KAMPO MEDICINE IN A MODERN CONTEXT: ETHNOPHARMACOLOGICAL PERSPECTIVES

EDITED BY: Kenny Kuchta, Silke Cameron, Yukihiro Shoyama, Kenji Watanabe and Munekazu Iinuma

PUBLISHED IN: Frontiers in Pharmacology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-227-3
DOI 10.3389/978-2-83250-227-3

About Frontiers

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

Frontiers Journal Series

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

Dedication to Quality

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

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

KAMPO MEDICINE IN A MODERN CONTEXT: ETHNOPHARMACOLOGICAL PERSPECTIVES

Topic Editors:
Kenny Kuchta, University Medical Center Göttingen, Germany
Silke Cameron, University Medical Center Göttingen, Germany
Yukihiro Shoyama, Nagasaki International University, Japan
Kenji Watanabe, Yokohama College of Pharmacy, Japan
Munekazu Iinuma, Gifu Pharmaceutical University, Japan

Citation: Kuchta, K., Cameron, S., Shoyama, Y., Watanabe, K., Iinuma, M., eds. (2022). Kampo Medicine in a Modern Context: Ethnopharmacological Perspectives. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-227-3

Table of Contents

frontiers | Frontiers in Pharmacology

TYPE Editorial
PUBLISHED 02 September 2022
DOI 10.3389/fphar.2022.971254

OPEN ACCESS

EDITED AND REVIEWED BY
Michael Heinrich,
University College London,
United Kingdom

*CORRESPONDENCE
Kenny Kuchta,
kenny.kuchta@med.uni-goettingen.de

SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

RECEIVED 16 June 2022
ACCEPTED 18 July 2022
PUBLISHED 02 September 2022

CITATION
Kuchta K and Cameron S (2022), Editorial: Kampo Medicine in a Modern Context: Ethnopharmacological Perspectives. *Front. Pharmacol.* 13:971254. doi: 10.3389/fphar.2022.971254

Editorial: Kampo Medicine in a Modern Context: Ethnopharmacological Perspectives

Kenny Kuchta[1]* and Silke Cameron[2,3]

[1]Forschungsstelle für Fernöstliche Medizin, Department of Vegetation Analysis and Phytodiversity, Albrecht von Haller Institute of Plant Sciences, Georg August University, Göttingen, Germany, [2]Clinic for Gastroenterology and GI-Oncology, University Medical Center Göttingen, Georg August University, Göttingen, Germany, [3]Department for Gastroenterology and General Internal Medicine, Clinic Hann. Münden, Hann. Münden, Germany

KEYWORDS

Kampo (traditional Japanese herbal medicine), Ethnopharmacology, cultural evolution, dementia—Alzheimer disease, supportive cancer therapy, personalised therapy, COVID-19 therapeutics, ICD-11 (international classification of diseases)

Editorial on the Research Topic
Editorial: Kampo Medicine in a Modern Context - Ethnopharmacological Perspectives

The word "Kampo" (漢方) is used to denote the traditional academic phytotherapy of Japan. This term, which can be translated as "Method of Han-Dynasty-China", was coined in the 19th century to distinguish the long established traditional medicine from the new influx of Western medicine.

In contrast to the pre-existing system of purely orally transmitted "Minkanyaku" (民間薬) practiced by non experts, Kampo medicine was established through the 6th to the 10th century AD as a specialised academic profession. This was possible through the import of books - such as the Shoukanron (傷寒論/Chin. Shanghan Lun) and the Shinnouhonzoukyou (神農本草経/Chin. Shennong Bencaojing)—and immigration of trained experts from China and Korea. In the following centuries–especially from the 17th to the 19th century during the seclusion of Japan, Japanese traditional medicine evolved largely independent from external influences. Thus, Ancient Chinese Medicine (ACM) was at the origin of a divergent cultural evolution leading to several distinct contemporary forms of Eastern Medicine such as Japanese Kampo, Traditional Chinese Medicine (TCM) and Korean Medicine (KM) [Kuchta K. Zeitschrift für Phytotherapie 2014; 35 (02): 79–84].

In biological evolution, the process of divergent evolution of one species into several daughter species as an adaptation to distinct environments is well known. The general preconditions of evolution 1) Replication, 2) Mutation, and 3) Selection of information [Darwin C (1871). The Descent of Man and Selection in Relation to Sex. Photoreproduction of the 1871 Edition (Princeton, New Jersey London: Princeton

University PressJ. Murray), 60–61] drive not only the evolutionary development of genetic information, but also of cultural systems: They are omnipresent in all aspects of human culture, where knowledge (information) can easily be replicated (learning), mutated (innovation), and is subject to selection. The resulting body of accumulated knowledge and engrained behaviours is referred to as "tradition" or "culture" and the process of its development and distribution can be referred to as "cultural evolution". In the case of traditional medicines and herbal remedies, the evolutionary pressure that drives their cultural evolution is the survival benefit of effective treatments. As any successful therapy depends on 1) human physiology and 2) the pathophysiology of the disease, these two factors form the equivalent of Darwinian evolutionary pressure in the cultural evolution of traditional medicine. Thus - although traditional medicine is not based on the modern knowledge of human physiology, biochemistry or genetics - the underlying information for safety and efficacy is "imprinted" onto traditional theories and practice by the cultural evolution process itself. Traditional medicines therefore exhibit an *a priori* internal structure that already corresponded to human pharmacology and physiology, long before these were scientifically understood. Whilst the "Bench to Bedside" and the "Bedside to Bench" approaches have been established also for medicinal (plant) research, we propose a third, novel approach: "From Tradition to Pathogenesis". Several examples are given where the understanding of empirical treatments leads to the understanding of the pathogenesis of disease. This understanding of cultural evolution in the context of traditional medicine and its application to modern pathophysiology might help to clarify as of yet unknown pathomechanisms of disease, or at least formulate a hypothesis which can be examined at a later stage of research [Kuchta and Cameron].

As Kampo has developed into its own distinct form of Eastern Medicine, Kampo diagnostics and anamnesis developed [Kuchta and Cameron]. E.g. in Kampo medicine, there exists an important system of abdominal diagnosis called Fukushin (腹診). By applying pressure to the abdomen of the patient, the physician can read the patients' physical state from the "patterns" of firmness or softness and thus choose a suitable Kampo formulation. Traditional Kampo anamnesis aims to correlate symptoms and abdominal "patterns" to the respective prescription formulas. In the present volume, Yakubo et al. report on the development of a Fukushin simulator, a teaching tool that reproduces the important abdominal "patterns" that doctors encounter in clinical practice. The various Fukushin models simplify the teaching of these inherently "hands on" concepts. In recent years, Kampo Fukushin "pattern" diagnosis and the corresponding anamnesis have found their way into the new ICD-11 framework of the WHO alongside several related concepts from TCM [Wu X. et al.].

With the establishment of the current form of the Japanese national health insurance system in the late 1960s the status of Kampo as standardized academic medicine was legally established. Currently, 148 Kampo prescriptions are covered by the national insurance system. The health insurance thus covers these Kampo medicines when prescribed by any medical doctors - not only by dedicated Kampo therapists. Further, official approval standards for over-the-counter Kampo products have been established for 294 formulations [Kuchta and Cameron].

As such, Kampo constitutes an integral part even of the most innovative forms of therapy such as the Japanese response to the current COVID-19 pandemic. In the present volume, Ogawa-Ochiai et al. present data from a retrospective observational study documenting the immunological and preventive effects of the Kampo prescriptions Hochuekkito (補中益気湯) and Kakkonto (葛根湯) against COVID-19 in healthcare workers.

Takayama et al. present a review of all currently available data from case reports and ongoing clinical trials on the prevention and treatment of COVID-19 patients with Kampo medicine as a whole. Building on the successful use of Kampo during the Spanish flu one century earlier, the authors were able to document numerous successful Kampo therapies for the current pandemic.

Moving from clinical research to laboratory research, Kakimoto et al. used an *in vitro* model system to demonstrate that several well established Kampo formulas that are commonly used in the treatment of respiratory symptoms of the common cold were able to successfully suppress SARS-CoV-2 infection. Here, Maoto (麻黄湯) was the most effective among the tested Kampo formulas, and Ephedrae herba according to the Japanese Pharmacopoeia (JPh XVIII) was the most effective among the tested individual herbal drugs. Ephedrae herba is also one of the main component drugs of Kakkonto (葛根湯/see above), a prescription that was demonstrated by Saito et al. to inhibit rhinovirus induced cytokine production in primary cultures of human nasal epithelial cells.

Whilst Kampo medicines enhance the immune systems' self-defence and might thus be used to treat acute infections, their main strength lies in the treatment (and prevention) of chronic disease, where microcirculation, the immune system and healing properties play a role.

In an ageing society an increased incidence of cancer constitutes a major challenge to the public health system. Even though Kampo medicine cannot replace chemotherapy, it has proven its invaluable potential as a supportive therapy.

Chemotherapy-induced oral ulcerative mucositis for instance is one of the most common and challenging side effects of anti-tumour chemotherapy. As Miyano et al. have demonstrated, the Japanese Kampo medicine Hangeshashinto (半夏瀉心湯) improves this condition markedly. In this volume, they provide evidence suggesting that Hangeshashinto enhances human oral keratinocyte migration by up-regulating the

chemokine protein stromal cell-derived factor 1, also known as C-X-C motif chemokine 12 (CXCL12), via extracellular signal-regulated kinase.

Furthermore, Kampo treatments for dementia - one typical case of a long term chronic disease - deserve special mention. In their review, Kuchta et al. demonstrate the efficacy of the traditional prescription Kamiuntanto (加味温胆湯) as a useful treatment option. One of its component drugs Polygala radix (JPh XVIII) appears to be the biggest individual contributor to this activity. As similar effects could also be demonstrated for Saffron - the dried red stigmata of the flowers of *Crocus sativus* L. - the combination of the two active principles as Kamiuntantokabankoka (加味温胆湯加番紅花) proved most promising as treatment option for dementia. As an alternative approach for the treatment of dementia Wenger et al. have reviewed Kampo prescriptions that traditionally contain cannabis (*Cannabis sativa* L.) as its major neurological active cannabinoids cannabidiol (CBD) and tetrahydrocannabinol (THC) have long been identified as important anti-dementia drug candidates. Consequently, Shakanzoto (炙甘草湯) the "long-term use [of which] is believed to promote youthfulness and lucidness" and which is further used against typical afflictions of the elderly such as "shortness of breath and palpitations with constipation-like symptoms, lack of nutrients, dry skin, and fatigue" seems to be one of the most promising candidates for future wide spread use in dementia therapy. It should be emphasised that Kampo prescriptions based on *Cannabis sativa* L. generally carry a far smaller risk of addiction than similar Western medicinal products as in Kampo only the relatively less addictive seeds are used.

Overall, undesired drug effects in Kampo are extremely rare and new methods to prevent and counteract such effects are constantly being developed. Liquorice (JPh XVIII) is one of the most common crude drugs in traditional Japanese Kampo medicine and famed for numerous anti-inflammatory and anti-infective properties. However, in high dosages its major constituent glycyrrhizin can cause pseudohyperaldosteronism as a side effect. Ishiuchi et al. have identified 18β-glycyrrhetyl-3-O-sulfate as a glycyrrhizin metabolite in a rat model and present convicting evidence that this compound, which is mainly detected in serum of pseudohyperaldosteronism patients, is the most promising causative agent of this adverse effect. They have successfully established an ELISA system able to monitor its blood concentration in patients taking liquorice. This new technology will further strengthen the predisposition of Kampo as a highly personalised safe and effective therapy.

In summary, Kampo medicine is well-established in conjunction with Western medicine in Japan, especially for the inflammation and infectious diseases, supportive in cancer care and in the elderly. In Kampo medicine (in contrast to most other forms of Eastern Medicines) philosophical considerations take a back seat to traditional clinical empiricism. This fact has stimulated the development of a lively dialogue and cooperation with Western forms of pharmacotherapy as well as clinical and basic research studies within the framework of "integrative medicine", called "Tougouiryou" (統合医療). Due to this development as well as the ongoing international efforts to place Kampo into the context of Eastern Medicine in the WHO's ICD-11 concept and the ISO TC-249 industrial standardisation process, Kampo is clearly the form of Eastern Medicine most easily accessible to Western thinking as this process has already happened in Japan during the adaptation of Western cultural techniques into Japanese civilisation in the Meiji reforms of the 19th century. It thus warrants a large-scale application in the West itself, where - especially in the context of our ageing society - new "integrative" forms of therapy for chronic diseases are urgently needed.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

The authors thank the "Förderkreis für Fernöstliche Medizin" for financial support.

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 May 2021
doi: 10.3389/fphar.2021.688508

Identification of an Alternative Glycyrrhizin Metabolite Causing Liquorice-Induced Pseudohyperaldosteronism and the Development of ELISA System to Detect the Predictive Biomarker

*Kan'ichiro Ishiuchi[1†], Osamu Morinaga[2†], Tetsuhiro Yoshino[3†], Miaki Mitamura[1], Asuka Hirasawa[1], Yasuhito Maki[1], Yuuna Tashita[1], Tsubasa Kondo[2], Kakuyou Ogawa[2], Fangyi Lian[3], Keiko Ogawa-Ochiai[4‡], Kiyoshi Minamizawa[5], Takao Namiki[6], Masaru Mimura[3], Kenji Watanabe[3] and Toshiaki Makino[1]**

[1]Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan, [2]Department of Natural Medicines, Daiichi University of Pharmacy, Fukuoka, Japan, [3]Center for Kampo Medicine, Keio University School of Medicine, Tokyo, Japan, [4]Department of Otorhinolaryngology and Head and Neck Surgery, Clinic of Japanese Oriental (Kampo) Medicine, Kanazawa University Hospital, Kanazawa, Japan, [5]Department of Oriental Medicine, Kameda Medical Center, Kamogawa, Japan, [6]Department of Japanese Oriental (Kampo) Medicine, Graduate School of Medicine, Chiba University, Chuo-ku, Japan

Liquorice is usually used as crude drug in traditional Japanese Kampo medicine and traditional Chinese medicine. Liquorice-containing glycyrrhizin (GL) can cause pseudohyperaldosteronism as a side effect. Previously, we identified 18β-glycyrrhetyl-3-*O*-sulfate (**3**) as a GL metabolite in Eisai hyperbilirubinuria rats (EHBRs) with the dysfunction of multidrug resistance-related protein (Mrp2). We speculated that **3** was associated with the onset of liquorice-induced pseudohyperaldosteronism, because it was mainly detected in serum of patients with suspected to have this condition. However, it is predicted that other metabolites might exist in the urine of EHBRs orally treated with glycyrrhetinic acid (GA). We explored other metabolites in the urine of EHBRs, and investigated the pharmacokinetic profiles of the new metabolite in EHBRs and normal Sprague-Dawley rats. We further analyzed the serum concentrations of the new metabolite in the patients of pseudohyperaldosteronism. Finally, we developed the analyzing method of these metabolites as a preventive biomarker for the onset of pseudohyperaldosteronism using an enzyme-linked immunosorbent assay (ELISA). We isolated a new GL metabolite, 18β-glycyrrhetyl-3-*O*-sulfate-30-*O*-glucuronide (**4**). Compound **4** significantly inhibited rat type-2 11β-hydroxysteroid dehydrogenase (11β-HSD2) and was a substrate of both organic anion transporter (OAT) 1 and OAT3. Compound **4** was also detected in the serum of patients with suspected pseudohyperaldosteronism at an approximately 10-fold lower concentrations than **3**, and these concentrations were positively correlated. Compound **4**

OPEN ACCESS

Edited by:
Wei Li,
Toho University, Japan

Reviewed by:
Decio Armanini,
University of Padua, Italy
Liqin Ding,
Tianjin University of Traditional Chinese Medicine, China

***Correspondence:**
Toshiaki Makino
makino@phar.nagoya-cu.ac.jp

‡Present address:
Kampo Clinical Center, Department of General Medicine, Hiroshima University Hospital, Hiroshima, Japan

†These authors have contributed equally to this work

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

Received: *30 March 2021*
Accepted: *29 April 2021*
Published: *17 May 2021*

Citation:
Ishiuchi K, Morinaga O, Yoshino T, Mitamura M, Hirasawa A, Maki Y, Tashita Y, Kondo T, Ogawa K, Lian F, Ogawa-Ochiai K, Minamizawa K, Namiki T, Mimura M, Watanabe K and Makino T (2021) Identification of an Alternative Glycyrrhizin Metabolite Causing Liquorice-Induced Pseudohyperaldosteronism and the Development of ELISA System to Detect the Predictive Biomarker. Front. Pharmacol. 12:688508. doi: 10.3389/fphar.2021.688508

showed a lower serum concentration and weaker inhibitory titer on 11β-HSD2 than **3**. We developed an enzyme-linked immunosorbent assay system using an anti-18β-glycyrrhetyl-3-*O*-glucuronide (3MGA) monoclonal antibody to measure the serum concentration of **3** to facilitate the measurement of biomarkers to predict the onset of pseudohyperaldosteronism. Although we found **4** as the secondary candidate causative agent, **3** could be the main potent preventive biomarker of liquorice-induced pseudohyperaldosteronism. Compound **3** was detected in serum at a higher concentration than GA and **4**, implying that **3** may be a pharmacologically active ingredient mediating not only the development of pseudohyperaldosteronism but anti-inflammatory effects in humans administered GL or other liquorice-containing preparations.

Keywords: kampo medicine, side effect, liquorice, glycyrrhizin, pseudoaldosteronism, sex differences

INTRODUCTION

Liquorice, the root or stolon of *Glycyrrhiza uralensis* or *G. glabra*, is a natural material commonly used as a sweetener for foods as well as a crude drug used in traditional Japanese Kampo medicine and traditional Chinese medicine (TCM). Its component glycyrrhizin (**5**) (GL; **Figure 1A**) has various pharmacological actions such as anti-allergy, anti-inflammation, anti-ulcer, anti-virus, anti-androgenic, and hepatorptective effects (Isbrucker and Burdock, 2006). Pseudohyperaldosteronism, a well-known side effect of liquorice, is characterized by hypertension, oedema, and hypokalaemia (Conn et al., 1968; Stewart et al., 1987). The onset of pseudohyperaldosteronism is associated with the inhibition of type-2 11β-hydroxysteroid dehydrogenase (11β-HSD2) in renal distal tubular epithelial cells by some GL

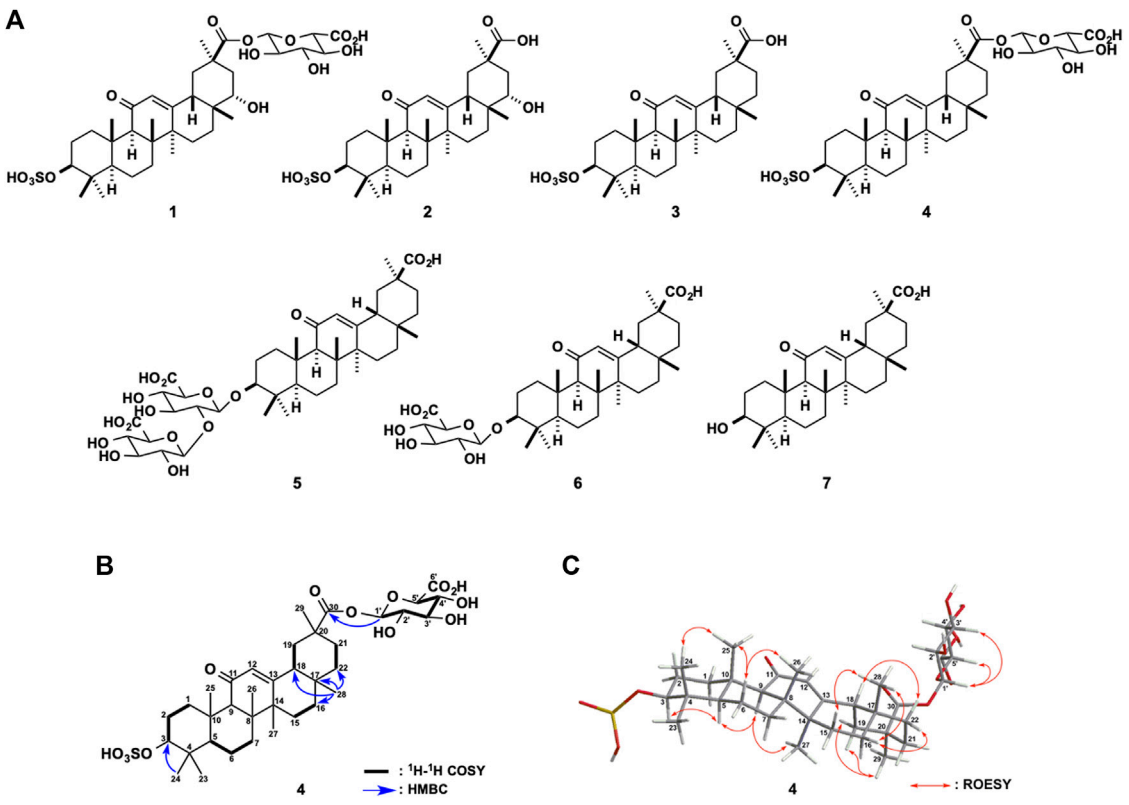

FIGURE 1 | (A) Chemical structures of glycyrrhizin (GL, **5**) and its metabolites (**1**–**4**), 18β-glycyrrhetyl-3-*O*-glucuronide (3MGA, **6**), and glycyrrhetinic acid (GA, **7**) **(B)** Key two-dimensional (2D) nuclear magnetic resonance (NMR) correlations for **4 (C)** Selected rotating frame overhause effect spectroscopy (ROESY) correlations and relative stereochemistry of **4**.

metabolites (Ploeger et al., 2001). Under normal conditions, cortisol, which has a similar affinity to the mineralocorticoid receptor as aldosterone, is converted by 11β-HSD2 to cortisone, which has a low affinity for the receptor. When 11β-HSD2 is inhibited by GL metabolites, the local cortisol concentration increases, which stimulates the mineralocorticoid receptor. Consequently, sodium reabsorption and potassium excretion are accelerated, resulting in symptoms such as hypokalaemia, hypertension, and oedema (Ploeger et al., 2001).

Recently, we found 22α-hydroxy-18β-glycyrrhetyl-3-*O*-sulfate-30-glucuronide (**1**), 22α-hydroxy-18β-glycyrrhetyl-3-*O*-sulfate (**2**), and 18β-glycyrrhetyl-3-*O*-sulfate (**3**; **Figure 1A**) as GL metabolites in the urine of Eisai hyperbilirubinuria rats (EHBRs) orally treated with glycyrrhetinic acid (**7**, GA; **Figure 1A**) (Morinaga et al., 2018; Ishiuchi et al., 2019). Orally administered GL is hydrolysed by intestinal bacteria to GA and appears in circulating blood (Akao et al., 1994). GA then translocates to the liver where it is metabolised to compounds **1**–**3**, and 18β-glycyrrhetyl-3-*O*-glucuronide (**6**, 3MGA; **Figure 1A**) by sulfotransferase (SULT) 2A1 (Takahashi et al., 2019), some cytochrome P450 (CYP) enzymes, and some glucuronyltransferases (Kato et al., 1995; Makino et al., 2008; Makino et al., 2012; Morinaga et al., 2018; Ishiuchi et al., 2019).

Previous studies suggested GA or 3MGA as causative agents of pseudohyperaldosteronism because they had a greater inhibitory effect on the activity of 11β-HSD2 than GL, which was not detected in the blood (Monder et al., 1989; Kato et al., 1995). Most of the GA, 3MGA, and compounds **1**–**3** are bound to albumin in the blood and cannot translocate to cells by simple diffusion. To inhibit 11β-HSD2, it has to be transported by some transporters into the renal tubular cells where it is actively expressed. Compounds **1**–**3** and 3MGA but not GA are substrates for organic anion transporter (OAT) 1 and OAT3 expressed on the vascular side of renal tubular epithelial cells. Since compounds **1**–**3** and 3MGA exhibit sufficient inhibitory effects on 11β-HSD2, we reported that all these compounds could cause pseudohyperaldosteronism (Makino et al., 2008; Makino et al., 2012; Morinaga et al., 2018; Ishiuchi et al., 2019).

Furthermore, we evaluated the association of the concentration of each GL metabolite in serum samples of patients with suspected pseudohyperaldosteronism with the laboratory data and various symptoms. Compound **3** but not **1**, **2**, and 3MGA were detected in the serum samples, which suggests the association of **3** with the onset of pseudohyperaldosteronism (Takahashi et al., 2019).

