: a promising food matrix for nutraceuticals development as a source of hydrogen sulfide. *J Funct Foods*. (2024) 116:106214. doi: 10.1016/j.jff.2024.106214

35. Reddy ST, Wadleigh DJ, Herschman HR. Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. *J Biol Chem*. (2000) 275:3107–13. doi: 10.1074/jbc.275.5.3107

36. Vo TS. Natural products targeting FcεRI receptor for anti-allergic therapeutics. *J Food Biochem*. (2020) 44:e13335–10. doi: 10.1111/jfbc.13335

37. Zhang T, Li S, Yang M, Li Y, Ma S, Zhang H, et al. The influence of unique interfacial networks based on egg white proteins for the stabilization of high internal phase Pickering emulsions: physical stability and free fatty acid release kinetics. *Food Chem*. (2024) 442:138448. doi: 10.1016/j.foodchem.2024.138448

38. Dufoo-hurtado E, Luzardo I, Wall-medrano A. Diseases linked to chronodisruption. *Curr Dev Nutr*. (2021) 5:581. doi: 10.1093/cdn/nzab044

39. Huang L, Pi J, Wu J, Zhou H, Cai J, Li T, et al. A rapid and sensitive assay based on particle analysis for cell degranulation detection in basophils and mast cells. *Pharmacol Res*. (2016) 111:374–83. doi: 10.1016/J.PHRS.2016.05.033

40. Kim YS, Go G, Yun CW, Yea JH, Yoon S, Han SY, et al. Topical administration of melatonin-loaded extracellular vesicle-mimetic nanovesicles improves 2,4-dinitrofluorobenzene-induced atopic dermatitis. *Biomol Ther*. (2021) 11:1450. doi: 10.3390/biom11101450

41. Cerrato S, Brazis P, Valle MF, Miolo A, Puigdemont A. Effects of palmitoylethanolamide on immunologically induced histamine, PGD2 and TNFα release from canine skin mast cells. *Vet Immunol Immunopathol*. (2010) 133:9–15. doi: 10.1016/J.VETIMM.2009.06.011

42. Pham L, Baiocchi L, Kennedy L, Sato K, Meadows V, Meng F, et al. The interplay between mast cells, pineal gland, and circadian rhythm: links between histamine, melatonin, and inflammatory mediators. *J Pineal Res*. (2021) 70:e12699–15. doi: 10.1111/jpi.12699

43. Frossi B, De CM, Calabrò A. Coeliac disease and mast cells. *Int J Mol Sci*. (2019) 20:143400. doi: 10.3390/ijms20143400

44. Uranga JA, Martínez V, Abalo R. Mast cell regulation and irritable bowel syndrome: effects of food components with potential nutraceutical use. *Molecules*. (2020) 25:184314. doi: 10.3390/molecules25184314

45. Keppel Hesselink JM. Evolution in pharmacologic thinking around the natural analgesic palmitoylethanolamide: from nonspecific resistance to PPAR-α agonist and effective nutraceutical. *J Pain Res*. (2013) 6:625–34. doi: 10.2147/JPR.S48653

46. Maldonado MD, García-Moreno H, González-Yanes C, Calvo JR. Possible involvement of the inhibition of NF-κB factor in anti-inflammatory actions that melatonin exerts on mast cells. *J Cell Biochem*. (2016) 117:1926–33. doi: 10.1002/jcb.25491

47. Alhouayek M, Muccioli GG. Harnessing the anti-inflammatory potential of palmitoylethanolamide. *Drug Discov Today*. (2014) 19:1632–9. doi: 10.1016/j.drudis.2014.06.007

48. Bergandi L, Apprato G, Silvagno F. Antioxidant and anti-inflammatory activity of combined Phycocyanin and Palmitoylethanolamide in human lung and prostate epithelial cells. *Antioxidants*. (2022) 11:20201. doi: 10.3390/antiox11020201

49. Bergandi L, Apprato G, Silvagno F, Jhaveri MD, Richardson D, Robinson I, et al. The inhibitory potential of Montmorency tart cherry on key enzymes relevant to type 2 diabetes and cardiovascular disease. *Food Chem*. (2018) 252:142–6. doi: 10.1016/j.foodchem.2018.01.084

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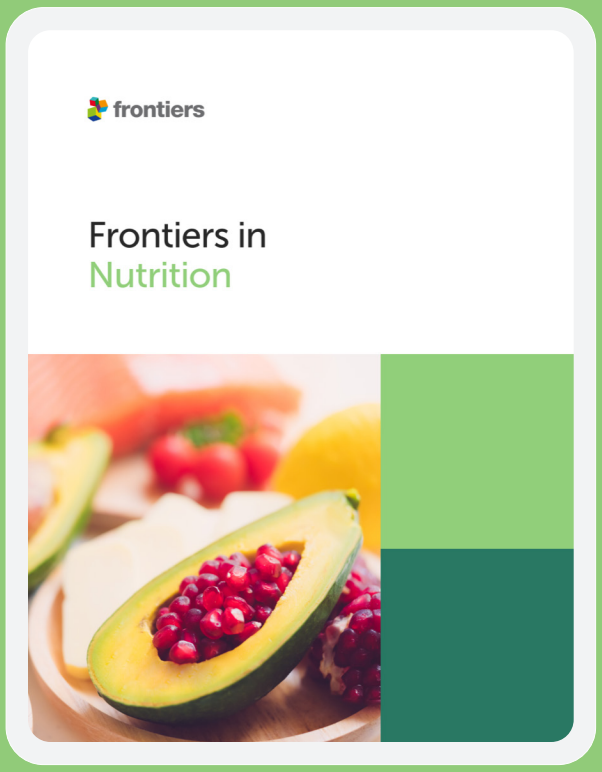

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