In this study, we isolated another new GL metabolite, 18β-glycyrrhetyl-3-*O*-sulfate-30-*O*-glucuronide (**4**), from the urine of EHBRs orally treated with GA and elucidated its structure based on spectroscopic data. We demonstrated the inhibitory effect of **4** on 11β-HSD2 and detected **4** in serum samples from patients with suspected pseudohyperaldosteronism. However, the concentration of **4** was lower than that of **3**, therefore, **3** was likely the major cause of pseudohyperaldosteronism. Then, we developed an enzyme-linked immunosorbent assay (ELISA) system to facilitate the measurement of the concentration of **3** in serum samples collected from the patients. In addition, we compared the differences in the pharmacokinetics of these GL metabolites between male and female EHBRs and humans.

MATERIAL AND METHODS

Chemicals, Reagents, Animals, and General Procedures

All chemicals, reagents, animals, and general procedures were the same as those described in our previous study (Morinaga et al., 2018; Ishiuchi et al., 2019). The animal experimental procedures were approved by the Animal Care Committee at the Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care.

Isolation of Compound 4

EHBRs (6 week-old, Japan SLC, Hamamatsu, Japan) were reared and provided with drinking water containing 1 mg/ml GA suspended in 1% carboxymethylcellulose solution for 3 months. The urine of the rats was collected, pooled, filtered, and then 2.5 L of the urine was evaporated under reduced pressure (dried weight, approximately 40 g). The concentrated urine (1.4 L) was partitioned with ethyl acetate and 1-buthanol. The 1-buthanol-soluble part (25 g) was separated by ODS silica gel column chromatography with a mixture of methanol/H_2O (1:4 → 1:0). A fraction eluted with methanol/H_2O (3:2) was further separated by silica gel column chromatography (chloroform/methanol/H_2O/trifluoroacetic acid 1:0:0:0 → 5:5:1:0.01), from which a fraction eluted with chloroform/methanol (1:1) was further purified using C_{18} high-performance liquid chromatography (HPLC, Cosmosil 5C_{18}-ARII (Nakalai Tesque, Kyoto, Japan), 5 μm, 4.6 mm *i. d.* × 250 mm) with the following parameters: solvent, acetonitrile/H_2O/trifluoroacetic acid (35:65:0.1); flow rate, 0.6 ml/min; detection 254 nm, to obtain compound **4** (3.2 mg).

Compound **4**: colorless amorphous solid $[\alpha]_D^{24}$ + 107 (*c* 0.3, methanol); ultraviolet (UV) (methanol) λ_{max} 248 (ε 9782) nm; electronic circular dichroism (ECD) (methanol) λ ($\Delta\varepsilon$) 230 (+8.3) nm; ^{1}H-nuclear magnetic resonance (NMR) (deuterated methanol, 500 MHz) and ^{13}C-NMR (deuterated methanol, 125 MHz), see **Table 1**; electrospray ionization tandem mass spectrometry (ESIMS) *m/z* 725 [M-H]$^-$; high resolution (HR) ESIMS *m/z* 725.3212 [M-H]$^-$ (calculated for $C_{36}H_{53}O_{13}S$, 725.3207).

Determination of *in vitro* 11β-HSD2 Activity Using Rat Kidney Microsomes

Assays were conducted as described by Diederich et al. (2000) with the slight modifications as described in our previous report (Makino et al., 2012).

TABLE 1 | ^{1}H and ^{13}C NMR Data (CD_3OD) of compounds **4** and **1**.

	4			1	
Position	**δ_H[a]**	**δ_C[b]**	**HMBC**	**δ_H[a]**	**δ_C[b]**
1a	2.74 (1H, brd 13.5 Hz)	40.1	9, 25	2.74 (1H, d 13.5 Hz)	40.1
1b	1.05 (1H, nd[c])			1.04 (1H, nd[c])	
2a	2.07 (1H, brd 13.5 Hz)	25.2		2.08 (1H, brd 11.5 Hz)	25.2
2b	1.80 (1H, nd[c])			1.78 (1H, nd[c])	
3	3.96 (1H, dd 12.0, 4.0 Hz)	87.3	23, 24	3.96 (1H, m)	87.4
4		39.9	3, 23, 24		39.9
5	0.86 (1H, nd[c])	56.6	1a, 7b, 23, 24, 25	0.86 (1H, nd[c])	56.6
6a	1.64 (1H, brd 13.0 Hz)	18.6	5	1.65 (1H, brd 13.5 Hz)	18.6
6b	1.49 (1H, nd[c])			1.49 (1H, nd[c])	
7a	1.76 (1H, nd[c])	33.8	6a, 26	1.76 (1H, nd[c])	33.7
7b	1.45 (1H, nd[c])			1.45 (1H, nd[c])	
8		46.7	6a, 7a, 9, 15a, 26, 27		46.7
9	2.47 (1H, s)	63.0	7b, 8, 12, 25, 26	2.47 (1H, s)	63.1
10		38.2	1a, 5, 6a, 9, 25		38.1
11		202.6	9, 12		202.5
12	5.62 (1H, s)	129.1	18	5.61 (1H, s)	129.2
13		172.7	12, 15b, 18, 19b, 27		171.1
14		44.6	9, 12, 15a, 18, 26, 27		45.1
15a	1.88 (1H, nd[c])	27.6	16a, 27	1.80 (1H, nd[c])	27.2
15b	1.25 (1H, nd[c])			1.29 (1H, m)	
16a	2.15 (1H, ddd 14.0, 14.0, 4.0 Hz)	27.4	15a, 18, 28	1.80 (1H, nd[c])	20.3
16b	1.03 (1H, nd[c])			1.48 (1H, nd[c])	
17		32.9	15b, 16a, 18, 19a, 19b 21a, 21b, 22b, 28		38.4
18	2.26 (1H, dd 14.0, 3.5 Hz)	49.3	12, 16b, 19a, 19b, 22b 28	2.21 (1H, brd 13.5 Hz)	49.0
19a	1.92 (1H, nd[c])	42.2	18, 21a, 29	1.89 (1H, brd 12.5 Hz)	41.6
19b	1.77 (1H, nd[c])			1.80 (1H, nd[c])	
20		45.2	19a, 19b, 21a, 22b, 29		45.1
21a	2.01 (1H, brd 9.0 Hz)	32.0	19a, 19b, 29	2.16 (1H, brd 11.0 Hz)	39.5
21b	1.45 (1H, nd[c])			1.51 (1H, nd[c])	
22a	1.45 (1H, nd[c])	38.7	21b, 28	3.42 (1H, nd[c])	76.1
22b	1.38 (1H, brd 10.5 Hz)				
23	1.06 (3H, s)	28.7	3, 24	1.07 (3H, s)	28.7
24	0.86 (3H, s)	16.9	3, 5, 23	0.86 (3H, s)	16.9
25	1.16 (3H, s)	17.0	9	1.16 (3H, s)	17.0
26	1.14 (3H, s)	19.3	7a, 7b, 9	1.14 (3H, s)	19.2
27	1.43 (3H, s)	23.8	15a, 15b	1.44 (3H, s)	23.9
28	0.82 (3H, s)	28.9	16a, 18, 22a	0.93 (3H, s)	25.5
29	1.21 (3H, s)	28.1	19b	1.25 (3H, s)	28.1
30		176.9	19b, 21b, 29, 1′		176.6
1′	5.53 (1H, d 8.0 Hz)	95.5	2′	5.53 (1H, d 7.5 Hz)	95.6
2′	3.40 (1H, dd 9.0, 8.0 Hz)	73.6	3′	3.42 (1H, nd[c])	73.6
3′	3.45 (1H, dd 9.0, 9.0 Hz)	77.7	2′, 4′	3.45 (1H, nd[c])	77.6
4′	3.59 (1H, dd 9.0, 9.0 Hz)	72.9	3′	3.60 (1H, dd 9.0, 9.0 Hz)	72.9
5′	3.90 (1H, d 9.0 Hz)	77.3	1′, 4′	3.90 (1H, d 9.0 Hz)	77.4
6′		171.9	4′, 5′		171.8

[a]*500 MHz.*
[b]*125 MHz.*
[c]*nd: J-values were not determined because of overlapping with other signals.*

Uptake of Compound 4 by Rat Kidney Slices and Cells Expressing OAT1 and 3

For the uptake study of **4** into rat kidney slices, we used the samples obtained in our previous study (Ishiuchi et al., 2019). For the uptake study of **4** into the cells transiently expressing OAT1 and OAT3, we used the same protocol as in our previous study (Makino et al., 2012). cDNAs encoding human OAT1 and OAT3 inserted into pGH19 were generously supplied by Prof. Mitsuru Sugawara (Hokkaido University, Sapporo, Japan), and then subcloned into a pCI-neo mammalian expression vector (Promega, Madison, WI, United States). We used the same pooled plasma of male EHBRs orally treated with GA for 12 h used in our previous study (Ishiuchi et al., 2019).

Binding of Compound 4 to Albumin

We measured the binding ratios of **4** to albumin in pooled plasma of female EHBRs using the same protocol used in our previous study (Ishiuchi et al., 2019).

Measurement of Compound 4 in Human Serum Samples

Human serum samples (100 μl) were pre-treated as described in the protocol reported in our previous study (Takahashi et al., 2019), and the concentration of **4** was measured using LC-tandem mass spectrometry (MS/MS) as described below. This study was conducted with the approval of the appropriate ethics committee at Keio University, Chiba University, Kanazawa University, and Kameda Medical Center.

Evaluation of the Pathological Relationship Between Compound 4 and Pseudohyperaldosteronism Pathology

We recorded the following available clinical symptoms and laboratory data when blood samples were drawing from patients: age, sex, complications, concomitant medications, daily liquorice dose, duration of administration of liquorice-containing herbal preparations, symptoms (blood pressure and pedal oedema), and laboratory test values, namely: total protein, albumin, total and direct bilirubin, aspartate amino transferase, alanine amino transferase, blood urea nitrogen, creatinine, sodium, potassium, chloride, calcium, magnesium, prothrombin time, plasma renin activity or activated renin concentration, and plasma aldosterone concentration (blood test data), and urine concentrations of potassium and creatinine (urinalysis data). Samples were processed according to the individual institutional protocols, and all data were measured at each institution.

Dot-Blot Analysis Using anti-3MGA-Monoclonal Antibody

An anti-3MGA-monoclonal antibody (mAb) and 3MGA-human serum albumin (HSA)-conjugate were developed using the same protocols established in our previous studies (Morinaga et al., 2018; Ishiuchi et al., 2019). GL, GA, 3MGA, compounds **1**–**4**, and bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, United States, 1 μg each) were spotted onto a Mustang E positively charged polyethersulfone membrane (Pall Co., East Hills, NY, United States), fixed, and stained with the anti-3MGA-mAb using the same method used in our previous study (Ishiuchi et al., 2019).

ELISA System Using anti-3MGA-mAb

An ELISA system to measure the cross-reactivity of compounds **3** and **4** by using 3MGA-HSA-conjugate and anti-3MGA-mAb was used according to the protocol in our previous study (Ishiuchi et al., 2019). To measure the concentration of **3** in human serum samples using the system ELISA, **3** was dissolved in normal human serum (Sigma) to prepare standard solutions (16 nM–2.0 μM) and the samples or standard solutions (each 10 μl) and ethanol (40 μl) were well mixed and centrifuged ($1.2 \times 10^4 \times g$ for 7 min). The supernatant was dried up under nitrogen flow at 40°C overnight, and the residue was dissolved in Can Get Signal® Solution 1 (Toyobo Co., Ltd: Osaka, Japan) (50 μl). The ELISA was conducted according to the protocol described in our previous study (Ishiuchi et al., 2019). When the observed values were more than 400 nM, the deproteinized serum samples were further diluted with Can Get Signal® Solution 1 for two or four times and analyzed again to observe values from 15.6 to 400 nM.

Pharmacokinetic Experiments of GA Metabolites in EHBRs Orally Treated With GA

Male and female EHBRs (9 week-old) were anaesthetized by an intraperitoneal injection of urethane (1 g/kg) and their jugular veins were exposed. GA suspended in 0.5% carboxymethylcellulose was then administered orally (200 mg/kg) to the unconscious rats, and blood samples were collected from the jugular vein while urine samples were collected using a metabolic cage at appropriate times over a 12 h period.

LC-MS/MS Analysis for GA Metabolites

The concentrations of compounds **1**–**4**, 3MGA, GA, and GL in serum and urine samples were measured using an LC-MS/MS system (Quattro Premier XE; Waters, Milford, MS, United States) as described in our previous studies (Takahashi et al., 2019). Briefly, after diluting serum and urine samples with water to obtain suitable concentrations, they were digested with subtilisin (0.91 U/ml), followed by the addition of astragaloside IV (used as an internal standard; Fujifilm Wako Pure Chemicals, Osaka, Japan), and deproteinized using 80% ethanol. The supernatant was transferred into a vial and the concentrations of compounds **1**–**4**, 3MGA, GA, and GL in the samples prepared from plasma and urine were measured under the following conditions: column, Scherzo SM-C18 (3 μm, 3 mm *i. d.* × 150 mm; Imtakt, Kyoto, Japan); mobile phase (A) 5 mM ammonium acetate in H_2O (B) 125 mM ammonium acetate in H_2O/acetonitrile (1:4), at a flow rate of 0.3 ml/min, with the following gradient profile: A:B = 50:50–0:100 (0–2 min) and 0:100 (2–21 min). The transitions (precursor to daughter) monitored and retention times were as follows: ESI(+), 743.4 to 567.5 m/*z* (35 and 25 V for cone voltage and collision energy, respectively) for compound **1** (8.1 min); ESI(−), 565.5 to 96.5 m/*z* (80 and 60 V) for **2** (9.6 min); ESI(−), 549.5 to 96.5 m/*z* (80 and 60 V) for **3** (15.4 min); ESI(+), 727.3 to 551.2 m/*z* (30 and 25 V) for **4** (11.3 min); ESI(+), 647.6 to 453.6 m/*z* (40 and 20 V) for 3MGA (15.0 min); ESI(+), 471.3 to 91.0 m/*z* (40 and 20 V) for GA (13.4 min); ESI(+), 823.5 to 453.6 m/*z* (30 and 20 V) for GL (18.4 min); and ESI(+), 785.4 to 143.0 m/*z* (30 and 20 V) for astragaloside IV (3.0 min). Linear regressions over the concentration range of 2 nM to 2 μM for each compound were examined using the peak-area ratio of the compounds to their internal standards and the least-squares method ($r^2 > 0.98$). The detection limits for **1**, GL, and GA were 6.4 nM, and those for **2**, **3**, and **4,** and 3MGA were 3.2 nM.

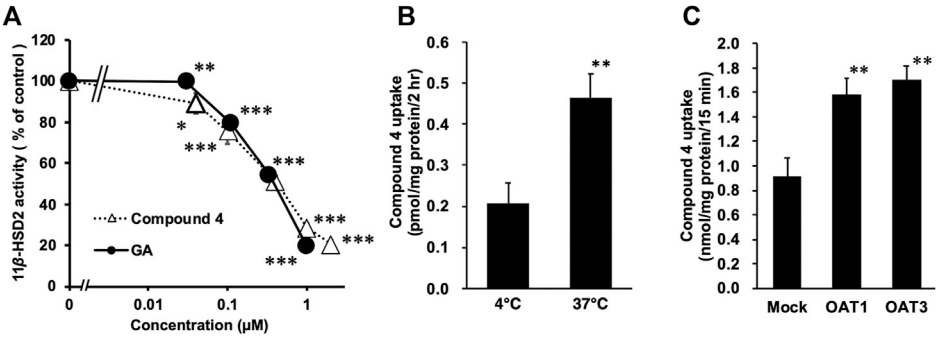

FIGURE 2 | Inhibitory effects of compound **4** on type-2 11β-hydroxysteroid dehydrogenase (11β-HSD2) using rat kidney microsome (A), and the uptake of **4** into rat kidney slices (B) or into HEK293 cells transfected with organic anion transporter (OAT) 1 (C) or OAT3 (D). **(A)** [^{3}H] cortisone and glycyrrhetinic acid (GA) or **4** were mixed with rat kidney microsome fractions, and incubated at 37°C for 30 min. Then, the amount of [^{3}H] cortisol was measured. Data are means ± standard error (S.E: n = 4) of percentage [^{3}H] cortisol in mixtures without samples. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with groups without samples using Dunnett's multiple t-test **(B)** Female Eisai hyperbilirubinuria rats (EHBRs) were orally treated with GA (200 mg/kg), and their plasma samples were collected 24 h after the treatment. Concentration of **4** in plasma sample was 115 μM. Kidneys were collected from normal Sprague-Dawley (SD) rats and slices were incubated with plasma samples of female EHBRs at 37°C or 4°C for 2 h, and then homogenized. Samples were those described in our previous study (Ishiuchi et al., 2019). Concentrations of **4** in kidney slice homogenates were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). **p < 0.01 *vs* 4°C group using Student's t-test **(C)** Male EHBRs were orally treated with GA (200 mg/kg) and their plasma samples were collected 24 h after treatment. Concentrations of **4** in plasma sample was 189 μM. HEK293 cells transfected with OAT1 or OAT3 were incubated with 1:2 diluted plasma samples of male EHBRs at 37°C for 15 min. Then, concentrations of **4** in cells were measured using LC-MS/MS. Data are means ± S.E. (n = 6). *p < 0.05 *vs* mock cells using Dunnett's multiple t-test.

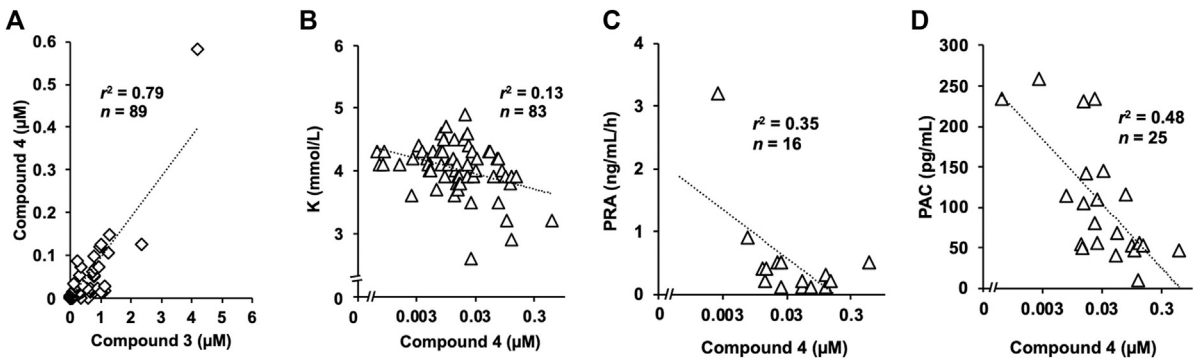

FIGURE 3 | Concentration of 4 in human serum samples and three or laboratory markers **(A)** Strong positive correlation was identified between serum concentrations of **3** and **4**. The concentration of **4** was approximately 10-fold lower than that of **3 (B)** Serum potassium concentration tended to be lower in participants with higher serum concentration of compound **4 (C)** Plasma renin activity and **(D)** plasma aldosterone concentration were also suppressed in patients with higher concentration of compound **4**, which were negatively correlated.

Statistics

Statistical analysis of the inhibitory effect of **4** on 11β-HSD2 (**Figure 2**) and the results of the uptake study using OAT1-and OAT3-induced cells (**Figure 2C**) were performed using a one-way analysis of variance (ANOVA), followed by Dunnett's multiple t-test using PASW Statistics (version 18, SPSS; IBM, Armonk, NY, United States). Statistical analysis of the uptake study using rat kidney slices (**Figure 2B**) was performed using the Student's t-test with the Microsoft Excel®. The calibrated line and the regression formula in **Figures 3**, **4D** were calculated by the linear least-squares method along with Pearson's product moment correlation coefficients using the Microsoft Excel®. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Isolation and Structural Elucidation of Compound 4 From EHBR Urine

From the urine of female EHBRs, we isolated compound **4** (3.2 mg) along with **2** and **3**. Compound **4** [$[\alpha]_D^{24}$ + 107 (c 0.3, methanol)] exhibited a deprotonated molecule at m/z 725 $(M\text{-}H)^-$ in the ESIMS, and the molecular formula, $C_{36}H_{54}O_{13}S$, was established by HRESIMS [m/z 725.3212 $(M\text{-}H)^-$, Δ+0.5 mmu]. The ^{1}H and ^{13}C NMR (**Table 1**) and heteronuclear single quantum coherence (HSQC) spectra of **4** shared similarity with those of 22α-hydroxy-18β-glycyrrhetyl-3-*O*-sulfate-30-glucuronide (**1**) except for five signals (δ_C 38.7, δ_H 1.45 and 1.38; δ_C 32.9; δ_C 32.0, δ_H 2.01 and 1.45; δ_C 28.9,

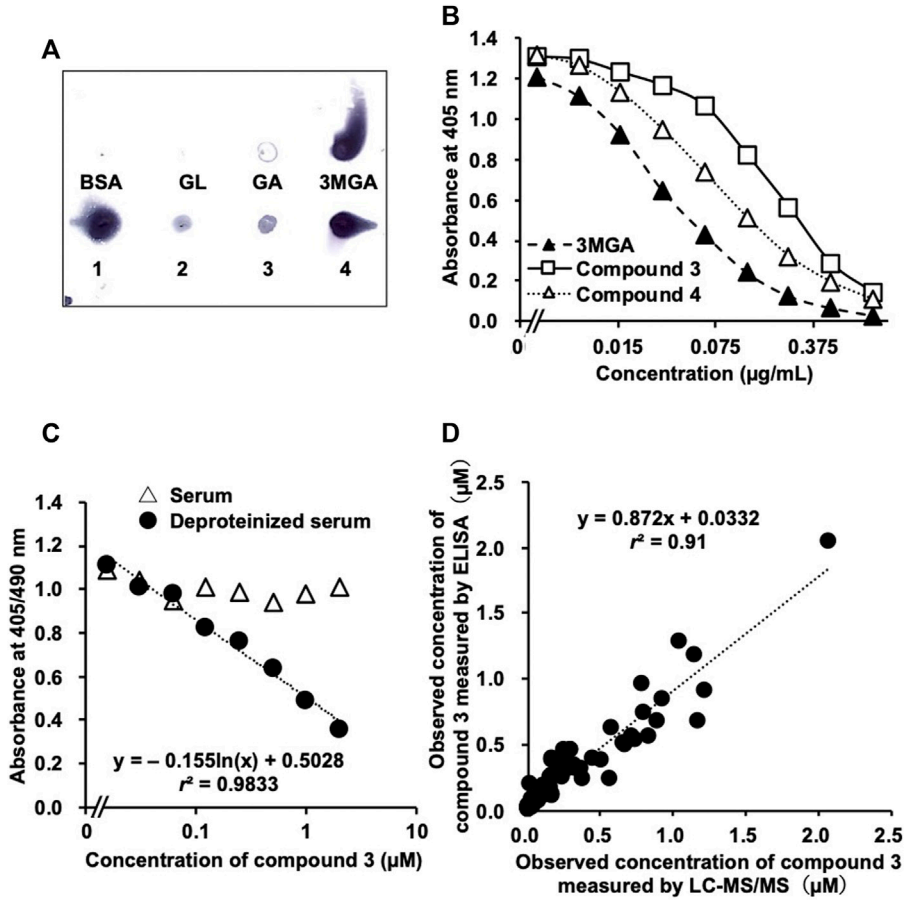

FIGURE 4 | Development of enzyme-linked immunosorbent assay (ELISA) system to measure concentrations of **3** in human serum samples using anti-18β-glycyrrhetyl-3-*O*-glucuronide (3MGA)-monoclonal antibody (mAb). **(A)** Dot-blot analysis of glycyrrhizin (GL) metabolites using anti-3MGA-mAb. GL metabolites and bovine serum albumin (BSA, 1 μg each) were spotted onto polyethersulfone membrane and stained using an anti-3MGA-mAb. **(B)** Competitive ELISA using an anti-3MGA-mAb for 3MGA, **3**, and **4** in aqueous solution was performed **(C)** Standard solutions of **3** (15.6 nM–2 μM) in normal human serum were prepared. Serum samples were deproteinized by treating with 80% ethanol, followed by centrifugation, and the supernatants were dried up under reduced pressure. Standard lines between absorbance and concentrations of compound **3** are shown **(D)** Accuracy of ELISA system in measuring concentrations of **3** in human serum samples using anti-3MGA-mAb. Human serum samples were deproteinized, and concentration of **3** was measured using ELISA system. Samples with observed values higher than 400 nM were diluted appropriately with H_2O to set detectable range from 10 to 400 nM. Values were compared with the result of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis from our previous study (Takahashi K, 2019). Significant relationship was observed between the values observed using ELISA and LC-MS/MS values (r^2 = 0.91).

δ_H 0.82; and δ_C 27.4, δ_H 2.15, and 1.03) in **4**. In particular, compound **4** shows the disappearance of one methine signal (δ_C 76.1, δ_H 3.42) at the C-22 of **1** and the difference in the molecular formulas between **4** and **1** indicated that compound **4** was a deoxygenated derivative of **1**.

The planar structure of **4** was elucidated by analysing the two-dimensional (2D) NMR data including the ^{1}H-^{1}H correlation spectroscopy (COSY), HSQC, and heteronuclear multiple bond correlation (HMBC) spectra in CD_3OD. The analysis ^{1}H-^{1}H COSY spectrum disclosed six structural units (C-1–C-3, C-5–C-7, C-15–C-16, C-18–C-19, C-21–C-22, and C-1′–C-5′). The observed HMBC correlations including a key HMBC cross-peaks of H_3-28 (δ_H 0.82) to C-16 (δ_C 27.4), C-17 (δ_C 32.9), C-18 (δ_C 49.3), and C-22 (δ_C 38.7) revealed that the planar structure of **4** was the same as that of **1** deoxygenated at C-22 (**Table 1** and **Figure 1B**). The connections of a β-glucuronic acid group to C-30 and of a sulfate group to C-3 were suggested by the HMBC cross-peaks of H-1′ (δ_H 5.53) to C-30 (δ_C 176.9) and H_3-24 (δ_H 0.86) to C-3 (δ_C 87.3), respectively (**Figure 1B**). The relative stereochemistry of **4** was revealed to be the same as that of **1**, except for the C-22 based on the rotating frame overhauser effect spectroscopy (ROESY) correlations for H-3/H-5, H_3-24/H_3-25, H-9/H-5, and H_3-27; H-6b/H_3-25 and H_3-26; H_3-28/H-15a, H-16b, and H-18; H_3-29/H-19a and H-19b; H-16b/H-22b, H-18/H-22a, and H-1′/H-3′; and H-5′ and based on $3J_{H\text{-}1'/H\text{-}2'}$ (8.0 Hz) and $3J_{H\text{-}4'/H\text{-}5'}$ (9.0 Hz, **Figure 1C**). The ECD spectrum of **4** showed a positive Cotton effect at 230 nm, similar to that of **1**. Thus, the absolute configuration of **4** was established as 3*S*, 5*R*, 8*R*, 9*R*, 10*S*, 17*R*, 18*S*, and 20*R*. A metabolite possessing the same structure as that of **4** was speculated in rat bile after intravenous injection of GA in a previous LC-MS analysis by Jing et al. (2008). However, to the best of our

knowledge, we successfully purified compound **4** and elucidated the structure comprehensively for the first time in this study.

Inhibitory Effects of Compound 4 on Rat 11β-HSD2

Compound **4** and GA (positive control) significantly inhibited 11β-HSD2 in the rat kidney microsome fraction in a concentration-dependent manner (**Figure 2A**) with half-maximum inhibitory concentration (IC_{50}) values of 0.38 and 0.35 μM, respectively.

Transport of Compound 4 Into Rat Kidney Slices and Cells Expressing OAT1 and 3

The pooled plasma samples collected from EHBRs 12 h after oral treatment with GA were loaded into ultracentrifuge filters with a 1×10^4 molecular weight cut-off and concentrated by centrifugation. The concentrations of **4** in the pooled plasma and the filtrate were 77 μM and undetectable, respectively. Based on the detection limits (3.2 nM) in the LC-MS/MS analysis, the albumin-binding ratios of **4** in the plasma was calculated to be more than 99.9%.

Kidney slices prepared from Sprague-Dawley (SD) rats were incubated with the pooled plasma at 4°C or 37°C for 2 h. **Figure 2B** shows the amounts of **4** accumulated in the kidney slices. The uptake of **4** by the kidney slices was significantly higher at 37°C ($p < 0.01$) than at 4°C (**Figure 2B**).

HEK293 cells transfected with OAT1, OAT3, or the mock plasmid were incubated with pooled plasma collected from EHBRs at 37°C for 15 min. The uptake of **4** into the cells was then measured (**Figure 2C**) and its level in cells expressing OAT1 or OAT3 was significantly higher ($p < 0.01$) than in mock cells.

Detection of Compound 4 in Human Serum Samples

Among the 89 serum samples stored, the daily dose of liquorice was available for samples from 86 participants, and 70 participants were taking liquorice-containing herbal preparations when we collected the serum samples. We detected compound **4** in 62 samples from the 70 participants. In contrast, we detected trace amounts of compound **4** in only one sample from the 16 participants who were not taking liquorice-containing herbal preparations. However, we found a weak positive correlation between the serum concentrations of compound **4** and the daily dosage of liquorice (**Supplementary Figure S1A**). On the other hand, we found a strong positive correlation between serum concentrations of **3** and **4**, and the concentration of **4** was approximately 10-fold lower than that of **3** (**Figure 3A**). We detected trace levels of compound **4** (3 nM) in one urine sample among the 20 available samples.

Relationship Between Laboratory Markers or Clinical Symptoms of Pseudohyperaldosteronism and Serum Concentration of Compound 4

Serum potassium concentration tended to be lower in participants with higher serum concentrations of compound **4** (**Figure 3B**). We confirmed a pseudohyperaldosteronism case where the patient had normal potassium concentration. The serum sample was collected from the patient who had been administered spironolactone which could increase potassium concentration. Other patients with higher serum concentrations of compound **4** had not been administered potassium-sparing medications. Additionally, there were negative correlations between the serum concentration of **4** and both plasma renin activity and aldosterone concentration (**Figures 3C,D** activated renin activity is shown in **Supplementary Figure S1B**). The relationship between compound **4** and clinical symptoms was not significant. The systolic and diastolic blood pressure were not measured on the day we collected the serum samples (**Supplementary Figure S1C**) and exacerbation of hypertension (data not shown) was not correlated with the concentration of compound **4**. No relationship was found between compound **4** and oedema grade (**Supplementary Figure S1D**) and exacerbation (data not shown).

Dot-Blot Analysis and the Cross-Reactivity of Compound 4 to anti-3MGA-mAb

We confirmed the cross-reactivity of anti-3MGA-mAb with GL, GA, 3MGA, and compounds **1**–**4** dissolved in aqueous solution. The anti-3MGA-mAb cross-reacted with **4** at a level similar to that of **1**, followed by **3** and **2** (**Figure 4A**). The concentration profiles of **3** and **4** to the absorbance in a competitive ELISA system using anti-3MGA-mAb were well characterised, and when the specificity of anti-3MGA-mAb to 3MGA was calibrated to 100%, the cross-reactivity for **3** and **4** was 23 and 40%, respectively (**Figure 4B**).

Measurement of the Concentration of Compound 3 Using ELISA With anti-3MGA-mAb

When **3** was dissolved in normal human serum and its concentration was measured using ELISA, no competitive reaction was observed between **3** and anti-3-MGA-mAb, and the standard line between the concentration of **3** and the absorbance could not be plotted. After deproteinization using 80% ethanol, a better standard line was obtained (**Figure 4C**). Using this protocol, we measured the concentration of **3** in 97 human serum samples and compared the data with those measured using the LC-MS/MS method described in our previous study (Takahashi et al., 2019). However, the r^2 value between the obtained data measured using ELISA and LC-MS/MS was 0.69, and inconsistencies appeared at higher concentrations. When the first measurements were more than 400 nM, the serum samples were diluted 5- or 10-fold with H_2O, and the concentration of **3** was measured again using the same ELISA system. This produced a better correlation ($r^2 = 0.91$) between the data measured using ELISA and LC-MS/MS (**Figure 4D**).

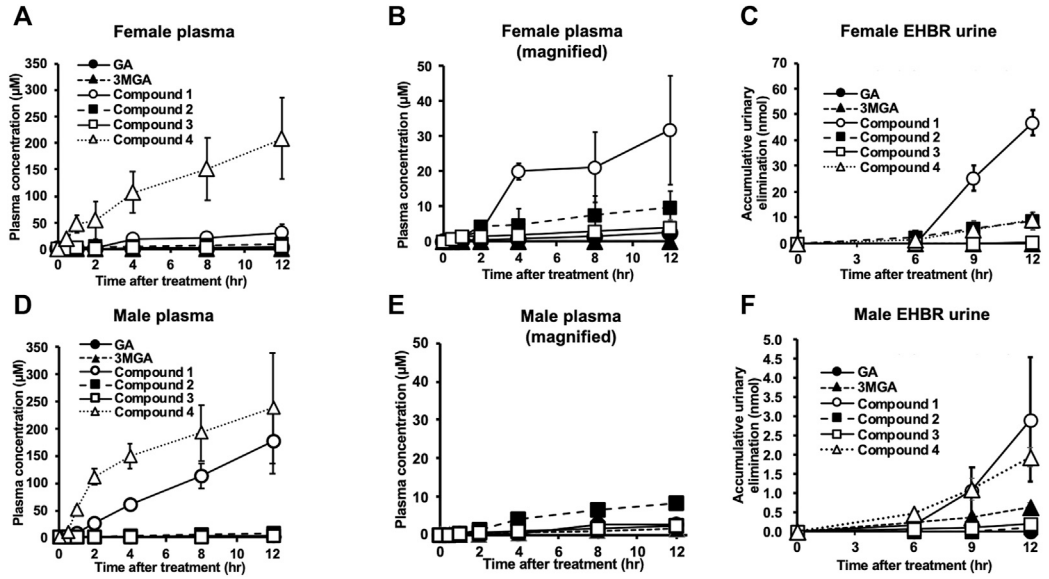

FIGURE 5 | Pharmacokinetic profiles of glycyrrhizin (GL) metabolites in female **(A–C)** and male **(D–F)** Eisai hyperbilirubinuria rats (EHBRs). Glycyrrhetinic acid (GA, 200 mg/kg) was administered orally to anesthetized EHBRs, and plasma and urine were collected for 12 h. Data of GA, 3MGA, **1**, **2**, and **3** in **(A)** and GA, 3MGA, **2**, and **3** for **(D)** are relatively small; their magnified graphs are shown in **(B,E)**, respectively. Concentrations of GA metabolites were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and data are means ± standard error (S.E: n = 4).

Pharmacokinetics of GA Metabolites in Female and Male EHBRs Orally Treated With GA

We successively collected plasma and urine samples from both male and female EHBRs that were orally administered GA (0.20 g/kg), and measured the concentrations of GA and its metabolites using LC-MS/MS. **Figure 5** shows the plasma concentration profiles and urinary elimination of GA and its metabolites. In female EHBRs orally treated with GA, **4** appeared in the plasma after 30 min and the concentration of **4** in plasma was sequentially increased over 12 h (**Figure 5A**). Next, the concentrations of **1**, **2**, and **3** were gradually increased after the oral treatment with GA (**Figure 5B**). The concentrations of GA and its metabolites 12 h after oral administration of GA in female EHBRs were 3.2 μM of GA, 0.1 μM of 3MGA, 14 μM of **1**, 4.3 μM of **2**, 6.6 μM of **3**, and 166 μM of **4**. The urine of female EHBRs showed the highest elimination levels of **1**, followed by **2** and **4**, which were at the same levels (**Figure 5C**). In male EHBRs, although the profiles of the plasma concentrations of **4**, **2**, and **3** exhibited curved similar to those of female EHBRs, the concentration curve of **1** was much higher than that of the female EHBRs (**Figures 5D,E**). The concentrations of GA and its metabolites 12 h after oral administration of GA in male EHBRs were 2.6 μM of GA, 1.2 μM of 3MGA, 102 μM of **1**, 4.1 μM of **2**, 1.2 μM of **3**, and 198 μM of **4**. In the urine of male EHBRs, the elimination of these metabolites was approximately 10-fold lower than it was in female EHBRs (**Figure 5F**). The eliminations of **1** and **4** were the highest and at similar levels, respectively. The plasma concentration and urinary elimination of 3MGA in male EHBRs was tended to be higher than those in female EHBRs.

These metabolites were gradually excreted into the bile in female SD rats with functional Mrp2 which were intravenously injected with GA, and the accumulation of **4** in the bile was the highest, followed by **3** and **2** (**Figure 6**). The accumulative elimination of **2**, **3**, and GA in faeces collected 24 h after the injection were 0.86 ± 0.1 nmol, 1.8 ± 0.3 nmol, and 9.8 ± 1.3 nmol, respectively. however, GL, 3MGA, **1** and **4** in faeces were below detectable levels. Compounds **1**–**4** were not detected in the serum of female SD rats collected 1, 2, 3, 4, and 24 h after GA injection.

DISCUSSION

We found compounds **1**, **2**, and **3** in the urine of EHBR orally treated with GA and proposed that these compounds could be candidates agents, along with the previously identified 3MGA (Kato et al., 1995; Makino et al., 2008; Makino et al., 2012), causing GL-induced pseudohyperaldosteronism (Morinaga et al., 2018; Ishiuchi et al., 2019). We analyzed 97 serum samples from patients suspected to have developed pseudohyperaldosteronism and found that **3** was the most probable causative agent (Takahashi et al., 2019). In this study, we isolated **4** as a new GL metabolite from the urine of EHBR orally treated with GA. Compound **4** exhibited 11β-HSD2 inhibitory activity equivalent to that of GA and high binding activity to serum albumin; additionally compound **4** was recognized as a substrate for OAT1 and OAT3 to transfer into renal tubular epithelial cells where 11β-HSD2 is expressed. When we measured the concentration of **4** in human serum samples from patients who developed pseudohyperaldosteronism, the concentration detected was sufficient but lower than that of **3**. This observation suggests that **4** is also a candidate causative agent of GL-induced pseudohyperaldosteronism.

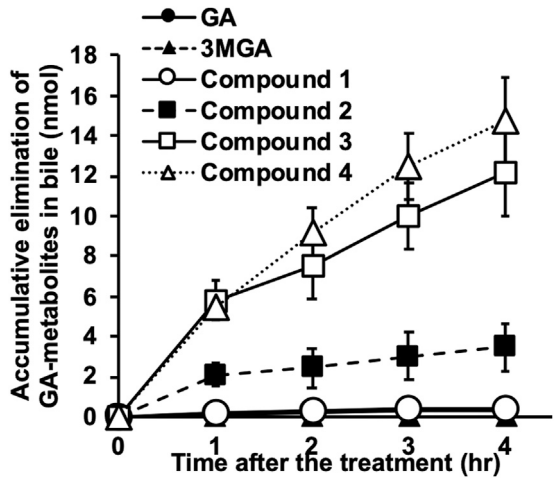

FIGURE 6 | Elimination of glycyrrhizin (GL) metabolites into bile in Sprague-Dawley (SD) rats. Female SD rats (10 week-old) were anesthetized using intraperitoneal injection of urethane (1 g/kg), and biliary tracts were cannulated. Glycyrrhetinic acid (GA, 0.2 mg/kg) was injected into jugular veins and bile was collected every hour for 4 h. Concentrations of GA metabolites were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and accumulated elimination of GA-metabolites are means ± standard error (S.E: n = 3).

Our results suggest that **3** is the major GL metabolite in human serum, followed by **4**, which can reach 11β-HSD2 in renal tubular epithelial cells. The analysis of serum samples from most of patients indicated that those with pseudohyperaldosteronism did not contain **1** or **2**, and the major GL metabolites in blood of these patients was **3** which was found at 1.5–2-fold higher than those of GA, and there was a positive correlation between the blood concentrations of **3** and GA (Takahashi et al., 2019). In the present study, **4** was detected in the same serum samples at concentrations of approximately 10-fold lower than those of **3**, and their concentrations were positively correlated. The serum concentration of **4** was negatively correlated with several blood markers of pseudohyperaldosteronism such as serum potassium concentration, plasma renin activity, and plasma aldosterone concentration. The serum concentration of **4** was lower than that of **3** in the patients and the inhibitory effect of **4** on 11β-HSD2 was also lower than that of **3** as the IC_{50} values of **3** and **4** on 11β-HSD2 were 0.10 μM (Ishiuchi et al., 2019) and 0.38 μM, respectively. Therefore, **3** would be more effective than **4** and could cause pseudohyperaldosteronism in patients using liquorice or GL-preparations.

To prevent the development of pseudohyperaldosteronism, early laboratory tests to detect the blood concentration of **3** in patients prescribed with liquorice-containing medicines are desirable. The analysis of **3** using LC-MS/MS has the disadvantage of requiring elaborate facilities and is not common in general hospitals, clinics, and pharmacies because of its high cost and long processing time. These disadvantages could be overcome with the ELISA system which is more convenient with a lower price and faster analysis in the detection of specific compounds in biological samples. In our previous study, we proposed that the blood concentration of **3** in the patients orally treated with liquorice could be measured using ELISA to prevent the onset of pseudohyperaldosteronism in early stages by using an anti-3MGA-mAb because of the enough affinity of anti-3MGA-mAb to **3** (Ishiuchi et al., 2019). However, we could not measure the concentration of **3** in the serum samples of the patients by using this ELISA system. Because major portions of **1**–**4** in serum are bound to albumin, the recognition site of this antibody to **3** has been predicted to likely the same as the binding site of **3** to albumin. Treating the serum samples with ethanol and deproteinizing them enabled the construction of a good calibration curve by using the standard human serum solution of **3** and the absorbance obtained using ELISA. Then, the concentrations of **3** in the serum samples of the patients were measured using ELISA after the serum samples were treated with ethanol. The comparative analysis of the methods showed higher values were detected using ELISA than LC-MS/MS, especially at high concentrations of **3**, and many outliers were observed in the correlation. The affinity of anti-3MGA-mAb to **4** was higher than to **3**, suggesting that the cross-reaction of anti-3MGA-mAb to **4**, as to the minor metabolite in the serum, could not be negligible in the samples with high concentration of **3**. To avoid the cross-reaction of this antibody to **4**, the measurable range of **3** in ELISA should be set to 10–400 nM. When compound **3** was obtained at more than 400 nM in the first measurement, the serum samples were appropriately diluted and re-measured. This process produced a good correlation of the values obtained using ELISA with those using LC-MS/MS. Furthermore, metabolites other than **3** and **4** that could cross-react with this antibody were considered likely not present in human serum. Then, the ELISA measurement system for the serum concentration of **3** was established and it is expected to contribute to the prevention of pseudohyperaldosteronism in patients administered liquorice-containing medicines.

In female SD rats, GA was excreted mainly in the bile as **4** and **3**, and as the minor metabolites **2** and **1** when GA was administered intravenously. These metabolites did not appear in the blood of these rats, whereas they appeared in that of EHBRs. EHBRs were originally found as mutant rats with chronic conjugated hyperbilirubinemia (Hosokawa et al., 1992). They were subsequently confirmed to express the dysfunctional Mrp2 protein by a point mutation in the open reading frame (Ito et al., 1997). Compounds **1** – **4** did not appear in the blood and urine of SD rats but in those of EHBRs, it is suggested that Mrp2 was involved in the bile excretion of these metabolites, and the reduction and/or dysfunction of Mrp2 by liver injury or mutation decreased bile excretion of these metabolites. In normal SD rats, GA can be sulfonated by Sult2A1 (Takahashi et al., 2019) to generate **3**, or sulfonated and glucuronidated to generate **4** in the liver, and then **3** and **4** can be excreted into the bile *via* Mrp2. In the gastrointestinal tract, **3** and **4** can be hydrolysed by intestinal bacteria to GA, which can then be partly absorbed from the gastrointestinal tract into the circulation, while the other part is excreted into faeces. Indeed, we found **3** and GA in the faeces of SD rats injected intravenously with GA (Ishiuchi et al., 2019). Although **4** was more highly excreted into the bile than **3**, it was not detected in faeces, suggesting that the hydrolysis reaction mediated by enterobacteria at C-30 was superior to that at the C-3 of **3** and **4**.

In EHBRs, compounds **1**–**4** appeared in the blood as GL metabolites, but there was a difference in their blood

concentrations between male and female EHBRs. The blood concentration of **1** in male rats was approximately 7-fold higher than that in female rats 12 h after oral administration of GA, indicating a marked sex difference. We then evaluated the possibility of sex differences in blood concentrations of **3** and **4** among the patients, but none was observed (data not shown). The possible mechanism of the sex difference observed in EHBRs may be related to the hydroxylating function of Cyps on GA-metabolites at C-22. The observation that the serum samples collected from patients did not contain **1** or **2** (Takahashi et al., 2019) indicated that the activity of CYPs involved in the hydroxylation of GA and its metabolites at C-22 could be much lower in human beings than that in rats, and, therefore, it was reasonable that we could not find sex differences in blood concentrations of **3** and **4** in serum samples of patients.

Although the functions of MRP2 were not directly evaluated in the present patients, their functions were not considered not to have varied significantly. This is because serum concentrations of direct bilirubin, as a potential surrogate marker of MRP2 function, were normal in all the participants in this study. Furthermore, there was a positive correlation between the daily dose of liquorice and blood concentrations of **3** and **4**. We detected very small amounts of **3** (Ishiuchi et al., 2019) and **4** (data not shown) in plasma samples collected from normal SD rats orally treated with GA. It is suggested that the functions of human MRP2 and rat Mrp2 may be different, and human MRP2 likely transports **3** and **4** less efficiently than rat Mrp2 does.

Previous studies suggested GA as the causative agent of pseudohyperaldosteronism (Ulmann et al., 1975; Armanini et al., 1983; Monder et al., 1989) with the binding activity (ED_{50}) of GA on mineralocorticoid receptor (MR) being about 100 μM. In our previous clinical study, the maximum concentrations of compound **3** and GA are 4.2 μM and 1.8 μM, respectively (Takahashi et al., 2019). The serum concentration of GA was too low not to inhibit MR directly. Although the affinity of compounds **3** and **4** for MR is unknown and the possible future studies are needed, it is estimated that the affinities would be low by the previous results of GA (Armanini et al., 1983). Indeed, there are several earlier reports (Molhuysen et al., 1950; Borst et al., 1953; Card et al., 1953; Elmadjian et al., 1956; Girerd et al., 1960) exhibiting that the adrenal gland must be present for liquorice to have mineralocorticoid activity, suggesting that glycyrrhizin and its metabolites itself including GA do not bind to MR directly *in vivo*. We have also addressed the capability of GA on transporting through the membrane of tubular cells, and shown that GA could not transfer into the cells under the existence of albumin (Makino et al., 2012). In the study using rat kidney slices, GA can only bind the surface of the tissue, and cannot exhibit any binding effect of mineralocorticoid receptor and the inhibition of 11β-HSD2 at 100 μM (Makino et al., 2012). Therefore, we suggest that GA might not be the causative agent of pseudohyperaldosteronism induced by liquorice *in vivo*.

The limitation of this study is the possibility that there are other GL metabolites than compounds **3** and **4** in the serum of patients who took liquorice. Ploeger et al. predicted 18β-glycyrrhetyl-30-*O*-glucuronide as another metabolite of GL than the metabolites evaluated in this study (Ploeger et al., 2001). However, the possibility of its presence may be low because we found no peaks in MS chromatogram of ESI(+) 647.6 to 453.6 m/*z* for 3MGA in all human samples. In MS chromatogram of ESI(+) 471.3 to 91.0 m/*z* for GA, we found other possible GL metabolites than compounds **3** and **4**, though the size of these peaks are quite smaller than **3** and **4** (data not shown). In order to reveal the causative agents of liquorice-induced pseudohyperaldosteronism completely, metabolomics studies will be demanded.

Collectively, our results led us conclude that compound **3** would be the most promising causative agent of pseudohyperaldosteronism. Furthermore, **3** was detected in the human serum at concentrations 1.5- to 2-fold higher than those of GA, implying that **3** also acted as the active ingredient exerting not only pseudohyperaldosteronism but also other pharmacological actions such as the anti-inflammatory effects of GL and liquorice preparations (Isbrucker and Burdock, 2006) in human beings. Further clinical studies are needed to investigate the pharmacologically and toxicologically active ingredients of GL and liquorice.

CONCLUSION

We identified **3** as the major metabolite and **4** as the secondary major GL metabolite, in the blood of patients administered GL and liquorice, which could reach 11β-HSD2 in renal tubular epithelial cells. We concluded that **3** was the most promising causative agent of pseudohyperaldosteronism induced by liquorice, and we successfully established an ELISA system to easily detect the blood concentration of **3** in patients taking liquorice, to prevent the onset of pseudohyperaldosteronism. Further pharmacological studies of **3** are required to determine the mechanisms of action of GL and liquorice preparations in humans.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The appropriate institutional review boards at Keio University, Chiba University, Kanazawa University, and Kameda Medical Center. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Animal Care Committee at the Graduate School of Pharmaceutical Sciences, Nagoya City University.

AUTHOR CONTRIBUTIONS

KI, OM, MiM, AH, YM, YT, TK, KO, and TM had full access to the basic research data used in this study and take responsibility

for the analysis of GL metabolites. TY and FL had full access to the clinical data used in this study and take responsibility for the data's integrity and the accuracy of the data analysis. KI, OM, TY, and TM wrote a draft of this manuscript. TY, KO-O, KM, TN, MaM, and KW participated in designing the study and collecting human samples and clinical data. All authors read and approved the final manuscript.

FUNDING

This work was supported by Grant-in-Aid of a Research Project for Improving Quality in Healthcare and Collecting Scientific Evidence on Integrative Medicine from AMED under Grant numbers, JP17lk0310036h0001, JP18lk0310049h0001, and JP19lk0310064h0001. This study is also supported by a grant of Oriental Medicine Research Foundation 2019. The funding source had no involvement in the interpretation of data, writing of the report, and the decision to submit the article for publication.

ACKNOWLEDGMENTS

We thank Prof. Mitsuru Sugawara (Laboratory of Pharmacokinetics, Faculty of Pharmaceutical Science, Graduate School of Hokkaido University) for providing plasmids containing the open reading frame of OAT1 and OAT3. We thank Kaori Sawai, Akiko Shirai, and Koh'ichi Ryu for helping with sample collection, Kanon Takahashi, Prof. Tomonoti Nakamura for helping the analysis of clinical data. We thank the Japan Agency for Medical Research and Development (AMED) and Oriental Medicine Research Foundation in data analysis and presentation for providing research grant support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.688508/full#supplementary-material

REFERENCES

Akao, T., Hayashi, T., Kobashi, K., Kanaoka, M., Kato, H., Kobayashi, M., et al. (1994). Intestinal Bacterial Hydrolysis Is Indispensable to Absorption of 18 Beta-Glycyrrhetic Acid after Oral Administration of Glycyrrhizin in Rats. *J. Pharm. Pharmacol.* 46, 135–137. doi:10.1111/j.2042-7158.1994.tb03756.x

Armanini, D., Karbowiak, I., and Funder, J. W. (1983). Affinity of Liquorice Derivatives for Mineralocorticoid and Glucocorticoid Receptors. *Clin. Endocrinol.* 19, 609–612. doi:10.1111/j.1365-2265.1983.tb00038.x

Borst, J. G. G., De Vries, L. A., Holt, S. P. T., and Molhuysen, J. A. (1953). Synergistic Action of Liquorice and Cortisone in Addison's and Simmonds's Disease. *Lancet* 261, 657–663. doi:10.1016/s0140-6736(53)91800-5

Card, W. I., Strong, J. A., Tompsett, S. L., Mitchell, W., Taylor, N. R. W., and Wilson, J. M. G. (1953). Effects of Liquorice and its Derivatives on Salt and Water Metabolism. *Lancet* 261, 663–668. doi:10.1016/s0140-6736(53)91801-7

Conn, J. W., Rovner, D., and Cohen, E. (1968). Licorice-Induced Pseudoaldosteronism. *JAMA* 205, 492–496. doi:10.1001/jama.1968.03140330034006

Diederich, S., Grossmann, C., Hanke, B., Quinkler, M., Herrmann, M., Bahr, V., et al. (2000). In the Search for Specific Inhibitors of Human 11beta-Hydroxysteroid-Dehydrogenases (11beta-HSDs): Chenodeoxycholic Acid Selectively Inhibits 11beta-HSD-I. *Eur. J. Endocrinol.* 142, 200–207. doi:10.1530/eje.0.1420200

Elmadjian, F., Hope, J. M., and Pincus, G. (1956). The Action of Mono-Ammonium Glycyrrhizinate on Adrenalectomized Subjects and its Synergism with Hydrocortisone. *J. Clin. Endocrinol. Metab.* 16, 338–349. doi:10.1210/jcem-16-3-338

Girerd, R. J., Rassaert, C. L., Dipasquale, G., and Kroc, R. L. (1960). Endocrine Involvement in Licorice Hypertension. *Am. J. Physiol.-Legacy Content* 198, 718–720. doi:10.1152/ajplegacy.1960.198.4.718

Hosokawa, S., Tagaya, O., Mikami, T., Nozaki, Y., Kawaguchi, A., Yamatsu, K., et al. (1992). A New Rat Mutant with Chronic Conjugated Hyperbilirubinemia and Renal Glomerular Lesions. *Lab. Anim. Sci.* 42, 27–34.

Isbrucker, R. A., and Burdock, G. A. (2006). Risk and Safety Assessment on the Consumption of Licorice Root (*Glycyrrhiza* sp.), its Extract and Powder as a Food Ingredient, with Emphasis on the Pharmacology and Toxicology of Glycyrrhizin. *Regul. Toxicol. Pharmacol.* 46, 167–192. doi:10.1016/j.yrtph.2006.06.002

Ishiuchi, K., Morinaga, O., Ohkita, T., Tian, C., Hirasawa, A., Mitamura, M., et al. (2019). 18*β*-glycyrrhetyl-3-*O*-sulfate Would Be a Causative Agent of Licorice-Induced Pseudoaldosteronism. *Sci. Rep.* 9, 1587. doi:10.1038/s41598-018-38182-2

Ito, K., Suzuki, H., Hirohashi, T., Kume, K., Shimizu, T., and Sugiyama, Y. (1997). Molecular Cloning of Canalicular Multispecific Organic Anion Transporter Defective in EHBR. *Am. J. Physiol.-Gastrointestinal Liver Physiol.* 272, G16–G22. doi:10.1152/ajpgi.1997.272.1.g16

Jing, J., Ren, W., Chen, X., Wang, Y., Yu, Q., Wang, G., et al. (2008). Glucuronide-sulfate Diconjugate as a Novel Metabolite of Glycyrrhetic Acid in Rat Bile. *Drug Metab. Pharmacokinet.* 23, 175–180. doi:10.2133/dmpk.23.175

Kato, H., Kanaoka, M., Yano, S., and Kobayashi, M. (1995). 3-Monoglucuronyl-glycyrrhetinic Acid Is a Major Metabolite that Causes Licorice-Induced Pseudoaldosteronism. *J. Clin. Endocrinol. Metab.* 80, 1929–1933. doi:10.1210/jc.80.6.1929

Makino, T., Ohtake, N., Watanabe, A., Tsuchiya, N., Imamura, S., Iizuka, S., et al. (2008). Down-regulation of a Hepatic Transporter Multidrug Resistance-Associated Protein 2 Is Involved in Alteration of Pharmacokinetics of Glycyrrhizin and its Metabolites in a Rat Model of Chronic Liver Injury. *Drug Metab. Dispos* 36, 1438–1443. doi:10.1124/dmd.108.021089

Makino, T., Okajima, K., Uebayashi, R., Ohtake, N., Inoue, K., and Mizukami, H. (2012). 3-Monoglucuronyl-Glycyrrhretinic Acid Is a Substrate of Organic Anion Transporters Expressed in Tubular Epithelial Cells and Plays Important Roles in Licorice-Induced Pseudoaldosteronism by Inhibiting 11β-Hydroxysteroid Dehydrogenase 2. *J. Pharmacol. Exp. Ther.* 342, 297–304. doi:10.1124/jpet.111.190009

Molhuysen, J. A., Gerbrandy, J., De Vries, L. A., Lenstra, J. B., De Jong, J. C., Turner, K. P., et al. (1950). A Liquorice Extract with Deoxycortone-like Action. *Lancet* 256, 381–386. doi:10.1016/s0140-6736(50)91341-9

Monder, C., Stewart, P. M., Lakshmi, V., Valentino, R., Burt, D., and Edwards, C. R. W. (1989). Licorice Inhibits Corticosteroid 1lβ-Dehydrogenase of Rat Kidney and Liver: In Vivo and in Vitro Studies. *Endocrinology* 125, 1046–1053. doi:10.1210/endo-125-2-1046

Morinaga, O., Ishiuchi, K., Ohkita, T., Tian, C., Hirasawa, A., Mitamura, M., et al. (2018). Isolation of a Novel Glycyrrhizin Metabolite as a Causal Candidate Compound for Pseudoaldosteronism. *Sci. Rep.* 8, 15568. doi:10.1038/s41598-018-33834-9

Ploeger, B., Mensinga, T., Sips, A., Seinen, W., Meulenbelt, J., and Dejongh, J. (2001). The Pharmacokinetics of Glycyrrhizic Acid Evaluated by Physiologically Based Pharmacokinetic Modeling. *Drug Metab. Rev.* 33, 125–147. doi:10.1081/dmr-100104400

Stewart, P., Valentino, R., Wallace, A. M., Burt, D., Shackleton, C. L., and Edwards, C. W. (1987). Mineralocorticoid Activity of Liquorice: 11-Beta-Hydroxysteroid Dehydrogenase Deficiency Comes of Age. *Lancet* 330, 821–824. doi:10.1016/s0140-6736(87)91014-2

Takahashi, K., Yoshino, T., Maki, Y., Ishiuchi, K. i., Namiki, T., Ogawa-Ochiai, K., et al. (2019). Identification of Glycyrrhizin Metabolites in Humans and of a Potential Biomarker of Liquorice-Induced Pseudoaldosteronism: a Multi-Centre Cross-Sectional Study. *Arch. Toxicol.* 93, 3111–3119. doi:10.1007/s00204-019-02588-2

Ulmann, A., Menard, J., and Corvol, P. (1975). Binding of Glycyrrhetinic Acid to Kidney Mineralocorticoid and Glucocorticoid Receptors1. *Endocrinology* 97, 46–51. doi:10.1210/endo-97-1-46

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

frontiers in Pharmacology

REVIEW
published: 23 June 2021
doi: 10.3389/fphar.2021.656246

Prevention and Recovery of COVID-19 Patients With Kampo Medicine: Review of Case Reports and Ongoing Clinical Trials

Shin Takayama[1,2,3], Takao Namiki[4], Hiroshi Odaguchi[5], Ryutaro Arita[1,2], Akito Hisanaga[6,7], Kazuo Mitani[8,9] and Takashi Ito[7]*

[1]Department of Kampo Medicine, Tohoku University Hospital, Sendai, Japan, [2]Department of Education and Support for Regional Medicine, Tohoku University Hospital, Sendai, Japan, [3]Department of Kampo and Integrative Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan, [4]Department of Japanese-Oriental (Kampo) Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan, [5]Oriental Medicine Research Center, Kitasato University, Minato-ku, Japan, [6]Hospital Bando, Ibaraki, Japan, [7]Akashi Clinic Kanda, Chiyoda-ku, Japan, [8]Department of Yamato Kampo Medicine and Pharmacy Center, Nara Medical University, Kashihara, Japan, [9]Mitani Family Clinic, Osaka, Japan

Coronavirus disease 2019 (COVID-19) spread to Japan in 2020, where the number of infected patients exceeded 250,000 and COVID-related deaths exceeded 3,500 in one year. Basic guidelines for infection control were implemented in Japan, and research and development of effective drugs and vaccines were promoted. This included considering Kampo medicine, which has a long history of treating recurring emerging viral infections. Considering the characteristics of the disease (inflammation of the upper and lower respiratory tract as well as potential neural damage and vasculitis), Kampo medicine could be considered as a treatment strategy due to its antiviral and anti-inflammatory effects induced by multiple active substances that could aid in disease prevention and recovery. In this study, case reports on the management of COVID-19 with Kampo medicine, which were published until March 31, 2021, were reviewed. The search strategy involved the use of Medline and hand-searching. Twenty two patients were treated using Kampo medicines with or without Western medicine, based on individual conditions. On the other hand, the effects of Kampo medicines as a potential preventive treatment (pre-infection), active treatment (especially in the acute and subacute stage), or treatment of sequelae to aid recovery (after infection) in the different stages of COVID-19 are being studied as research projects in the Japan Society for Oriental Medicine (JSOM). JSOM has also organized a pioneering project of clinical trials for COVID-19, some of which are now in progress.

Keywords: COVID-19, SARS-CoV-2, Japan society for oriental medicine, Kampo, treatment, prevention, recovery

OPEN ACCESS

Edited by:
Kenny Kuchta,
University Medical Center Göttingen, Germany

Reviewed by:
Lei Chen,
Guangdong Ocean University, China
Shailendra S. Gurav,
Goa College of Pharmacy, India

****Correspondence:***
Shin Takayama
takayama@med.tohoku.ac.jp

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

Received: *20 January 2021*
Accepted: *31 May 2021*
Published: *23 June 2021*

Citation:
Takayama S, Namiki T, Odaguchi H, Arita R, Hisanaga A, Mitani K and Ito T (2021) Prevention and Recovery of COVID-19 Patients With Kampo Medicine: Review of Case Reports and Ongoing Clinical Trials. Front. Pharmacol. 12:656246. doi: 10.3389/fphar.2021.656246

INTRODUCTION

Spread of Coronavirus Disease 2019 (COVID-19) in Japan

The mass occurrence of an undefined pneumonia was first reported in Wuhan, China, to the World Health Organization (WHO) on December 31, 2019 (World Health Organization, 2019). Thereafter, the cause of this pneumonia was reported to be severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the disease was named coronavirus disease 2019 (COVID-19). COVID-19 rapidly spread worldwide, including Asia, in a year. At the end of March 2021, the number of

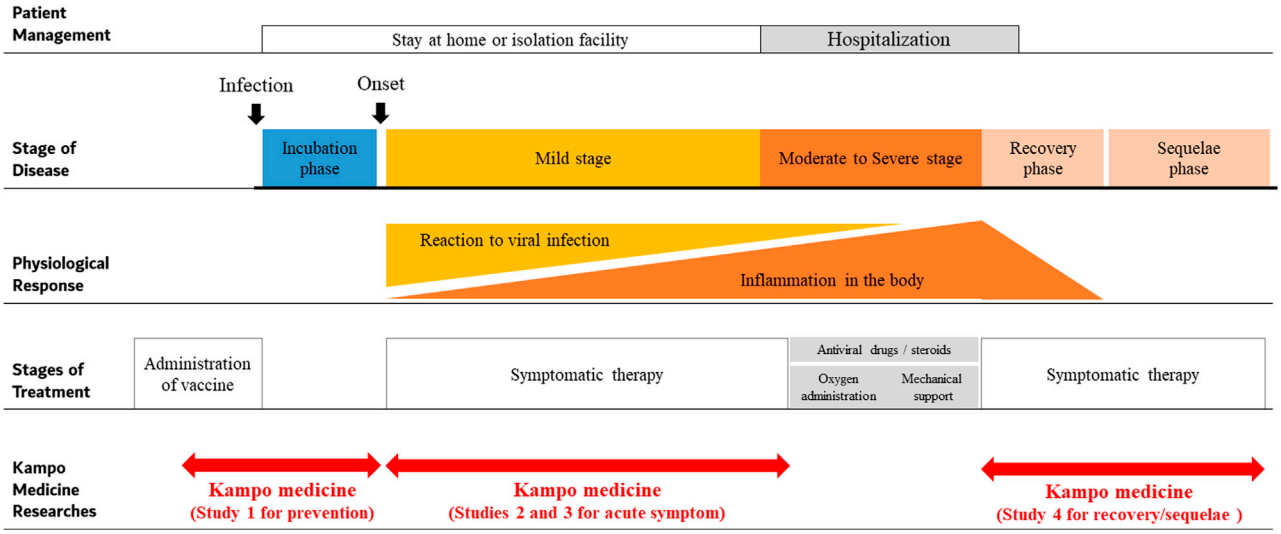

FIGURE 1 | Concept and clinical trials of Kampo medicine for COVID-19.

confirmed COVID-19 patients were 90,159 in China, 474,641 in Japan, and 103,639 in Korea, with the number of deaths being 4,636 in China, 9,155 in Japan, and 1,735 in Korea; these numbers have been increasing.

The first case of COVID-19 in Japan was confirmed on January 15, 2020. The Japanese government was quick to classify COVID-19 as a serious infectious disease on February 1, 2020. Following which, basic guidelines for infection control against COVID-19 were published on February 25, 2020 (Ministry of Health, Labour and Welfare of Japan, 2020a). The number of patients continued to increase, thus increasing the burden on hospitals, and creating a shortage of beds due to many patients requiring hospitalization. Efforts were made to follow patients via course observation at home in asymptomatic cases, while mild stage patients were cared for in isolation facilities (**Figure 1**). On March 11, 2020, the WHO declared COVID-19 global pandemic based on the spread and severity of the infection (World Health Organization, 2020). Additional guidance for basic infection control against COVID-19 in Japan was published on March 28, 2020, stating to avoid "enclosed and dense spaces and close contact with others." In addition, the guide stated that ensuring the healthcare system and promoting research and development of effective drugs and vaccines for COVID-19 was important (National Institute of Infectious Diseases, 2020).

History of an Emerging Viral Infection as a Pandemic

The Spanish flu, a global pandemic, affected Japan from 1918 to 1919, and is now known to be caused by the H1N1 influenza virus. Its fatality rate was reported to be approximately 2.5% worldwide and 1.6% in Japan (Influenza, 2008). In Japan, 23.8 million people had been infected and 388,000 people had died by the end of 1919. Its symptoms are similar to that of a common cold, and include fever, headache, muscle aches, and joint pain; however, it spreads to the lungs and causes severe pneumonia in severe cases. During this period, famous Japanese Kampo doctors treated patients with the Spanish flu using Kampo medicines. Saikatsugekito, daiseiryuto, chikujountanto, kososan, shoseiryuto, shomakakkonto, and additional or reductional formulae were applied according to the patient's symptoms (Yasui 2007; Irie and Nakae, 2020).

Additionally, SARS-CoV in 2003 (World Health Organization, 2015), influenza A (H1N1) pdm09 in 2009, Middle East respiratory syndrome (MERS)-CoV in 2012 (Zaki et al., 2012), and SARS-CoV-2 in 2019 have affected the world, and the contribution of Kampo medicine for the treatment of these emerging viral infections need to be evaluated using clinical studies. According to a clinical trial, Nabeshima et al. reported the effects of maoto on seasonal influenza compared with neuraminidase inhibitors and found that the time to fever resolution was shorter with maoto than with oseltamivir in a randomized controlled trial (RCT) (Nabeshima et al., 2012). Yoshino et al. reported that maoto may decrease the duration of fever when used alone or in combination with neuraminidase inhibitors in a systematic review (Yoshino et al., 2019). Arita et al. described pharmacological activities of saikatsugekito against viral infection and respiratory inflammation. In this review, some components of saikatsugekito demonstrated therapeutic effect in the infection processes of single-strand RNA viruses. The therapeutic effect encompasses the enhancement of the immunomodulating activities against experimental inflammation, including cytokine production, regulation of immune cells, and protection against lung tissue injury (Arita et al., 2020). This information could be useful for developing a treatment approach to target COVID-19.

Features of SARS-CoV-2 and COVID-19

Coronavirus is a single-stranded RNA virus. Six coronaviruses, especially SARS and MERS, cause severe pneumonia. The other four types of coronaviruses are known to cause symptoms more akin to the common cold. SARS-CoV-2 is genetically similar to SARS-CoV and causes severe pneumonia and various related symptoms (Lu et al., 2020). SARS-CoV-2 infects humans through the human angiotensin-converting enzyme two receptor, which is located at the surface of the nasal mucosa and type 2 lung epithelial cells (Hoffmann et al., 2020; Zhou et al., 2020).

The infectious period is from two days before onset to 7–10 days after onset, and the infectivity is extremely high just before and shortly after onset (He et al., 2020). The main route of infection is via airborne droplet transmission; however, contact transmission through patients or contaminated surfaces is also contributory. Of note, it has been confirmed that erosols can be generated and cause infection (Jarvis, 2020).

The typical clinical course of COVID-19 is shown in **Figure 1**. After infection with SARS-CoV-2, an incubation period of 1–14 days (mean 5.8 days) occurs before onset of common cold symptoms (Wu and McGoogan 2020). As per a study, among the sources of infection, 45% of individuals are infectious before the onset of symptoms and 5% are infectious and completely asymptomatic (Ferretti et al., 2020). The source of infection was also reported to be asymptomatic individuals in 50% of the cases, while 40% of the cases were from symptomatic individuals and 10% came from environmental sources, i.e., contact transmission (Ferretti et al., 2020). Another study indicated that approximately 80% of the patients in the mild stage recovered from the disease; however, 20% of the patients required hospitalization, of which 5% required treatment in the intensive care unit (Wu and McGoogan 2020). The following criteria were used to determine staging: mild stage, $SpO_2 \geq 96\%$ with cough without dyspnea; moderate I stage, SpO_2 93–96% with dyspnea and pneumonia findings; moderate II stage, SpO2 ≤ 93% and required oxygen administration and treatment; and severe stage, required intensive care or mechanical ventilation. Age (≥65 years) and underlying diseases such as chronic respiratory disease, chronic renal disease, and diabetes mellitus are risk factors for severe disease in COVID-19 patients. Significantly, in severe-stage COVID-19 patients, levels of interleukin-6, interleukin-1beta, and soluble tumor necrosis factor receptor one are elevated, suggesting that a strong inflammatory response is related to severe disease (McElvaney et al., 2020). Symptoms persist even after stabilization of patients and can be problematic. Post-acute COVID-19 follow-up showed that 87.4% of patients complained of symptoms, especially fatigue (53.1%), dyspnea (43.4%), joint pain (27.3%), and chest pain (21.7%) even 60 days after the onset of COVID-19 in one study (Carfì et al., 2020).

Vaccine for COVID-19

In Japan, an agreement to obtain a supply of the mRNA vaccine with Pfizer Inc. and the adenovirus vector vaccine with AstraZeneca Inc. was announced on July 31 and August 7, 2020, respectively.

Treatment of COVID-19

In Japan, remdesivir was approved on May 7, 2020, and dexamethasone was approved on July 21, 2020, for the treatment of COVID-19. Remdesivir and dexamethasone were approved for patients in the moderate to severe stage of COVID-19; however, favipiravir, ciclesonide, or lopinavir-ritonavir were not approved as of December 31, 2020.

Review of Clinical Reports on Kampo Treatment for COVID-19

Case reports on the management of COVID-19 using Kampo medicine, published until March 31st, 2021, were reviewed. The search strategy involved the use of Medline and hand-searching. In 2020 and 2021, several case reports and case series of patients with COVID-19 treated with Kampo medicines were reported (**Table 1**). Kampo medicine listed in **Table 1** are described in detail in **Table 2**. Plant names and part of each ingredient composed in Kampo medicine are listed in **Supplementary Table S1**.

Niitsuma et al. reported two cases (case 1 and 2) of COVID-19 pneumonia (Niitsuma et al., 2020). In case 1, a 78-year-old male patient with a history of stroke and hypertension presented with complains of fever, dyspnea, malaise, and loss of appetite with a decreased oxygen saturation. Computed tomography (CT) revealed bilateral interstitial shadow, and a diagnosis of moderate stage II COVID-19 was made. The patient was administered Western medication including lopinavir/ritonavir, hydrocortisone, ceftriaxone, sulbactam/ampicillin, and acetaminophen. For the fever, cough, and sputum, Kampo medicine maoto, daiseiryuto, and chikujountanto were also sequentially administered. This multidisciplinary treatment approach relieved symptoms within 10 days. Case 2 was a 74-year-old woman with complains of joint pain, fever, and loss of appetite. CT revealed interstitial shadow in the left lung, and a diagnosis of moderate stage I COVID-19 was made. Additionally, the patient was administered Western medication including baloxavir marboxil, moxifloxacin, lopinavir/ritonavir, and warfarin. Kampo medicine maoto, followed by keishito and eppikajutsuto were also administered. This multidisciplinary approach relieved patient symptoms within 10 days.

Kashima et al. reported two cases of COVID-19 in which Kampo medicine may have contributed toward the suppression of a severe stage (cases 3 and 4) (Kashima et al., 2020). Case 3 was a 50-year-old woman with complains of fever, malaise, headache, blocked nose, lumbago, and loss of appetite. CT showed interstitial shadow in the right lung, and a diagnosis of moderate stage I COVID-19 was made. Western medicines such as acetaminophen and ciclesonide were prescribed. Kampo medicine kakkonto and shosaikoto, followed by shosaikoto and bukuryoingohangekobokuto were administered. All symptoms alleviated within 4 days. Whereas case 4 was a 53-year-old woman with systemic lupus erythematosus, who presented with cold and heat sensation, malaise, cough, lumbago, appetite loss, and diarrhea. CT showed interstitial shadow in the bilateral lung with a decrease in oxygen saturation, and a diagnosis of moderate stage II COVID-19

TABLE 1 | Review of clinical reports on the management of COVID-19 with Kampo medicine.

Author	Cases		Age (y.o.)/ gender	Pre-existing conditions	Symptoms	Stage in COVID-19	Treatment			Outcome
	No.	Diagnosis					Oxygen administration	Others	Kampo medicine	
Niitsuma et al. (2020)	1	Pneumonia	78/M	Post stroke, hypertension	Fever, dyspnea, malaise, appetite loss	Moderate II	Yes	Lopinavir/ritonavir, hydrocortisone, ceftriaxone, sulbactam/ampicillin, acetaminophen	maoto, daiseiryuto, chikujountanto	Improved
	2	Pneumonia	74/F	None	Joint pain, fever, appetite loss	Moderate I	No	Baloxavir marboxil, moxifloxacin, lopinavir/ritonavir, warfarin	maoto, keishito + eppikajutsuto	Improved
Kashima et al. (2020)	3	Pneumonia	50/F	None	Fever, malaise, headache, block nose, lumbago, appetite loss	Moderate I	No	Acetaminophen, ciclesonide, favipiravir	kakkonto + shosaikoto, shosaikoto + bukuryoingohangekobokuto	Improved
	4	Pneumonia	53/F	Systemic lupus erythematosus treated with prednisolone	Cold and heat sensation, malaise, cough, lumbago, appetite loss, diarrhea	Moderate II	Yes	Ceftriaxone, azithromycin, acetaminophen, ciclesonide, favipiravir	shosaikoto, saikanto, chikujountanto, bukuryoingohangekobokuto, jinsoin, hochuekkito, gokoto, kikyosekko, keishibukuryogan, saffron; combination of the above according to the condition	Improved
Homma et al. (2020a)	5	Pneumonia	30/M	Atopic dermatitis	Headache, fever, throat pain, coldness, cough, malaise	Moderate II	Yes	Acetaminophen	bakumondoto + hangekobokuto	Improved
Homma et al. (2020b)	6	Pneumonia with facial paralysis and olfactory disturbance	35/F	None but smoker	Cough, malaise, sore throat, nausea, fever, headache, smell impairment, taste impairment, facial paralysis	Moderate II	Yes	Acetaminophen, ciclesonide, favipiravir	maoto	Improved
Irie et al. (2020)	7	Common cold with taste disorder	41/F	N/A	Headache, sore throat, nausea, taste disorder, fever	Mild	No	None	kakkonto + shosaikotokakikyosekko	Improved
	8	Common cold with taste disorder	16/F	N/A	Nasal congestion, taste disorder	Mild	No	None	kakkonto + shosaikotokakikyosekko	Improved
	9	Common cold with taste disorder	12/M	N/A	Nasal congestion, taste disorder	Mild	No	None	kakkonto + shosaikotokakikyosekko	Improved
Watanabe and Watanabe. (2020)	10	Common cold with smell impairment	59/M	None	Fever, sore through, smell impairment, abdominal fullness	N/A	No	Acetaminophen, clarithromycin, tranexamic acid, L-carbocisteine	Qing Fei Pai Du Tang, hochuekkito	Improved

(Continued on following page)

TABLE 1 | (*Continued*) Review of clinical reports on the management of COVID-19 with Kampo medicine.

Author	Cases		Age (y.o.)/ gender	Pre-existing conditions	Symptoms	Stage in COVID-19	Treatment			Outcome
	No.	Diagnosis					Oxygen administration	Others	Kampo medicine	
	11	Common cold	36/F	Hashimoto's disease	Fever, cough, sputum, headache, nasal bleeding	N/A	No	Acetaminophen, clarithromycin, tranexamic acid, L-carbocisteine	Qing Fei Pai Du Tang, kakkoshokisankakikukakyouninrengyohakka, hochuekkito	Improved
Kyo (2020)	12	Pneumonia	40/F	N/A	High fever, cough, dyspnea, chest pain, chest oppression, malaise, headache	Moderate II	Yes	Intravenous hydration	saikokeishito, hangekobokuto, maobushisaishinto	Improved
	13	Pneumonia	64/F	N/A	High fever, cough, dyspnea, chest pain, chest oppression, malaise, headache	Moderate II	Yes	Intravenous hydration	saikokeishito, hangekobokuto, maobushisaishinto	Improved
	14	Pneumonia	47/M	N/A	High fever, dyspnea, chest pain, malaise	Moderate II	Yes	Intravenous hydration	saikatsugekitogokoto, daiseiryuto	Improved
Yamasaki (2020)	15	Pneumonia	67/F	Post operation of lung cancer	Fever, malaise	Moderate I	No	—	makyokansekito + ireito + shosaikotokakikyosekko	Improved
	16	Pneumonia	69/M	Type II diabetes mellitus	Fever, cough	Moderate I	No	Favipiravir	makyokansekito + ireito + shosaikotokakikyosekko	Improved
Takayama et al. (2021b)	17	Pneumonia	52/M	None	Cough, sputum, joint pain	Moderate I	No	None	gokoto	Improved
Takayama et al. (2021a)	18	Common cold with smell and taste impairment	36/F	None	Nasal discharge, blocked nose, smell and taste impairment	N/A	No	None	kakkontokasenkyushin'i	Improved
	19	Common cold with smell and taste impairment	18/F	None	Nasal discharge, smell and taste impairment	N/A	No	None	kakkontokasenkyushin'i	Improved
	20	Common cold with smell and taste impairment	24/F	None	Blocked nose, smell and taste impairment	N/A	No	None	kakkontokasenkyushin'i	Improved
	21	Common cold with smell and taste impairment	44/M	None	Blocked nose, smell and taste impairment	N/A	No	None	kakkontokasenkyushin'i	Improved
	22	Common cold and diarrhea with smell and taste impairment	24/M	None	Nasal discharge, blocked nose, cough, sputum, diarrhea, fatigue, smell and taste impairment	N/A	No	None	kakkontokasenkyushin'i, makyokansekito, saireito	Improved

Notes: M; male, F; female. N/A; not assigned.

Mild stage; $SpO_2 \geqq 96\%$ with cough without dyspnea. Moderate I stage; $93\% < SpO_2 < 96\%$ with dyspnea and pneumonia findings. Moderate II stage; $93\% \leqq SpO_2$ needed oxygen administration, treatment with steroid and antiviral agent. Severe stage; needed intensive care or mechanical ventilation.

TABLE 2 | Kampo medicine and Chinese medicine described in the review of case reports.

Japanese names in roman characters	Chinese characters	Chinese name for Pinyin	Ingredients and daily dosage (JP: The Japanese Pharmacopoeia)
Bakumondoto	麦門冬湯	Mai men dong tang	JP Ophiopogon Tuber 10.0 g, JP Brown Rice 5.0 g, JP Pinellia Tuber 5.0 g, JP Jujube 3.0 g, JP Glycyrrhiza 2.0 g, JP Ginseng 2.0 g
Bukuryoingohangekobokuto	茯苓飲合半夏厚朴湯	Fu ling yin he ban xia hou po tang	JP Pinellia Tuber 6.0 g, JP Poria Sclerotium 5.0 g, JP Atractylodes Lancea Rhizome 4.0 g, JP Magnolia Bark 3.0 g, JP Citrus Unshiu Peel 3.0 g, JP Ginseng 3.0 g, JP Perilla Herb 2.0 g, JP Immature Orange 1.5 g, JP Ginger 1.0 g
Chikujountanto	竹茹温胆湯	Zhu ru wen dan tang	JP Pinellia Tuber 5.0 g, JP Bupleurum Root 3.0 g, JP Ophiopogon Tuber 3.0 g, JP Poria Sclerotium 3.0 g, JP Platycodon Root 2.0 g, JP Immature Orange 2.0 g, JP Cyperus Rhizome 2.0 g, JP Citrus Unshiu Peel 2.0 g, JP Coptis Rhizome 1.0 g, JP Glycyrrhiza 1.0 g, JP Ginger 1.0 g, JP Ginseng 1.0 g, Bamboo Culm 3.0 g
Daiseiryuto	大青竜湯	Da qing long tang	Application with Kampo medicine combination i. e. maoto + keishito, eppikajutsuto + keishito
Eppikajutsuto	越婢加朮湯	Yue bi jia zhu tang	JP Gypsum 8.0 g, JP Ephedra Herb 6.0 g, JP Atractylodes Lancea Rhizome 4.0 g, JP Jujube 3.0 g, JP Glycyrrhiza 2.0 g, JP Ginger 1.0 g
Gokoto	五虎湯	Wu hu tang	JP Gypsum 10.0 g, JP Apricot Kernel 4.0 g, JP Ephedra Herb 4.0 g, JP Mulberry Bark 3.0 g, JP Glycyrrhiza 2.0 g
Hangekobokuto	半夏厚朴湯	Ban xia hou po tang	JP Pinellia Tuber 6.0 g, JP Poria Sclerotium 5.0 g, JP Magnolia Bark 3.0 g, JP Perilla Herb 2.0 g, JP Ginger 1.0 g
Hochuekkito	補中益気湯	Bu zhong yi qi tang	JP Astragalus Root 4.0 g, JP Atractylodes Lancea Rhizome 4.0 g, JP Ginseng 4.0 g, JP Japanese Angelica Root 3.0 g, JP Bupleurum Root 2.0 g, JP Jujube 2.0 g, JP Citrus Unshiu Peel 2.0 g, JP Glycyrrhiza 1.5 g, JP Cimicifuga Rhizome 1.0 g, JP Ginger 0.5 g
Ireito	胃苓湯	Wei ling tang	JP Magnolia Bark 2.5 g, JP Atractylodes Lancea Rhizome 2.5 g, JP Alisma Tuber 2.5 g, JP Polyporus Sclerotium 2.5 g, JP Citrus Unshiu Peel 2.5 g, JP Atractylodes rhizome 2.5 g, JP Poria sclerotium 2.5 g, JP Cinnamon Bark 2.0 g, JP Ginger 1.5 g, JP Jujube 1.5 g, JP Glycyrrhiza 1.0 g
Jinsoin	参蘇飲	Shen su tang	JP Pinellia Tuber 3.0 g, JP Poria Sclerotium 3.0 g, JP Pueraria Root 2.0 g, JP Platycodon Root 2.0 g, JP Peucedanum Root 2.0 g, JP Citrus Unshiu Peel 2.0 g, JP Jujube 1.5 g, JP Ginseng 1.5 g, JP Glycyrrhiza 1.0 g, JP Immature Orange 1.0 g, JP Perilla Herb 1.0 g, JP Ginger 0.5 g
Kakkonto	葛根湯	Ge gen tang	JP Pueraria Root 4.0 g, JP Jujube 3.0 g, JP Ephedra Herb 3.0 g, JP Glycyrrhiza 2.0 g, JP Cinnamon Bark 2.0 g, JP Peony Root 2.0 g, JP Ginger 2.0 g
Kakkontokasenkyushin'i	葛根湯加川芎辛夷	Ge gen tang jia chuan xiong xin yi	JP Pueraria Root 4.0 g, JP Jujube 3.0 g, JP Ephedra Herb 3.0 g, JP Glycyrrhiza 2.0 g, JP Cinnamon Bark 2.0 g, JP Peony Root 2.0 g, JP Magnolia Flower 2.0 g, JP Cnidium Rhizome 2.0 g, JP Ginger 1.0 g
Kakkoshokisankagen	霍香正気散加減	Huo xiang zheng qi san jia jian	JP Atractylodes Rhizome 3.0 g, JP Pinellia Tuber 3.0 g, JP Poria Sclerotium 3.0 g, JP Magnolia Bark 2.0 g, JP Citrus Unshiu Peel 2.0 g, JP Platycodon Root 1.5 g, JP Angelica Dahurica Root 1.5 g, JP Perilla Herb 1.0 g, JP Pogostemon Herb 1.0 g, Areca Pericarp 1.0 g, JP Jujube 1.0 g, JP Glycyrrhiza 1.0 g, JP Ginger 0.5 g, JP Chrysanthemum Flower 3.0 g, JP Apricot Kernel 3.0 g, JP Forsythia Fruit 3.0 g, JP Mentha Herb 2.0 g
Keishibukuryogan	桂枝茯苓丸	Gui zhi fu ling wan	JP Cinnamon Bark 3.0 g, JP Peony Root 3.0 g, JP Peach Kernel 3.0 g, JP Poria Sclerotium 3.0 g, JP Moutan Bark 3.0 g
Keishito	桂枝湯	Gui zhi tang	JP Cinnamon Bark 4.0 g, JP Peony Root 4.0 g, JP Jujube 4.0 g, JP Glycyrrhiza 2.0 g, JP Ginger 1.5 g
Kikyosekko	桔梗石膏	Jie geng shi gao	JP Gypsum 10.0 g, JP Platycodon root 3.0 g
Makyokansekito	麻杏甘石湯	Ma xing gan shi tang	JP Gypsum 10.0 g, JP Apricot Kernel 4.0 g, JP Ephedra Herb 4.0 g, JP Glycyrrhiza 2.0 g
Maobushisaishinto	麻黄附子細辛湯	Ma huang fu zi xi xin tang	JP Ephedra Herb 4.0g, JP Asiasarum Root 3.0g, JP Powdered Processed Aconite Root 1.0 g
Maoto	麻黄湯	Ma huang tang	JP Apricot Kernel 5.0 g, JP Ephedra Herb 5.0 g, JP Cinnamon Bark 4.0 g, JP Glycyrrhiza 1.5 g
Renkaseiun	連花清瘟	Lianhua qingwen	Forsythia Fruit (Lianqiao) 170 g, Lonicera Flower (Jinyinhua) 170 g, Ephedra Herb (Mahuang) 57 g, Bitter Apricot Seed (Kuxingren) 57 g, Gypsum (Shigao) 170 g, Indigo Woad Root (Banlangen) 170 g, Male Fern Rhizome (Mianmaguanzhong) 170 g, Heartleaf Houttuynia Herb (Yuxingcao) 170 g, Pogostemon Herb (Guanghuoxiang) 57 g, Chinese Rhubarb Rhizome (Dahuang) 34 g, Roseroot (Hongjingtian) 57 g, Mentha haplocalyx Herb (Bohe) 5 g, Glycyrrhiza Root (Gancao) 57 g
Saikanto	柴陥湯	Chai xian tang	JP Bupleurum Root 5.0 g, JP Pinellia Tuber 5.0 g, JP Scutellaria Root 3.0 g, JP Jujube 3.0 g, JP Ginseng 2.0 g, JP Coptis Rhizome 1.5 g, JP Glycyrrhiza 1.5 g, JP Ginger 1.0 g, Trichosanthes Seed 3.0 g
Saikatsugekito	柴葛解肌湯	Chai ge jie ji tang	Application with Kampo medicine combination i. e. kakkonto + shosaikotokakikyosekko
Saikokeishito	柴胡桂枝湯	Chai hu gui zhi tang	JP Bupleurum Root 5.0 g, JP Pinellia Tuber 4.0 g, JP Scutellaria Root 2.0 g, JP Glycyrrhiza 2.0 g, JP Cinnamon Bark 2.0 g, JP Peony Root 2.0 g, JP Jujube 2.0 g, JP Ginseng 2.0 g, JP Ginger 1.0 g

(Continued on following page)

TABLE 2 | (*Continued*) Kampo medicine and Chinese medicine described in the review of case reports.

Japanese names in roman characters	Chinese characters	Chinese name for Pinyin	Ingredients and daily dosage (JP: The Japanese Pharmacopoeia)
Saireito	柴苓湯	Chai ling tang	JP Bupleurum Root 7.0 g, JP Alisma Tuber 5.0 g, JP Pinellia Tuber 5.0 g, JP Scutellaria Root 3.0 g, JP Atractylodes Lancea Rhizome 3.0 g, JP Jujube 3.0 g, JP Polyporus Sclerotium 3.0 g, JP Ginseng 3.0 g, JP Poria Sclerotium 3.0 g, JP Glycyrrhiza 2.0 g, JP Cinnamon Bark 2.0 g, JP Ginger 1.0 g
Seihaihaidokuto	清肺排毒湯	Qing fei pai du tang	Original Chinese formula Ephedra Herb (Mahuang) 9.0 g, Glycyrrhiza Root and Rhizome processed with honey (Zhigancao) 6.0 g, Apricot Seed (Xingren) 9.0 g, Gypsum (Shengshigao) 15.0–30.0 g, Cinnamon Twig (Guizhi) 9.0 g, Alisma Tuber (Zexie) 9.0 g, Polyporus Sclerotium (Zhuling) 9.0 g, Atractylodes Rhizome (Baizhu) 9.0 g, Poria Sclerotium (Fuling) 15.0 g, Bupleurum Root (Chaihu) 16.0 g, Scutellaria Root (Huangqin) 6.0 g, Pinellina Rhizome processed with ginger (Jiangbanxia) 9.0 g, Ginger (Shengjiang) 9.0 g, Aster Root (Ziwan) 9.0 g, Common Coltsfoot Flower (Kuandonghua) 9.0 g, Blackberrylily Rhizome (Shegan) 9.0 g, Asiasarum Root and Rhizome (Xixin) 6.0 g, Dioscorea Rhizome (Shanyao) 12.0 g, Immature Orange Fruit (Zhishi) 6.0 g, Dried Tangerine Peel (Chenpi) 6.0 g, Pogostemon Herb (Huoxiang) 9.0 g — Japanese modification fomula JP Ephedra Herb 3.0 g, JP Glycyrrhiza 2.0 g, JP Apricot Kernel 3.0 g, JP Gypsum 10.0 g, JP Cinnamon Bark 3.0 g, JP Alisma Tuber 3.0 g, JP Poluporus Sclerotium 3.0 g, JP Atractylodes Rhizome 3.0 g, JP Poria Sclerotium 5.0 g, JP Bupleurum Root 5.3 g, JP Scutellaria Root 2.0 g, JP Pinellia Tuber 3.0 g, JP Ginger 3.0 g, Aster Root 3.0 g, Coltsfoot Flower 3.0 g, Blackberrylily Rhizome 3.0 g, JP Asiasarum Root 2.0 g, JP Discorea Rhizome 4.0 g, JP Immature Orange 2.0 g, JP Citrus Unshiu Peel 2.0 g, JP Pogostemon Herb 3.0 g
Shosaikoto	小柴胡湯	Xiao chai hu tang	JP Bupleurum Root 7.0 g, JP Pinellia Tuber 5.0 g, JP Scutellaria Root 3.0 g, JP Jujube 3.0 g, JP Ginseng 3.0 g, JP Glycyrrhiza 2.0 g, JP Ginger 1.0 g
Shosaikotokakikyosekko	小柴胡湯加桔梗石膏	Xiao chai hu tang jia jie geng shi gao	JP Gypsum 10.0 g, JP Bupleurum Root 7.0 g, JP Pinellia Tuber 5.0 g, JP Scutellaria Root 3.0 g, JP Platycodon Root 3.0 g, JP Jujube 3.0 g, JP Ginseng 3.0 g, JP Glycyrrhiza 2.0 g, JP Ginger 1.0 g

was made. Western medications such as ceftriaxone, azithromycin, acetaminophen, ciclesonide, and favipiravir were administered to the patient. Kampo medicine shosaikoto, saikanto, chikujountanto, bukuryoingohangekobokuto, jinsoin, hochuekkito, gokoto, kikyosekko, and keishibukuryogan were also prescribed according to the patients symptoms and condition. Finally, the patient recovered 20 days after admission.

Homma et al. reported a case of COVID-19 pneumonia treated with bakumondoto and hangekobokuto combined with Western medicine (case 5) (Homma et al., 2020b). Case 5 was a 30-year-old man with atopic dermatitis who presented with complains of headache, high fever, throat pain, cold cough, and malaise. CT showed interstitial shadow in the bilateral lungs with a decrease in oxygen saturation, which resulted in the diagnosis of moderate stage II COVID-19. Kampo medicine bakumondoto and hangekobokuto for cough were administered, following which all symptoms alleviated within 7 days. They also reported a case of pneumonia with facial paralysis and olfactory disturbance treated by maoto combined with Western medicine (case 6) (Homma et al., 2020a). Case 6 was a 35-year-old woman with complains of cough, malaise, sore throat, nausea, fever, headache, anosmia, dysgeusia, and facial paralysis. CT showed multiple ground-glass opacities in both the lungs which was diagnosed as moderate stage I COVID-19. Western medication acetaminophen, ciclesonide, and favipiravir were prescribed. Kampo medicine maoto was also used for respiratory and neural symptoms. The patients symptoms improved 11 days after admission.

Irie et al. reported three mild cases of COVID-19 treated with saikatsugekito (cases 7, 8, and 9) (Irie et al., 2020). All three cases recovered after treatment with Kampo medicine alone. Case 7 was a mild stage COVID-19 patient who was a 41-year-old woman with complains of headache, sore throat, nausea, taste disorder, and fever. She was treated with kakkonto and shosaikotokakikyosekko, and her condition improved within 10 days. While case 8 was a mild stage COVID-19 patient, a 16-year-old woman who presented with nasal congestion and taste disorder. She was treated with kakkonto and shosaikotokakikyosekko, and the patient's condition improved within 4 days. Case 9 was a mild stage COVID-19 patient, a 12-year-old girl with complains of nasal congestion and taste disorder. She was also treated with kakkonto and shosaikotokakikyosekko, and the patient's condition improved within 3 days.

Watanabe et al. reported two cases of COVID-19 with symptoms similar to a common cold, treated using modified Qing Fei Pai Du Tang (QFPDT) followed by hochuekkito under treatment with Western medicine (cases 10 and 11) (Watanabe and Watanabe, 2020). Case 10 was a 59-year-old man with complains of fever, sore through, anosmia, and abdominal fullness. The patient was administered Western medication including acetaminophen, clarithromycin, tranexamic acid, and

L-carbocisteine, and administration of modified QFPDT for 1 week improved the patient's symptoms. Following which, hochuekkito was administered to promote recovery. While case 11 was a 36-year-old woman with Hashimoto's disease who presented with fever, cough, sputum, headache, and nasal bleeding. The patient was prescribed Western medication such as acetaminophen, clarithromycin, tranexamic acid, and L-carbocisteine. Following which, although she defervesced 1 week after the administration of modified QFPDT, complains of cough and sputum persisted. Kakkoshokisankagen were used thereafter, and symptoms such as cough, sputum, and malaise gradually improved. Finally, hochuekkito was administered to promote recovery.

Kyo reported three cases of pneumonia treated by a combination of Kampo medicine and Western medicine. These patients are presented as cases 12, 13, and 14 (Kyo 2020), and are a 40-year-old woman, a 64-year-old woman, and a 47-year-old man, respectively, with complains of high fever. They were diagnosed as having moderate stage II COVID-19. After the administration of Kampo formula, the patients defervesced within 3 days.

Yamasaki reported three cases of pneumonia treated by Kampo medicine in combination with Western medicine (cases 15 and 16) (Yamasaki 2020). Case 15 was a 67-year-old woman with a history of surgically resected lung cancer who complained of fever and malaise. CT showed infiltration shadow in the left lung, and the patient was diagnosed as having moderate stage I COVID-19. Administration of Kampo medicine combined with makyokansekito, ireito, and shosaikotokakikyosekko improved fever and malaise within 3 and 5 days, respectively. Case 16 was a 69-year-old man with type II diabetes mellitus with complains of fever and cough. CT showed infiltration shadow in the right lung that was diagnosed as moderate stage I COVID-19. Administration of Kampo medicine combined with makyokansekito, ireito, and shosaikotokakikyosekko improved fever and cough within 3 and 6 days, respectively.

Takayama et al. reported a case of pneumonia treated by Kampo medicine gokoto (case 17) (Takayama et al., 2021b). A 52-year-old male patient presented with cough, sputum, and joint pain. Chest radiography revealed interstitial shadow in the right lung that was diagnosed as moderate stage I COVID-19. After administration of gokoto, a Kampo medicine for cough and sputum, for symptom relief, cough and sputum improved within 4 days. Takayama et al. also reported 5 cases (cases 18–22) of COVID-19-related olfactory disorder treated by kakkontokasenkyushin'i (Takayama et al., 2021a). The symptoms improved within 3–5 days after administration of kakkontokasenkyushin'i. Kakkontokasenkyushin'i can be used for treating nasal congestion, rhinitis, and inflammation in the nasal mucosa. Olfactory disorder in COVID-19 has been reported to be associated with inflammation and congestion, especially in the olfactory bulb and olfactory cleft. They concluded that kakkontokasenkyushin'i may be considered as a treatment alternative for the olfactory disorder related to COVID-19.

On the other hand, some reports have suggested the clinical efficacy of Chinese medicine in COVID-19. Ke et al. have reported the efficacy of Lianhua Qingwen (LH) capsules in patients with COVID-19, using a multi-center, prospective, randomized controlled trial (Hu et al., 2020). Certain LH components overlap in the Kampo medicine maoto or maotokasekko that were administered to cases 1–4, 6–11, and 14–22, as shown in **Tables 1**, **2**. The efficacy of LH was compared between conventional treatment alone or in combination with LH for 14 days. The rate of recovery of symptoms including fever, fatigue, and cough was significantly shortened by LH administration. Zheng et al. reported that LH treatment modulates the inflammatory process, exerts antiviral effects and repairs lung injury in the network pharmacology analysis of the therapeutic mechanisms of LH in COVID-19 (Zheng et al., 2020).

Furthermore, a national retrospective registry study reported by Lihua et al. suggested that QFPDT was associated with a substantially lower risk of in-hospital mortality (Zhang et al., 2021). Modified QFPDT in Japan was administered to cases 10, 11, 15, and 16, as shown in **Tables 1**, **2**. Although these reports have revealed the efficacy of traditional medicine, further clinical trials are required to evaluate the efficacy of Kampo medicine on COVID-19-related symptoms and conditions.

JSOM RESEARCH PROJECT ON THE USE OF KAMPO MEDICINE IN TREATING COVID-19

The JSOM has prepared a research project for clinical trials of Kampo medicine in patients with COVID-19 (Takayama et al., 2020a; Takayama et al., 2020c; Namiki et al., 2021), some of which are currently in progress. **Table 3** shows the list of the clinical studies being planned or conducted in the JSOM projects. In particular, studies one through three are referred to as an Integrative Management in Japan for Epidemic Disease (IMJEDI).

Prevention (Pre-Disease)

Study 1 is a multi-centered, randomized trial to test our hypothesis that the Kampo medicine, hochuekkito, has a preventive effect on the symptoms of COVID-19 among healthy hospital workers (Namiki et al., 2021). While we hope for an effective and widely available vaccine, the efficacy of the current vaccines has not been shown in a large number of patients. Furthermore, the efficacy of the vaccines may be reduced if SARS-CoV-2 persistently and consistently undergoes mutations (Awadasseid et al., 2021; Lauring and Hodcroft 2021). The possible mechanisms of the preventive effect of hochuekkito (bu Zhong yi qi tang) against COVID-19 have reported by Takayama et al. (Takayama et al., 2020b). Thus, clinical trials are being conducted to assess the efficacy of Kampo medicine for preventing COVID-19. Medical staff, overextended from the increase in critical patient care and the other circumstances involved with the COVID-19 pandemic, are thought to have decreased immunity along with physical and mental health issues. Furthermore, they are at an increased risk of infection when providing medical care. Therefore, we considered

TABLE 3 | Outline of ongoing research on the prevention and treatment for COVID-19 in Kampo medicine.

Study of JSOM research	Trial design	Subjects	Number of subjects	Intervention	Comparison	Outcome	Aim	Registration
Study 1 (Prevention)	A multi-center, interventional, parallel-group, randomized (1:1 ratio), investigator-sponsored, two-arm study	Healthy hospital workers	Set at 6,000	Participants receive hochuekkito in 9 tablets 2 times per day for 8 weeks	Participants receive placebo in the same dosage as the intervention group	Primary outcomes: Number of patients with a SARS-CoV-2 RNA by polymerase chain reaction (PCR) positive result with at least one symptom (fever, cough, sputum, malaise, shortness of breath) during the 12 weeks study period (including the 4 weeks observation period after oral administration)	To test our hypothesis that hochuekkito has a preventive effect on the symptoms of COVID-19	jRCTs031200150 registered on October 14, 2020
Study 2 (Acute treatment)	Multicenter, retrospective observational study	Mild to moderate COVID-19 patients treated with conventional medicines/Kampo medicines	Set at 1,000	N/A	N/A	Primary outcomes: Treatment, symptom course, critical illness outcome	To investigate the efficacy of the actual treatment (the efficacy of conventional and Kampo medicines) in patients with mild to moderate or suspected COVID-19	UMIN000041301 registered on August 4, 2020
Study 3 (acute treatment)	A multi-center, interventional, parallel-group, randomized (1:1 ratio), investigator-sponsored, two-arm study	Mild to moderate COVID-19 patients	Set at 150	Patients will receive 2.5 g of KT (TJ-1@ TSUMURA and Co.) and 2.5 g of SSKKS (TJ-109@TSUMURA and Co.) 3 times a day, orally, for 14 days in addition to the conventional treatment	Patients will receive conventional treatment	Primary outcomes: The number of days till at least one of the symptoms (fever, cough, sputum, malaise, shortness of breath) improves in the first 14 days of treatment	To test our hypothesis that additional administration of kakkonto and shosaikotokakikyosekko is more effective in relieving symptoms and preventing the onset of severe infection in mild-to-moderate COVID-19 patients compared to treatment with only conventional treatment	jRCTs021200020. registered on August 25, 2020
Study 4 (Sequelae treatment)	Multicenter, prospective observational study	Patients with COVID-19 related sequelae	N/A	N/A	N/A	Outcomes: visual analogue scale (VAS) in each symptom (fatigue, short of breathing, joint pain, chest pain, cough, dysgeusia, anosmia, etc), SF-12 (evaluation scale for health related quality of life), five-grade evaluation in overall treatment efficacy, safety evaluation	To investigate the efficacy and safety of Kampo treatment in patients with COVID-19 related sequelae	UMIN000044318 registered on May 25, 2021

Notes: University Hospital Medical Information Network; UMIN.
Japan Registry of Clinical Trials; jRCT.

it necessary to verify the effectiveness of Kampo medicine in preventing illness among staff members who continue to provide medical care despite these challenges.

Treatment (Especially in the Acute and Subacute Stages of Disease)

Study 2 is a multi-centered, retrospective, observational study to investigate the efficacy of the actual treatment (conventional and Kampo medicine) in patients with mild-to-moderate or even suspected COVID-19 (Takayama et al., 2020a). Several clinical trials on antiviral drugs are currently in progress; however, evidence on its efficacy remains limited. Kampo medicine has a long history of repeated use in viral epidemics and was also used during the Spanish influenza pandemic, approximately 100 years ago. Several cases of COVID-19 treated with Kampo medicine have already been reported (Homma et al., 2020a; Homma et al., 2020b; Irie et al., 2020; Kashima et al., 2020; Kyo 2020; Niitsuma et al., 2020; Watanabe and Watanabe, 2020; Yamasaki 2020; Takayama et al., 2021a; Takayama et al., 2021b). The next step in establishing Kampo medicine as a treatment strategy for COVID-19 is organizing a clinical study with a large number of cases. In Japan, many COVID-19 patients are treated with a combination of Kampo medicine and Western medicine, and an observational study that would collect data from a multi-center setting would be ideal.

Study 3 is a multi-centered, randomized trial to test our hypothesis that additional administration of Kampo medicines kakkonto and shosaikotokakikyosekko is more effective in relieving symptoms and preventing the onset of severe infection in mild-to-moderate COVID-19 patients compared to those treated with conventional treatment alone (Takayama et al., 2020c). The symptoms and signs associated with acute infectious diseases were categorized into six-stage patterns in the original concept of Kampo medicine described in the Shanhanlun, which is a traditional Asian medical textbook of infectious diseases written by Zhan Zhongjing (Takayama et al., 2017; The Dictionary of Kampo Medicine, 2020). The Shanhanlun explains that infectious diseases progress through six-stage patterns: early, middle, and late yang patterns and early, middle, and late yin patterns. Characteristic symptoms are chill and fever in the early yang pattern, alternating chill and fever with respiratory and intestinal symptoms in the middle yang pattern, and marked fever with thirst in the late yang pattern. The symptoms also include coldness with intestinal symptoms in the early yin pattern, coldness with marked fatigue in the middle yang pattern, and coldness with circulation failure with disturbance of consciousness in the late yang pattern. To study the efficacy and safety of Kampo medicines for COVID-19 patients, it is necessary to focus on a certain type of Kampo medicine. COVID-19 symptoms include chills, fever, muscle pain, joint pain, nasal congestion and discharge, sore throat, a cough producing sputum, loss of appetite, diarrhea, impaired smell and taste, and malaise, which progress from the early yang pattern to the middle and late yang patterns described in Shanhanlun. The type of Kampo formula depends on the stage of clinical manifestation: mao formula in the early yang pattern, saiko formula in the middle yang pattern, and sekko in the late yang pattern. A combination of mao formula, saiko formula, and sekko is an ideal choice for treating diseases such as COVID-19, which transition from the early to the middle and late yang patterns in the Kampo concept. Thus, these combinations were included in saikatsugekito, which was used for the Spanish flu, and case series have already reported the use of saikatsugekito for COVID-19 (Irie et al., 2020). Saikatsugekitois applied in combination with kakkonto and shosaikotokakikyosekko under the permissions of the national health insurance. The possible mechanisms of saikatsugekito against SARS-CoV-2 and COVID-19 have been reported by Arita et al. (Arita et al., 2020).

Recovery and Sequelae (Post-Disease)

While study 4 is a multi-centered, prospective, observational study to investigate the efficacy of Kampo medicines in patients with COVID-19-related sequelae. After the stabilization of COVID-19 patients, continued support for recovery and management of sequelae is needed. Future research interests include epidemiological investigations of sequelae and case series that summarize the experience of patients who used Kampo medicine for treatment.

DISCUSSION

Prospects of a Clinical Trial of Kampo Medicine for COVID-19

The contribution and prospects of the present projects on Kampo medicine and its efficacy as a COVID-19 treatment are as follows:

- Kampo medicine may act on viruses, human tissues, and organs via multiple mechanisms that differ from those of other drugs being developed in Western medicine (Arita et al., 2020; Takayama et al., 2020b).
- Kampo medicines for common cold are well established; their possible side effects are generally known and safety information has already been acquired (AuthorAnonymous, 2005; Kubo et al., 2013; Shimamoto et al., 2014; Takayama et al., 2017; Arai et al., 2018; Takayama et al., 2018; Takayama et al., 2020d).
- Kampo medicines are inexpensive, and if the clinical studies show their efficacy, it would be possible to provide care to many health care workers and patients, thus reducing medical expenses and providing medical economic benefits (Arita et al., 2015).
- If Kampo medicine is shown to be effective in alleviating symptoms and reducing the severity of disease, it could help to address the depletion of medical resources. There are currently no reports on effective treatments for the sequelae of COVID-19; if a clinical study could help develop a treatment strategy for sequelae, this could benefit many patients with such issues.
- Since viral mutations are now rapidly progressing, Kampo medicine could serve as an option in cases of resistance to Western medicine (Awadasseid et al., 2021; Lauring and Hodcroft 2021).

The quality control of Kampo medicines in Japan is highly regarded internationally, with very low variability from product to product. In this respect, conducting clinical research using Japanese Kampo medicines also has the advantage of maintaining uniformity of treatment for consistency and accuracy of research results.

Attention to Use Qing Fei Pai Du Tang From China

QFPDT is recommended for use in patients as an option for the treatment of COVID-19 in the seventh edition of the Chinese COVID-19 guidelines (National Health Commission and National Administration of Traditional Chinese Medicine 2020). In an observational study, 98 patients were treated with QFPDT for 9 days, and the efficiency of relief in major symptoms was 91.6% (Wang et al., 2020). However, randomized controlled trials of QFPDT have never been reported previously. Furthermore, QFPDT includes crude drugs which are not approved by the regulatory authorities in Japan. Thus, it is difficult to reproduce and use the same products in Japan. On the other hand, it is possible to reproduce similar Kampo medicines by combining certain Kampo extract preparations such as gokoto and saireito in Japan. The original QFPDT includes 21 crude drugs; however, their dosages are much higher than the usual dosage in Japan. The JSOM reminds us that the duration of administration should be evaluated every 3 days for a maximum of 2 weeks. It is also important to note that while the drug is indicated for use in young people, it should be used with sufficient caution in the elderly (The Japan Society for Oriental Medicine, 2021).

Side Effects of Kampo Medicine

Guidelines for the management of hypertension (2014) introduced the side effect associated with glycyrrhizin, which has the potential to induce pseudo-aldosteronism (Shimamoto et al., 2014). A consensus statement for the diagnosis and treatment of drug-induced lung injuries (Kubo et al., 2013) reported the side effect of interstitial pneumonia with the use of shosaikoto (AuthorAnonymous, 2005). The prevalence of shosaikoto-induced interstitial pneumonia is reported to be under 0.1% (Arai et al., 2018); however, when limited in patients with no prior sensitization to shosaikoto, there were no reports of new-onset shosaikoto-induced interstitial pneumonia within 2 weeks after its administration. Ephedrae Herba also includes ephedrine, which induces an adrenergic reaction. Thus, several clinical guidelines recommend that this medicine should be cautiously prescribed by the Mao and Saiko formula in those with hypokalemia, heart disease, and prior sensitization of allergy to shosaikoto (Arita et al., 2015; Takayama and Iwasaki, 2017; Takayama et al., 2020d).

CONCLUSION

Several cases treated with Kampo medicine have reported in COVID-19. The JSOM research will enable us to propose a wide range of treatments for emerging viral infections, i.e., not only for COVID-19 but also for viruses we may encounter in the future.

AUTHOR CONTRIBUTIONS

ST wrote the manuscript and conducted studies 2 and 3. TN revised the draft manuscript and conducted study 1. HO have prepared study 4. RA revised **Table 2** and made the additional **Supplementary Table S1**. AH, KM, and TI gave suggestions to this project.

FUNDING

The authors declare that this study received funding from JSOM and TSUMURA and Co. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank the members of Japan Society for Oriental Medicine for the many proposals and suggestions for this project.

Rie Ono named this project as the "IMJEDI study." Natsumi Saito created the symbol for the "IMJEDI study." Akiko Kikuchi provided suggestions for **Figure 1**. Tetsuharu Kamiya translated the name of Kampo medicines to Chinese. Soichiro Kaneko confirmed the references in Japanese, Chinese, and English. Akiko Kuwabara coordinated the clinical research process in Studies 2 and 3. Emiko Yoshida coordinated the clinical research process in Study 1. Akino Wakasugi and Mariko Sekine coordinated the clinical research process in Study 4.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.656246/full#supplementary-material

REFERENCES

Arai, I., Hagiwara, Y., and Motoo, Y. (2018). Estimated Incidence of Adverse Reactions to Kampo Medicines in Randomized Controlled Clinical Trials. *Traditional Kampo Med.* 5, 106–112. doi:10.1002/tkm2.1200

Arita, R., Ono, R., Saito, N., Takayama, S., Namiki, T., Ito, T., et al. (2020). Kakkonto, Shosaikoto, Platycodon Grandiflorum Root, and gypsum (A Japanese Original Combination Drug Known as Saikatsugekito): Pharmacological Review of its Activity against Viral Infections and Respiratory Inflammatory Conditions and a Discussion of its Applications to COVID -19. *Traditional Kampo Med.* 7, 115–127. doi:10.1002/tkm2.1258

Arita, R., Yoshino, T., Hotta, Y., Miyamoto, Y., Osawa, I., Takemoto, O., et al. (2015). National Cost Estimation of Maoto, a Kampo Medicine, Compared with Oseltamivir for the Treatment of Influenza in Japan. *Traditional Kampo Med.* 3, 59–62. doi:10.1002/tkm2.1027

Awadasseid, A., Wu, Y., Tanaka, Y., and Zhang, W. (2021). SARS-CoV-2 Variants Evolved during the Early Stage of the Pandemic and Effects of Mutations on Adaptation in Wuhan Populations. *Int. J. Biol. Sci.* 17, 97–106. doi:10.7150/ijbs.47827

Carfì, A., Bernabei, R., and Landi, F. (2020). Persistent Symptoms in Patients after Acute COVID-19. *JAMA* 324, 603–605. doi:10.1001/jama.2020.12603

Ferretti, L., Wymant, C., Kendall, M., Zhao, L., Nurtay, A., Abeler-Dörner, L., et al. (2020). Quantifying SARS-CoV-2 Transmission Suggests Epidemic Control with Digital Contact Tracing. *Science* 368, eabb6936. doi:10.1126/science.abb6936

Goto, S., Lauda, D., Katai, S., and Yasui, H. (2005). "Appendix - Composition and Indications of 148 Prescriptions," *Current Kampo Medicine*. Berkeley: International Institute of Health and Human Services, 85–101.

He, X., Lau, E. H. Y., Wu, P., Deng, X., Wang, J., Hao, X., et al. (2020). Temporal Dynamics in Viral Shedding and Transmissibility of COVID-19. *Nat. Med.* 26, 672–675. doi:10.1038/s41591-020-0869-5

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271–e8. doi:10.1016/j.cell.2020.02.052

Homma, Y., Inoue, K., and Moritaka, T. (2020a). A Case of New Coronavirus (SARS-CoV-2) Pneumonia that Required Oxygen Administration but Improved with Symptomatic Treatment Alone. *Infect. Dis*, 1–2. (in Japanese). Available at: https://www.kansensho.or.jp/uploads/files/topics/2019ncov/covid19_casereport_200422_2.pdf.

Homma, Y., Watanabe, M., Inoue, K., and Moritaka, T. (2020b). Coronavirus Disease-19 Pneumonia with Facial Nerve Palsy and Olfactory Disturbance. *Intern. Med.* 59, 1773–1775. doi:10.2169/internalmedicine.5014-20

Hu, K., Guan, W. J., Bi, Y., Zhang, W., Li, L., Zhang, B., et al. (2020). Efficacy and Safety of Lianhuaqingwen Capsules, a Repurposed Chinese Herb, in Patients with Coronavirus Disease 2019: A Multicenter, Prospective, Randomized Controlled Trial. *Phytomedicine* 85, 153242. doi:10.1016/j.phymed.2020.153242

Influenza (2008). *Department of Health, Ministry of Home Affairs in Japan*. Tokyo, Japan: Heibonsha.

Irie, Y., and Nakae, S. (2020). The Role of Kampo Medicines in the Pandemic of Viral Infections: Learning from the Spanish Flu. *Kampo Med.* 71, 272–283.

Irie, Y., Nakae, H., and Fukui, S. (2020). Three Mild Cases of Coronavirus Disease 2019 Treated with Saikatsugekito, a Japanese Herbal Medicine. *Traditional Kampo Med.* 8, 111–114. doi:10.1002/tkm2.1261

Jarvis, M. C. (2020). Aerosol Transmission of SARS-CoV-2: Physical Principles and Implications. *Front. Public Health* 8, 590041. doi:10.3389/fpubh.2020.590041

Kashima, M., Hayano, S., and Iwagoe, H. (2020). Two Cases of COVID-19 in Which Kampo Medicine May Have Contributed to the Suppression for Severe Stage. Home page of the Japanese Association for. *Infect. Dis*, 1–5. (in Japanese). Available at: https://www.kansensho.or.jp/uploads/files/topics/2019ncov/covid19_casereport_200430_3.pdf.

Kubo, K., Azuma, A., Kanazawa, M., Kameda, H., Kusumoto, M., Genma, A., et al. (2013). Consensus Statement for the Diagnosis and Treatment of Drug-Induced Lung Injuries. *Respir. Investig.* 51, 260–277. doi:10.1016/j.resinv.2013.09.001

Kyo, S. (2020). Thirty Nine Case Reports of Kampo Treatment in COVID-19. *J. Kampo Med.* 67, 953–963.

Lauring, A. S., and Hodcroft, E. B. (2021). Genetic Variants of SARS-CoV-2—What Do They Mean?. *JAMA* 325, 529. doi:10.1001/jama.2020.27124

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic Characterisation and Epidemiology of 2019 Novel Coronavirus: Implications for Virus Origins and Receptor Binding. *Lancet* 395, 565–574. doi:10.1016/S0140-6736(20)30251-8

McElvaney, O. J., McEvoy, N. L., McElvaney, O. F., Carroll, T. P., Murphy, M. P., Dunlea, D. M., et al. (2020). Characterization of the Inflammatory Response to Severe COVID-19 Illness. *Am. J. Respir. Crit. Care Med.* 202 (6), 812–821. doi:10.1164/rccm.202005-1583OC

Ministry of Health, Labour and Welfare of Japan (2020a). A Basic Guidelines for Infection Control against COVID-19 25 February 2020. [cited 7 Jan 2021] Available at: https://www.mhlw.go.jp/content/10900000/000599698.pdf.

Ministry of Health, Labour and Welfare of Japan (2020b). COVID-19 Guide to Medical Treatment 2020, Version 2.2. 2020. (in Japanese) Available at: https://www.mhlw.go.jp/content/000646531.pdf.

Ministry of Health, Labour and Welfare of Japan (2020c). COVID-19 Guide to Medical Treatment 2020, Version 4.1, P29. (in Japanese) Available at: https://www.mhlw.go.jp/content/000712473.pdf.

Nabeshima, S., Kashiwagi, K., Ajisaka, K., Masui, S., Takeoka, H., Ikematsu, H., et al. (2012). A Randomized, Controlled Trial Comparing Traditional Herbal Medicine and Neuraminidase Inhibitors in the Treatment of Seasonal Influenza. *J. Infect. Chemother.* 18, 534–543. doi:10.1007/s10156-012-0378-7

Namiki, T., Takayama, S., Arita, R., Ishii, T., Kainuma, M., Makino, T., et al. (2021). A Structured Summary of a Study Protocol for a multi-center, Randomized Controlled Trial (RCT) of COVID-19 Prevention with Kampo Medicines (Integrative Management in Japan for Epidemic Disease by Prophylactic Study: IMJEDI P1 Study). *Trials* 22, 23. doi:10.1186/s13063-020-04939-2

National Institute of Infectious Diseases (2020). *COVID-19. Infectious Agents Surveillance Report 41*. Japan: National Institute of Infectious Diseases, 103–105. Available at: https://www.niid.go.jp/niid/images/idsc/iasr/41/485.pdf.

Niitsuma, K., Suzuki, T., and Saito, M. (2020). Two Cases of Novel Coronavirus (COVID-19) Pneumonia that Had Occurred Asymptomatically Including a Severe Case of Organized Pneumonia Pattern. Home page of the Japanese Association for. *Infect. Dis* , 1–5. (in Japanese). Available at: https://www.kansensho.or.jp/uploads/files/topics/2019ncov/covid19_casereport_200331_3.pdf.

Shimamoto, K., Ando, K., Fujita, T., Hasebe, N., Higaki, J., Horiuchi, M., et al. (2014). The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2014). *Hypertens. Res.* 37, 253–390. doi:10.1038/hr.2014.20

Takayama, S., Arita, R., and Iwasaki, K. (2017). How Do You TreatUpper Respiratory Infectionsin the Elderly in Your Practice? *Med. Acupuncture* 29, 105–113. doi:10.1089/acu.2017.29047.cpl

Takayama, S., Arita, R., Kikuchi, A., Ohsawa, M., Kaneko, S., and Ishii, T. (2018). Clinical Practice Guidelines and Evidence for the Efficacy of Traditional Japanese Herbal Medicine (Kampo) in Treating Geriatric Patients. *Front. Nutr.* 5, 66. doi:10.3389/fnut.2018.00066

Takayama, S., Arita, R., Ono, R., Saito, N., Suzuki, S., Kikuchi, A., et al. (2021a). Treatment of COVID-19-Related Olfactory Disorder Promoted by Kakkontokasenkyushin'i: a Case Series. *Tohoku J. Exp. Med* 254, 71–80. doi:10.1620/tjem.254.71

Takayama, S., and Iwasaki, K. (2017). Systematic Review of Traditional Chinese Medicine for Geriatrics. *Geriatr. Gerontol. Int.* 17, 679–688. doi:10.1111/ggi.12803

Takayama, S., Kashima, M., Namiki, T., Ito, T., Ono, R., Arita, R., et al. (2020a). Conventional and Kampo Medicine in the Treatment of Mild to Moderate COVID-19: A Multicenter, Retrospective Observational Study Protocol by the Integrative Management in Japan for Epidemic Disease (IMJEDI Study-Observation). *Tradit Kampo Med.* 8, 106–110. doi:10.1002/tkm2.1271

Takayama, S., Kikuchi, A., Makino, T., Kainuma, M., Namiki, T., and Ito, T. (2020b). Basic Pharmacological Mechanisms and Clinical Evidence of the Efficacy of Hochuekkito against Infectious Diseases and its Potential for Use against COVID-19. *Tradit Kampo Med.* 8, 3–21. doi:10.1002/tkm2.1264

Takayama, S., Namiki, T., Ito, T., Arita, R., Nakae, H., Kobayashi, S., et al. (2020c). A multi-center, Randomized Controlled Trial by the Integrative Management in Japan for Epidemic Disease (IMJEDI Study-RCT) on the Use of Kampo Medicine, Kakkonto with Shosaikotokakikyosekko, in Mild-To-Moderate COVID-19 Patients for Symptomatic Relief and Prevention of Severe Stage: a Structured Summary of a Study Protocol for a Randomized Controlled Trial. *Trials* 21, 827. doi:10.1186/s13063-020-04746-9

Takayama, S., Ono, R., Aria, R., Saito, N., Suzuki, S., Tadano, Y., et al. (2021b). Usefulness of Portable Chest Radiography and Blood Sampling for Prompt Medical Response in COVID-19 Isolation Facilities: Two Cases of Moderate Stage I COVID-19. *J. Hosp. Gen. Med.* 3(3), 92–96.

Takayama, S., Tomita, N., Arita, R., Ono, R., Kikuchi, A., and Ishii, T. (2020d). Kampo Medicine for Various Aging-Related Symptoms: A Review of Geriatric Syndrome. *Front. Nutr.* 7, 86. doi:10.3389/fnut.2020.00086

The Dictionary of Kampo Medicine (2020). *The Japan Society for Oriental Medicine*. Kyoto: Medical Yukon Publishing Co., Ltd..

The Japan Society for Oriental Medicine (2021). *Notice of Alert on the Use of a New Herbal Medicine Product from China*. Tokyo: Home page of The Japan Society for Oriental Medicine. . [cited 7 Jan 2021]. Available at: http://www.jsom.or.jp/medical/notice/pdf/covid-attention-medical.pdf.

Wang, R., Sijin, Y., Chunguang, X., Qilin, S., Minqing, L., Liao, L., et al. (2020). *Clinical Efficacy of Qing Lung Detoxification in the Treatment of Novel Coronavirus Pneumonia*. Beijing: Pharmacology and Clinics of Chinese Materia Medica; (in Chinese). doi:10.13412/j.cnki.zyyl.20200303.002

Watanabe, K., and Watanabe, N. (2020). Experience in the Treatment of COVID-19 with Qingfei Paidu Decoction. *J. Kampo Med.* 67, 785–790.

Wei, P-F. (2020). Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7). *Chin. Med. J. (Engl)* 133 (9), 1087–1095. doi:10.1097/CM9.0000000000000819

World Health Organization (2019). Novel Coronavirus (2019-nCOV) SITUATION REPORT-1. 21 January 2020. [cited 7 Jan 2021]. Available at: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf.

World Health Organization (2015). Summary of Probable SARS Cases with Onset of Illness from 1 November 2002 to 31 July 2003. [cited 7 Jan 2021]. Available at: https://www.who.int/publications/m/item/summary-of-probable-sars-cases-with-onset-of-illness-from-1-november-2002-to-31-july-2003.

World Health Organization (2020). WHO Director-General's Opening Remarks at the media Briefing on COVID-19 – 11 March 2020. [cited 7 Jan 2021]. Available at: https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020.

Wu, Z., and McGoogan, J. M. (2020). Characteristics of and Important Lessons from the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases from the Chinese Center for Disease Control and Prevention (COVID-19) Outbreak in China: Summary of a Report of 72,314 Cases from the Chinese Center for Disease Control and Prevention. *JAMA* 323, 1239–1242. doi:10.1001/jama.2020.2648

Yamasaki, G. (2020). Two Cases of COVID-19 Treated with Makyokansekitogoireitogo and Shosaikotokakikyosekko. *J. Kampo Med.* 67, 933–998.

Yasui, H. (2007). History of the Schools of Kampo Medicine. *Kampo Med.* 58, 177–202. doi:10.3937/kampomed.58.177 Available at: https://www.jstage.jst.go.jp/article/kampomed/58/2/58_2_177/_pdf/-char/ja.

Yoshino, T., Arita, R., Horiba, Y., and Watanabe, K. (2019). The Use of Maoto (Ma-Huang-Tang), a Traditional Japanese Kampo Medicine, to Alleviate Flu Symptoms: a Systematic Review and Meta-Analysis. *BMC. Complement. Altern. Med.* 19, 68. doi:10.1186/s12906-019-2474-z

Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D., and Fouchier, R. A. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *N. Engl. J. Med.* 367, 1814–1820. doi:10.1056/NEJMoa1211721

Zhang, L., Zheng, X., Bai, X., Wang, Q., Chen, B., Wang, H., et al. (2021). Association between Use of Qingfei Paidu Tang and Mortality in Hospitalized Patients with COVID-19: A National Retrospective Registry Study. *Phytomedicine* 85, 153531. doi:10.1016/j.phymed.2021.153531

Zheng, S., Baak, J. P., Li, S., Xiao, W., Ren, H., Yang, H., et al. (2020). Network Pharmacology Analysis of the Therapeutic Mechanisms of the Traditional Chinese Herbal Formula Lian Hua Qing Wen in Corona Virus Disease 2019 (COVID-19), Gives Fundamental Support to the Clinical Use of LHQW. *Phytomedicine* 79, 153336. doi:10.1016/j.phymed.2020.153336

Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A Pneumonia Outbreak Associated with a New Coronavirus of Probable Bat Origin. *Nature* 579, 270–273. doi:10.1038/s41586-020-2012-7

Conflict of Interest: ST and TI belong to the Department of Kampo and Integrative Medicine, Tohoku University Graduate School of Medicine, a joint research course with TSUMURA and Co., which is a paratheatrical company of Kampo medicine in Japan.

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.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 31 August 2021
doi: 10.3389/fphar.2021.687818

Kakkonto Inhibits Cytokine Production Induced by Rhinovirus Infection in Primary Cultures of Human Nasal Epithelial Cells

Natsumi Saito[1], Akiko Kikuchi[1,2], Mutsuo Yamaya[3], Xue Deng[2], Mitsuru Sugawara[4], Shin Takayama[1,2], Ryoichi Nagatomi[5] and Tadashi Ishii[1,2]*

[1]Department of Education and Support for Regional Medicine, Tohoku University Hospital, Sendai, Japan, [2]Department of Kampo and Integrative Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan, [3]Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan, [4]Department of Otolaryngology, Tohoku Kosai Hospital, Sendai, Japan, [5]Laboratory of Health and Sports Science, Division of Biomedical Engineering for Health and Welfare, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan

Rhinovirus (RV) is a primary etiologic agent of common cold that can subsequently acutely exacerbate bronchial asthma or chronic obstructive pulmonary disease. Kakkonto (Ge-gen-tang in Chinese), one of the most frequently prescribed traditional Japanese (Kampo) medicines, is used for treating common cold, shoulder stiffness, or inflammatory diseases of the upper body. Previous experimental studies have indicated that kakkonto exerts antiviral and anti-inflammatory effects on the influenza virus and the human respiratory syncytial virus. However, there is a lack of reports investigating the efficacy of kakkonto in RV infection. Hence, the aim of the current study was to investigate the effects of kakkonto on RV infection of human nasal epithelial (HNE) cells. HNE cells obtained via endoscopic sinus surgery were cultured and infected with RV14, with or without kakkonto treatment. The supernatants from the cells were collected, and the RV14 titer and cytokine levels were assessed. Reverse transcription-polymerase chain reaction was performed to determine the amount of viral RNA, while the level of nuclear factor kappa B (NF-κB) subunits in the nucleus was assessed by enzyme-linked immunosorbent assay. Although kakkonto treatment did not reduce RV14 titer or RNA levels, indicating that it did not inhibit RV14 proliferation, it was found to reduce the production of specific pro-inflammatory cytokines, including interleukin (IL)-8, tumor necrosis factor (TNF)-α, and monocyte chemotactic protein-1 (MCP-1). Unlike that observed with the kakkonto extract, none of the crude drugs contained in kakkonto reduced IL-8 level. Furthermore, though kakkonto treatment significantly reduced p50 levels, it did not impact the p65 subunit of NF-κB. These results indicated that kakkonto can inhibit inflammation caused by RV infection and may exert an immunomodulatory effect on HNE cells. This is the first report to elucidate the effects of kakkonto extract on RV infection in primary cultures of HNE cells,

OPEN ACCESS

Edited by:
Kenji Watanabe, Yokohama College of Pharmacy, Japan

Reviewed by:
Cheng-Peng Sun, Dalian Medical University, China
Kenny Kuchta, University Medical Center Göttingen, Germany

***Correspondence:**
Akiko Kikuchi
akikokik@med.tohoku.ac.jp

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

Received: *30 March 2021*
Accepted: *05 August 2021*
Published: *31 August 2021*

Citation:
Saito N, Kikuchi A, Yamaya M, Deng X, Sugawara M, Takayama S, Nagatomi R and Ishii T (2021) Kakkonto Inhibits Cytokine Production Induced by Rhinovirus Infection in Primary Cultures of Human Nasal Epithelial Cells. Front. Pharmacol. 12:687818. doi: 10.3389/fphar.2021.687818

Abbreviations: BA, bronchial asthma; COPD, chronic obstructive pulmonary disease; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HNE, human nasal epithelial cells, HEF, human embryonic fibroblasts; IL, interleukin; MCP-1, monocyte chemotactic protein-1; RT-PCR, reverse transcription-polymerase chain reaction; RV, Rhinovirus; TNF, tumor necrosis factor.

providing evidence that kakkonto may act as an effective therapy for RV infection and subsequent airway inflammation.

Keywords: kakkonto, Kampo, traditional herbal medicine, Rhinovirus, human airway epithelial cells, cytokine

INTRODUCTION

Rhinoviruses (RVs) were the most common cause of the common cold prior to the pandemic outbreak of the severe acute respiratory syndrome coronavirus 2. Specifically, RVs were reported to account for 30–50% of all respiratory illnesses in 2003 (Heikkinen and Järvinen, 2003). The common cold can cause acute exacerbation of chronic respiratory illnesses, such as bronchial asthma (BA) or chronic obstructive pulmonary disease (COPD). We have previously reported that hochuekkito, a traditional Japanese (Kampo) medicine, inhibits RV proliferation and cytokine production in human tracheal epithelial cells (Yamaya et al., 2007). Hochuekkito is commonly administered for the treatment of fatigability and anorexia. In a clinical trial, Tatsumi et al. reported that administration of hochuekkito to patients with COPD for 6 months reduced the number of common cold episodes and acute exacerbation events, improved systemic inflammation, and increased body weight (Shinozuka et al., 2007; Tatsumi et al., 2009). Other reports have indicated that hochuekkito may have a prophylactic effect on the common cold due to its immunomodulatory and immunostimulatory activities (Takayama et al., 2021). However, evidence supporting the efficacy of other Kampo medicines for the treatment of acute respiratory infections is limited.

Kakkonto (Ge-gen-tang in Chinese), one of the most frequently prescribed Kampo medicines in Japan, is used for the treatment of the common cold, shoulder stiffness, or inflammatory diseases of the upper body (DPPN, 2011). Previous studies using animal and *in vitro* models have reported the antiviral and anti-inflammatory effects of kakkonto (Arita et al., 2020). However, most experiments that have investigated the antiviral effect of kakkonto have focused on influenza viruses (Kurokawa et al., 2002; Wu et al., 2011; Shirayama et al., 2016; Geng et al., 2019) or on the human respiratory syncytial virus (Chang et al., 2012). In murine studies, administration of kakkonto has been reported to inhibit proliferation of influenza viruses in bronchoalveolar lavage fluid (Kurokawa et al., 2002) and lung tissue (Geng et al., 2019), prolong survival, and reduce mortality rates (Kurokawa et al., 2002; Geng et al., 2019). Kakkonto also reduces the expression of pro-inflammatory cytokines, including interleukin (IL)-1α, IL-6, and tumor necrosis factor (TNF)-α in H1N1 influenza-infected mice (Geng et al., 2019). Furthermore, kakkonto treatment reduces the rate of human respiratory syncytial virus infection in a human type II lung cell line (A549 cells) and human upper respiratory tract epithelial cell line (Hep-2 cells) (Chang et al., 2012). Cumulatively, these reports indicate that kakkonto might exert antiviral and anti-inflammatory effects on respiratory viral infections, although the effect of kakkonto on RV infection, both *in vivo* and *in vitro,* remains unclear. Therefore, the aim of the current study was to investigate the effect of kakkonto on RV infection of human nasal epithelial (HNE) cells, while also elucidating the underlying mechanism of action.

MATERIALS AND METHODS

Human Nasal Epithelial Cell Culture

Nasal polyps were obtained from 22 subjects aged 55.2 ± 15.1 years with chronic rhinosinusitis who underwent endoscopic sinus surgery. Among the 22 patients, 13 (59.1%) were ex-smokers; 13 had allergic disease (nine patients with BA, three with allergic rhinitis, and one with eosinophilic sinusitis); 10 (45.5%) were prescribed nasal corticosteroids; 10 (45.5%) were treated with L-carbocisteine; 14 (63.6%) were treated with macrolides (13 patients with clarithromycin and 1 with erythromycin); 9 (40.9%) were treated with Montelukast sodium; and 9 (40.9%) were treated with antihistamine agents (**Table 1**). All participants provided informed consent prior to enrolment in the study. This study was approved by the Tohoku University Ethics Committee (Institutional Review Board number: 2021–1–088).

Isolation and culturing of HNE cells from the nasal polyps was performed as described previously (Suzuki et al., 2001; Lusamba Kalonji et al., 2015). The HNE cells were plated at a density of 7.5×10^5 viable cells/mL in round-bottom plastic tubes (16 mm diameter × 125 mm length, Corning Incorporated, Corning, NY, United States) coated with human placental collagen. Cells were cultured in a 1 ml mixture of Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) medium (50:50, v/v) containing 2% Ultroser G (USG) (BioSepa, Cergy-Saint-Christophe, France),

TABLE 1 | Patient characteristics (*n* = 22).

Characteristics and medications	Value
Characteristics	
Age (years, mean ± SD)	55.2 ± 15.1
Males, *n* (%)	14 (63.6)
Smoker, *n* (%)	13 (59.1)
Brinkman index (mean ± SD)	284.8 ± 334.8
Laboratory data	
Eosinophil (%, mean ± SD)	6.8 ± 5.8
Allergic disease	
None, *n* (%)	9 (42.9)
Bronchial asthma, *n* (%)	9 (42.9)
Allergic rhinitis, *n* (%)	3 (14.3)
Eosinophilic sinusitis, *n* (%)	1 (4.5)
Medications, *n* (%)	
Inhaled corticosteroid	5 (22.7)
Nasal steroids	10 (45.5)
L-carbocisteine	10 (45.5)
Ambroxol hydrochloride	2 (9.1)
Long-acting β2 agonist	5 (22.7)
Macrolide	14 (63.6)
Montelukast sodium	9 (40.9)
Antihistamine agent	9 (40.9)

SD: standard deviation.

TABLE 2 | Original plants of kakkonto extract used in the study.

Common name	Latin name	Scientific name of the original plants
Pueraria Root	*Puerariae Radix*	*Pueraria lobata* (Willd.) Ohwi
Ephedra Herb	*Ephedrae Herba*	*Ephedra sinica* Stapf / *Ephedra intermedia* Schrenk & C.A. Mey. / *Ephedra equisetina* Bunge
Jujube	*Zizyphi Fructus*	*Zizyphus jujuba* Mill. var. *inermis* (Bunge) Rehder
Cinnamon Bark	*Cinnamomi Cortex*	*Cinnamomum cassia* Blume
Peony Root	*Paeoniae Radix*	*Paeonia lactiflora* Pall.
Glycyrrhiza	*Glycyrrhizae Radix*	*Glycyrrhiza uralensis* Fisch. / *Glycyrrhiza glabra* L.
Ginger	*Zingiberis Rhizoma*	*Zingiber officinale* Roscoe

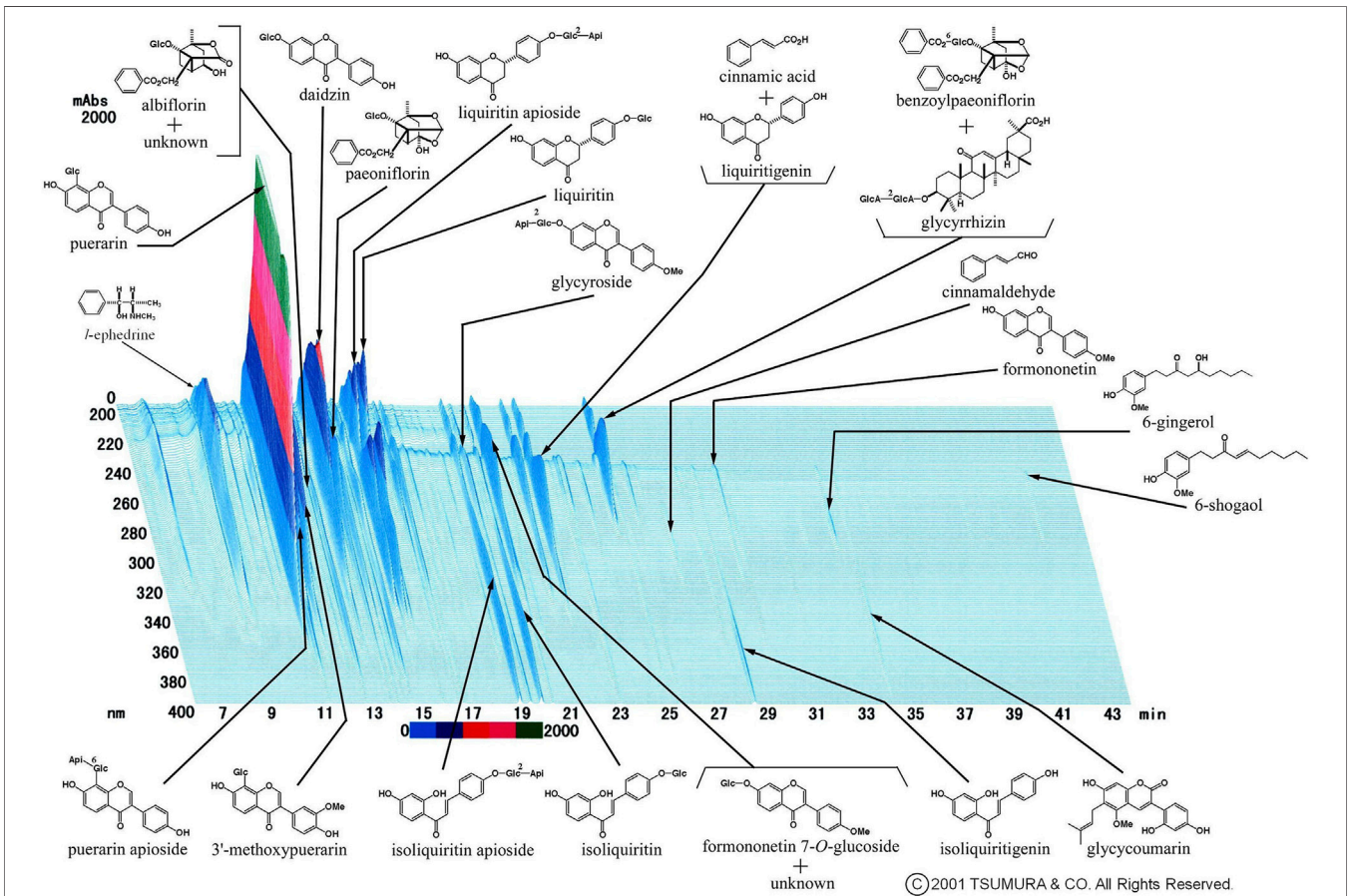

FIGURE 1 | Representative 3D-HPLC image of kakkonto extract. Kakkonto contains ephedrine from *Ephedra Herba*, puerarin or daidzin from *Puerariae Radix*, paeoniflorin from *Paeoniae Radix*, cinnamic acid from *Cinnamomi Cortex*, and glycyrrhizin or liquiritin from *Glycyrrhizae Radix*.

10^5 U/L penicillin, 100 mg/L streptomycin, 5 mg/L amphotericin B, and 100 mg/L gentamicin. The tubes, loosely covered with screw caps, were placed at a 5° angle and cultured at 37°C in a 5% CO_2 atmosphere.

Human Embryonic Fibroblast Cell Culture

HEF cells (HEF-III cells, Riken Bio Resource Center Cell Bank, Cell No: RCB0523; Tsukuba, Japan) were cultured as previously described (Yamaya et al., 2011) with slight modifications. In brief, HEF cells were cultured in T25 flasks in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (10^5 U/L penicillin and 100 mg/L streptomycin). The cells were then plated in 96-well plates or plastic tubes and cultured.

Viral Stocks

A stock of clinically isolated RV14 was prepared by infecting HEF cells as described previously (Numazaki et al., 1987; Yamaya et al., 2016). Briefly, HEF cells were exposed to RV14 at 10^5 tissue culture infective dose ($TCID_{50}$)/mL for 1 h, rinsed with phosphate buffered saline (PBS), and cultured in plastic tubes in 1 ml MEM supplemented with 2% ultra-low IgG FBS or 10%

FBS and antibiotics (10^5 U/L penicillin and 100 mg/L streptomycin) in a 33°C incubator (HDR-6-T, Hirasawa, Tokyo, Japan) with rotation (12 rotations per hour). The cells were incubated for approximately 3 days until cytopathic effects were observed. The virus-containing fluid obtained from these cells was frozen in aliquots at −80°C.

Preparations of Kakkonto Extract and Crude Drugs

The Japanese Pharmacopeia, 17th Edition defines kakkonto as being composed of seven crude drugs: 4 g *Puerariae Radix*, 3 g *Ephedrae Herba*, 3 g *Zizyphi Fructus*, 2 g *Cinnamomi Cortex*, 2 g *Paeoniae Radix*, 2 g *Glycyrrhizae Radix*, and 2 g *Zingiberis Rhizoma* (DPPN, 2011; The Ministry of Health, Labor and Welfare, 2016). The extracts of kakkonto (lot No. 2160001010) and the seven crude drugs were obtained from Tsumura and Co., (Tokyo, Japan). The plants of origin of these crude drugs are shown in **Table 2**.

Figure 1 shows a representative Three-Dimensional High-Performance Liquid Chromatographic(3D-HPLC) image of the kakkonto extract, provided by Tsumura and Co., (Tokyo, Japan). Due to it being composed of these seven crude drugs, Kakkonto contains many active agents, including ephedrine from the *Ephedrae Herba*, puerarin and daidzin from *Puerariae Radix*, paeoniflorin from *Paeoniae Radix*, cinnamic acid from *Cinnamomi Cortex*, and glycyrrhizin or liquiritin from *Glycyrrhizae Radix*. The CAS (Chemical Abstracts Service) registry numbers of components in kakkonto extract, shown in **Figure 1**, are provided as **Supplementary Table**.

The Japanese Pharmacopeia defines the kakkonto formula as containing the following indexed components: 9–27 mg of ephedrine or pseudoephedrine ($C_{10}H_{15}NO$: 165.23), 19–57 mg of glycyrrhizic acid ($C_{42}H_{62}O_{16}$: 822.93), and 14–56 mg of paeoniflorin ($C_{23}H_{28}O_{11}$: 480.46) per extract prepared according to the previously reported method (The Ministry of Health, Labor and Welfare, 2016).

The kakkonto extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml by vortexing for 5 min at room temperature (23°C). The mixture was then dispensed in microcentrifuge tubes and frozen at −80°C until experimentation. Kakkonto stock solution was diluted in culture medium (DMEM/F-12 with 2% USG) at a concentration of 100 μg/ml and the concentrations of ephedrine, pseudoephedrine, and glycyrrhizic acid were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) performed by Sekisui Chemistry, Tokyo, Japan.

Rhinovirus Infection and Treatment

Infection of HNE cells with RV14 was performed using previously described methods (Yamaya et al., 2007). A stock solution of RV was added to the cells in round-bottom plastic tubes (1 ml in each tube, 1.0×10^5 $TCID_{50}$/mL, 0.13 $TCID_{50}$/cell of the multiplicity of infection). After 1 h of incubation, the viral solution was removed, and cells were rinsed with PBS. The cells were then cultured in 1 ml fresh medium (2% USG in DMEM/F12) in the presence, or absence, of kakkonto extracts diluted in culture medium. The kakkonto treatments were initiated 1 h after infection and continued until the end of the experimental period. To investigate the concentration-dependent effects, cells were treated with 10, 20, or 50 μg/ml kakkonto. The supernatants (1 ml) were collected before infection, as well as 1, 3, 5, and 7 days after infection, and rapidly frozen in ethanol at −80°C. The specific kakkonto concentrations used in this study were determined based on the detected concentrations of ephedrine/pseudoephedrine and glycyrrhizic acid in the kakkonto solution. The specific details of these calculations can be found in *Concentration of Index Components in Kakkonto Extracts*.

Detection and Titration of Viruses

The detection and titration of RV14 in culture supernatants were performed using the endpoint method by infecting proliferating HEF cells in plastic 96-well plates containing 10-fold dilutions of virus-containing supernatants with MEM supplemented with 2% ultra-low IgG calf serum and antibiotics (10^5 U/L penicillin and 100 mg/L streptomycin), as described previously (Suzuki et al., 2001; Yamaya et al., 2014). The typical cytopathic effects of RV were then examined. The viral titers in the supernatants were expressed as $TCID_{50}$/mL.

Quantification of Rhinovirus RNA

The levels of RV14 RNA were determined using quantitative reverse transcription-polymerase chain reaction (RT-PCR) with TaqMan gene expression master mix (Applied Biosystems, Foster City, CA, United States), as described previously (Yamaya et al., 2011). The amount of RV14 RNA was normalized to that of β-actin, which was used as a housekeeping gene.

Determination of Cytokine Concentration

IL-6 and IL-8 level were assessed using an enzyme-linked immunosorbent assay (ELISA) performed by LSI Medience Corporation (Tokyo, Japan) with a solid phase chemiluminescent ELISA kit (QuantinGlo®; ELISA, R&D Systems, MN, United States), and an IL-8 EIA kit (Invitrogen, CA, United States). The levels of other cytokines were determined by a multiplex suspension array (Genetic Lab Co., Ltd., Sapporo, Japan) using a Luminex® assay system (Luminex Corp., Austin, TX, United States) with a magnetic Luminex® assay human premixed multi-analyte kit (R&D Systems Inc., Minneapolis, MN, United States). The assay was performed according to the manufacturer's instructions. Briefly, all samples were diluted using Calibrator Diluent RD6-52, and 50 μL of the diluted samples were used for the assay customized to detect and quantify TNF-α, IL-1β, IL-17C, IL-17E/IL25, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), thymic stromal lymphopoietin (TSLP), thymus and activation-regulated chemokine (TARC), eotaxin, and regulated on activation normal T cell expressed and secreted (RANTES). The fluorescence intensity of each sample was measured using a Luminex® 100/200TM system (Luminex Corp., Austin, TX, US), and the concentrations of each analyte were calculated using MILLIPLEX® Analyst 5.1 (Merck Millipore Corp., Burlington, MA, United States).

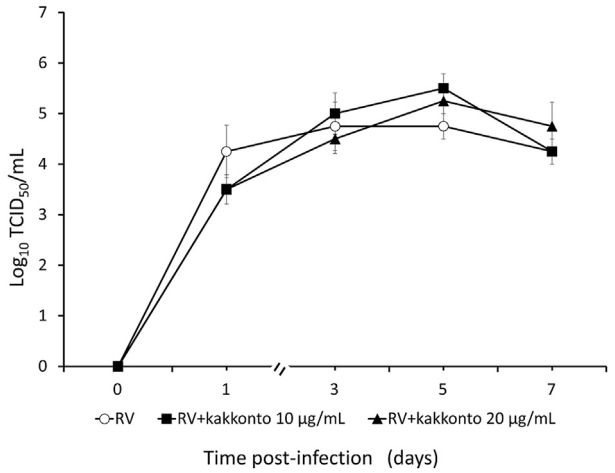

FIGURE 2 | Time course of viral release into supernatants of HNE cells infected with RV14 and treated with kakkonto (10 μg/ml, closed squares), kakkonto (20 μg/ml, closed triangles), or medium only (open circles). Results are presented as the mean ± standard error from four subjects.

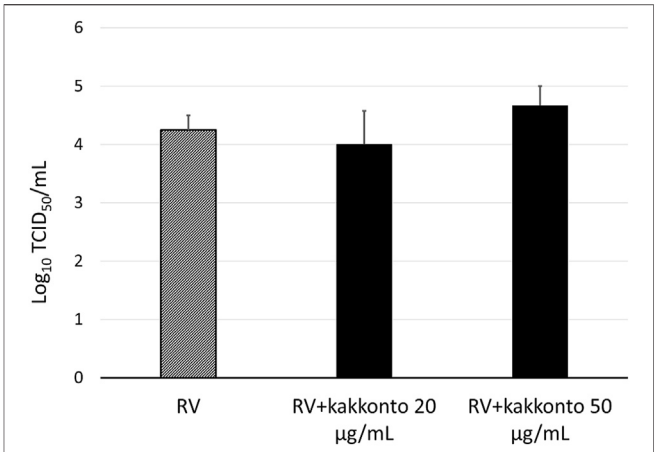

FIGURE 3 | Viral release into supernatants of HNE cells treated with 20 μg/ml and 50 μg/ml kakkonto or cultured in medium alone and collected 24 h after RV infection. Results are presented as the mean ± standard error from four subjects.

Measurement of Changes in Acidic Endosomes

The distribution and fluorescence intensity of acidic endosomes in the cells were measured with LysoSensor DND-189 dye (Molecular Probes, Eugene, OR, United States) using live-cell imaging (Lusamba Kalonji et al., 2015). The cells on coverslips in Petri dishes were observed under a fluorescence microscope (Olympus IX70; Olympus Co., Ltd., Tokyo, Japan). The excitation wavelength was 443 nm, and the light emitted from the cells was detected using a 505 nm filter. Fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®; Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope. HNE cells were treated with kakkonto (20 μg/ml) or media alone. The fluorescence intensity of the acidic endosomes was measured in 100 cells, and the mean value of the fluorescence intensity was expressed as a percentage of the control value compared to the fluorescence intensity of the cells prior to treatment.

Assessment of NF-κB Activation

Nuclear proteins were extracted from the nuclear extract kit of the epithelial cells (Active Motif, Carlsbad, CA, United States). The presence of translocated p50 and p65 subunits in the nuclear extracts was assessed using a TransAM NFκB family kit (Active motif, Carlsbad, CA, United States), according to the manufacturer's instructions. To examine the effect of kakkonto on the NF-κB subunits in the HNE cells, the cells were pretreated with kakkonto (20 μg/ml) or medium alone at 33°C for 24 h, after which the nuclear proteins were extracted.

Statistical Analysis

Results were expressed as mean ± standard error (SE). Statistical analysis was performed using two-way repeated-measures analysis of variance (ANOVA). Student's *t*-test or Mann-Whitney U-test was used for comparison between two groups according to the distribution patterns of the evaluated variables. Statistical significance was set at $p < 0.05$. In all experiments, n refers to the number of donors from whom the epithelial cells were obtained. All analyses were performed using SPSS version 21 (IBM Japan, Tokyo, Japan).

RESULTS

Concentration of Index Components in Kakkonto Extracts

LC-MS/MS analysis revealed that the concentrations of ephedrine/pseudoephedrine and glycyrrhizic acid in the kakkonto solution (100 μg/ml) were 363.0 ± 13.9 ng/ml and 867.3 ± 22.2 ng/ml, respectively. We then estimated the concentration of ephedrine/pseudoephedrine in human serum after administration of kakkonto to be up to 40–60 ng/ml (Yafune and Cyong, 2001; Inotsume et al., 2009), and that of glycyrrhetinic acid to be 100 ng/ml (provided by Tsumura and Co.,) based on a previous study (Yamaya et al., 2007). The 40–60 ng/ml of ephedrine/pseudoephedrine was calculated to apply 11.0–16.5 μg/ml of kakkonto extract and 100 ng/ml of glycyrrhetinic acid was 20.2 μg/ml of kakkonto extract. Therefore, we determined the concentration of kakkonto extract to be 10 or 20 μg/ml in our experiments.

Effects of Kakkonto on Rhinoviral Replication

The RV14 titer increased significantly until 7 days after infection ($p = 0.001$; **Figure 2**). Treatment of HNE cells with 10 and 20 μg/ml kakkonto 1 h after infection did not reduce RV titers ($p = 0.36$). However, the RV titer in the supernatants of the cells treated with kakkonto tended to decrease slightly by 24 h post-infection (h.p.i.). We then measured viral titers in the

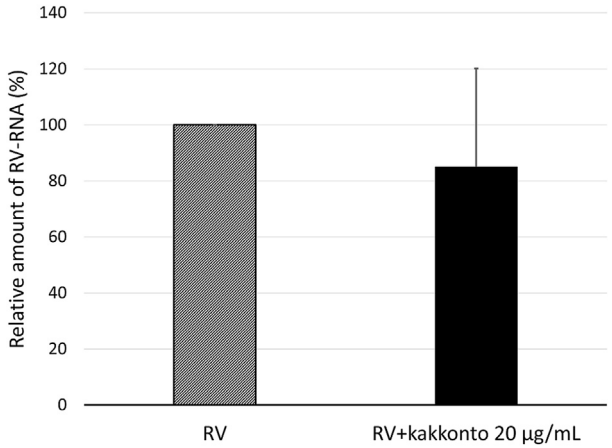

FIGURE 4 | Relative amount of RV14 RNA in HNE cells treated with 20 μg/ml kakkonto compared to cells cultured in medium alone 24 h after infection. Results are expressed as the relative amount (%) compared to that of cells cultured in medium alone and presented as mean ± standard error from five subjects. Kakkonto treatment did not reduce the relative amount of RV14 RNA (p = 0.69).

supernatants of HNE cells treated with 50 μg/ml kakkonto (**Figure 3**) and found that RV14 titers in the supernatants collected 24 h.p.i. did not differ significantly from that of cells cultured in medium alone or treated with 20 μg/ml kakkonto. These results indicate that kakkonto extract did not inhibit RV proliferation.

Effects of Kakkonto on RV14 RNA Expression

To investigate the effect of kakkonto on the proliferation of RV14, we determined the level of RV14 RNA in the cells 1 day post-infection (d.p.i., **Figure 4**). Treatment with 20 μg/ml kakkonto tended to decrease the amount of RV14 RNA; however, the difference between kakkonto treatment and medium alone was not statistically significant (p = 0.69).

Effects of Kakkonto on Endosome Acidification

The treatment of HNE cells with 20 μg/ml kakkonto for 24 h significantly reduced the number of green-fluorescent acidic endosomes (**Figures 5A,B**), as well as the fluorescence intensity of acidic endosomes compared to that of cells cultured in medium alone (p = 0.027, **Figure 5C**).

Effects of Kakkonto on Cytokine Production

The concentration of IL-6 in the supernatants of untreated, infected cells increased 24 h.p.i. (**Figure 6A**); whereas treatment with kakkonto tended to reduce IL-6 levels in a dose-dependent manner 24 h.p.i., however, this reduction was

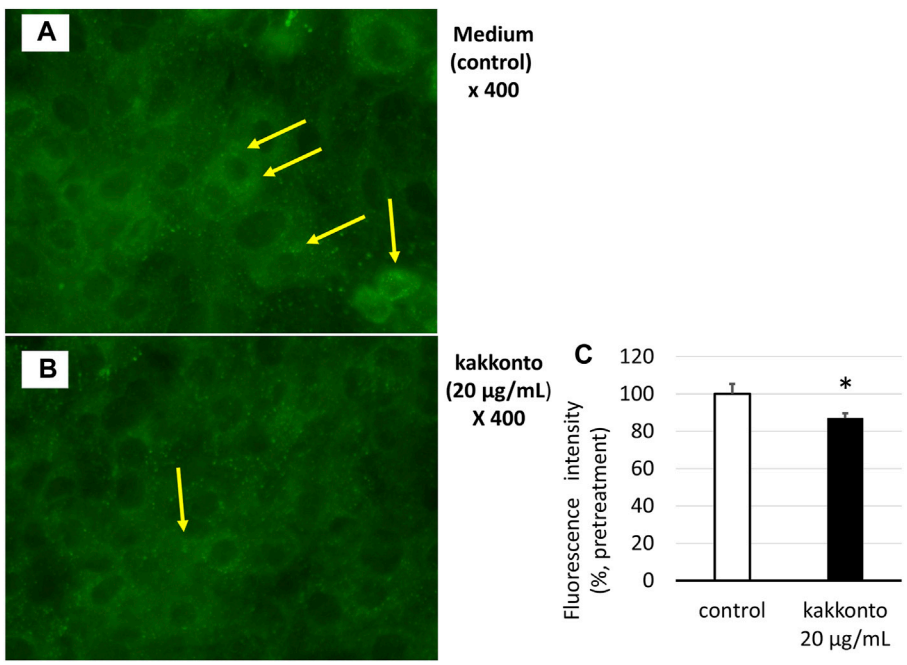

FIGURE 5 | (A,B) Distribution of acidic endosomes exhibiting green fluorescence in HNE cells 24 h after treatment with kakkonto (20 μg/ml) **(B)** or medium **(A)**. Yellow arrows indicate acidic endosomes in HNE cells. Data are representative of four individual experiments. Magnification, ×400. **(C)** Fluorescence intensity of acidic endosomes in HNE cells 24 h after treatment with kakkonto (20 μg/ml) or medium (control). The mean fluorescence intensity of the control cells was set at 100%. *p < 0.05.

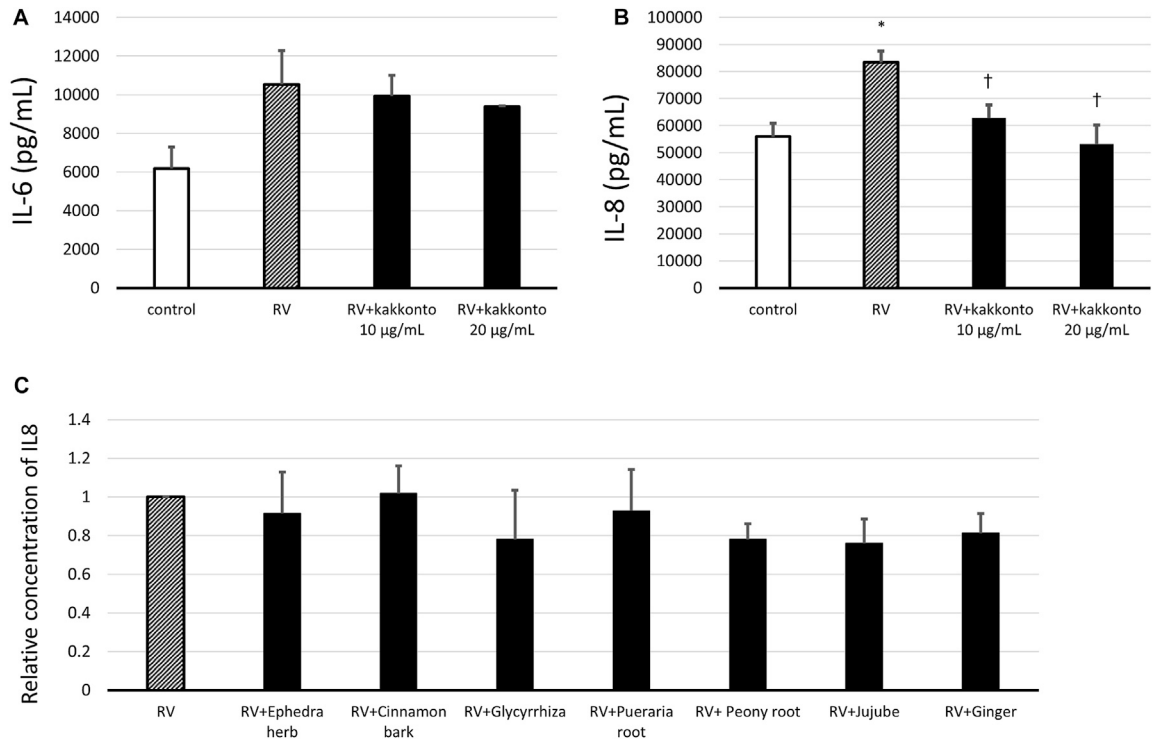

FIGURE 6 | (A,B) Release of IL-6 **(A)** and IL-8 **(B)** into culture supernatants 24 h after RV14 infection in the presence of 10 μg/ml or 20 μg/ml kakkonto, or after culturing in medium alone (RV) or mock infection (control). *$p < 0.05$ significant differences compared to control cells in the absence of RV infection (control); **(B)**. †$p < 0.05$ significant differences compared to RV infection alone (RV). **(C)** Relative abundance of IL-8 released in culture supernatants 24 h after RV14 infection in the presence of each crude drug of the kakkonto extract. Results are expressed as the relative amount (%) compared to cells cultured in medium alone (RV) and are presented as the mean ± SE from three **(A,B)** and seven **(C)** HNE cultures. The concentration of each crude drug was calculated as the weight % of 20 μg/ml kakkonto extract.

not statistically significant. We also measured the concentration of IL-6 3 d.p.i and found the concentration of IL-6 in the supernatants of uninfected cells (control), untreated infected cells, and kakkonto-treated infected cells to be 9,027 ± 1,260, 9,773 ± 1,026, and 7,933 ± 570 pg/ml, respectively. Kakkonto treatment tended to reduce the level of IL-6 compared to that in the supernatants of the cells cultured in medium alone, although this reduction was not statistically significant ($p = 0.19$).

In untreated, RV-infected cells, we also observed that the level of IL-8 significantly increased 24 h.p.i. compared to uninfected controls ($p = 0.013$; **Figure 6B**). Meanwhile, treatment with 10 or 20 μg/ml kakkonto significantly reduced IL-8 production compared to the untreated, infected cells ($p = 0.031$ and 0.021, respectively). We also assessed IL-8 concentration 3 d.p.i. and found it to 142,667 ± 1,453, 149,333 ± 7,216, and 122,000 ± 7,638 pg/ml in the supernatants collected from uninfected cells, infected and untreated cells, and infected cells treated with kakkonto (20 μg/ml), respectively. Thus, 20 μg/ml kakkonto treatment tended to reduce IL-8 concentration 3.d.p.i compared to RV14 infection alone, although the difference was not statistically significant ($p = 0.059$).

We also assessed the IL-8 level in the supernatant of cells 24 h.p.i. treated with individual extracts of the crude drugs (**Figure 6C**). The concentration of each crude drug was calculated as weight percentage of 20 μg/ml of kakkonto extract; that is 4 μg/ml of *Puerariae Radix*, 3 μg/ml of *Ephedrae Herba* and *Zizyphi Fructus*, and 2 μg/ml of *Cinnamomi Cortex*, *Glycyrrhizae Radix*, *Paeoniae Radix*, and *Zingiberis Rhizoma*, were applied. However, none of the crude drugs significantly reduced IL-8 concentration in the supernatants of RV14-infected cells (**Figure 6C**).

Furthermore, RV14 infection was found to increase the concentration of TNF-α, IL-1β, GM-CSF, and MCP-1 (**Table 3**), while kakkonto treatment (20 μg/ml) tended to reduce these effects. However, these differences were not statistically significant due to the relatively large standard errors (SE). We, therefore, elected to re-analyze this data by comparing concentrations to those of uninfected control supernatants (**Table 4**). RV14 infection was found to not affect the supernatant concentration of IL-17C, IL-25, or TARC. Meanwhile, TNF-α and MCP-1 levels in RV14-infected samples treated with kakkonto were significantly lower than those in untreated, RV-infected cultures ($p = 0.036$ and 0.033, respectively). RV infection also increased the levels of IL1β, GM-CSF, TSLP, while kakkonto treatment only slightly reduced these effects, however, the differences were not statistically significant. Also, RV infection tended to increase eotaxin levels, however, kakkonto treatment did not impact

TABLE 3 | Cytokine concentration in the supernatants of HNE cells 24 h after RV infection.

Cytokines and chemokines	Control (n = 3)	RV (n = 3)	RV + kakkonto (n = 3)
TNF-α (pg/mL, mean ± SE)	8.2 ± 2.9	9.8 ± 4.4	5.2 ± 1.2
p value vs. control	—	0.79	0.39
p value vs. RV	—	—	0.41
IL-1β (pg/mL, mean ± SE)	8.1 ± 3.8	19.6 ± 15.5	12.4 ± 8.6
p value vs. control	—	0.83	0.83
p value vs. RV	—	—	0.83
GM-CSF (pg/mL, mean ± SE)	13.7 ± 3.4	19.2 ± 7.7	9.8 ± 2.5
p value vs. control	—	0.55	0.40
p value vs. RV	—	—	0.31
MCP-1 (pg/mL, mean ± SE)	1,170 ± 349	1,372 ± 537	628 ± 167
p value vs. control	—	0.77	0.24
p value vs. RV	—	—	0.26
IL-17C (pg/mL, mean ± SE)	122.0 ± 31.3	125.0 ± 51.9	124.2 ± 70.9
p value vs. control	—	0.56	0.70
p value vs. RV	—	—	0.31
IL-25 (pg/mL, mean ± SE)	89.0 ± 0.0	90.5 ± 8.0	90.4 ± 3.5
p value vs. control	—	0.86	0.74
p value vs. RV	—	—	0.99
TSLP (pg/mL, mean ± SE)	1.5 ± 0.2	1.7 ± 0.2	1.4 ± 0.1
p value vs. control	—	0.51	0.83
p value vs. RV	—	—	0.27
TARC (pg/mL, mean ± SE)	54.5 ± 1.8	56.5 ± 3.7	57.5 ± 3.0
p value vs. control	—	0.82	0.49
p value vs. RV	—	—	0.64
Eotaxin (pg/mL, mean ± SE)	22.3 ± 2.1	25.4 ± 4.6	24.9 ± 2.3
p value vs. control	—	0.64	0.49
p value vs. RV	—	—	1.0

RANTES data is not presented as it was below the level of detection in seven of nine samples.
HNE, human nasal epithelial; RV, Rhinovirus; SE, standard error; TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; TSLP, thymic stromal lymphopoietin; TARC, thymus and activation-regulated chemokine; RANTES, regulated on activation normal T cell expressed and secreted.

these effects. The levels of RANTES were below the level of detection in seven of the nine samples.

Effects of Kakkonto on NF-κB

To investigate the mechanism underlying kakkonto-induced cytokine production, we quantified the nuclear levels of NF-κB subunits (p65 and p50) in HNE cells. Compared to the untreated control, Kakkonto treatment (20 μg/ml) did not impact the levels of p65 (**Figure 7A**) but significantly reduced the levels of p50 (**Figure 7B**) 24 h.p.i. ($p < 0.05$).

DISCUSSION

In the present study, we investigated the effect of kakkonto extract on RV infection in primary cultures of HNE cells. Kakkonto treatment did not reduce the RV14 titers or RNA levels, however, it did significantly reduce the number, and fluorescence intensity of, acidic endosomes, through which RV RNA enters the cytoplasm of epithelial cells (Perez and Carrasco 1993; Casasnovas and Springer 1994). These results indicate that the effect of kakkonto on the modulation of endosomal pH might not be sufficient for inhibiting RV14 replication.

RV infection induces the production of various cytokines, including IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and MCP-1 (Message and Johnston, 2004; Jacobs et al., 2013; Schuler et al., 2014). These pro-inflammatory cytokines and chemokines play important roles in the subsequent innate and adaptive immune responses. Despite their beneficial effects on viral clearance from the respiratory tract, the generation of pro-inflammatory mediators and the recruitment of inflammatory cells results in immunopathological changes in the in airway and may exacerbate airway inflammation (Message and Johnston, 2004). In the current study we observed that kakkonto treatment decreased production of certain cytokines, namely, IL-8, TNFα, and MCP-1 at 24 h. p.i., whereas it did not significantly reduce the enhanced IL-6 level post-infection. Hence, Kakkonto extract may exert an inhibitory effect on airway inflammation induced by RV infection.

IL-8 plays an important role in neutrophil migration to sites of inflammation. In fact, IL-8 concentration in sputum is reportedly related to the severity of COPD and BA, as well as accelerated reduction in forced expiratory volume (Shannon et al., 2008; Dong et al., 2020; Marc-Malovrh et al., 2020). Meanwhile, IL-8 is not only associated with neutrophilic, but also eosinophilic inflammation, as IL-8-stimulated neutrophils lead to accumulation of eosinophils (Kikuchi et al., 2006; Nakagome and Nagata, 2018). IL-8 level was markedly reduced when HNE cells were treated with kakkonto extract. Thus, considering that the concentration of extracellular IL-8 was markedly reduced

TABLE 4 | Cytokine concentration in the supernatants of HNE cells compared to control samples 24 h after RV infection.

Cytokines and chemokines	Control (*n* = 3)	RV (*n* = 3)	RV + kakkonto (*n* = 3)
TNF-α (means ± SE)	1	1.11 ± 0.12	0.69 ± 0.07
p value vs. control	–	0.38	0.051
p value vs. RV	–	–	0.036
IL-1β (means ± SE)	1	1.73 ± 0.77	1.22 ± 0.34
p value vs. control	–	0.44	0.57
p value vs. RV	–	–	0.58
GM-CSF (means ± SE)	1	1.31 ± 0.23	0.72 ± 0.05
p value vs. control	–	0.30	0.028
p value vs. RV	–	–	0.12
MCP-1 (means ± SE)	1	1.14 ± 0.18	0.56 ± 0.04
p value vs. control	–	0.47	0.009
p value vs. RV	–	–	0.033
IL-17C (means ± SE)	1	0.96 ± 0.20	0.93 ± 0.35
p value vs. control	–	0.49	0.49
p value vs. RV	–	–	0.51
IL-25 (means ± SE)	1	1.02 ± 0.09	1.01 ± 0.04
p value vs. control	–	0.86	0.74
p value vs. RV	–	–	0.99
TSLP (means ± SE)	1	1.13 ± 0.02	0.95 ± 0.07
p value vs. control	–	0.027	0.52
p value vs. RV	–	–	0.067
TARC (means ± SE)	1	1.04 ± 0.07	1.06 ± 0.06
p value vs. control	–	0.62	0.41
p value vs. RV	–	–	0.84
Eotaxin (means ± SE)	1	1.16 ± 0.21	1.16 ± 0.23
p value vs. control	–	0.53	0.56
p value vs. RV	–	–	1.00

Data are expressed as the ratio of cytokine concentration in the supernatants of RV-infected HNE cells to that in uninfected HNE cells (control).
HNE, human nasal epithelial; RV, Rhinovirus; SE, standard error; TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; TSLP, thymic stromal lymphopoietin; TARC, thymus and activation-regulated chemokine; RANTES, regulated on activation normal T cell expressed and secreted.

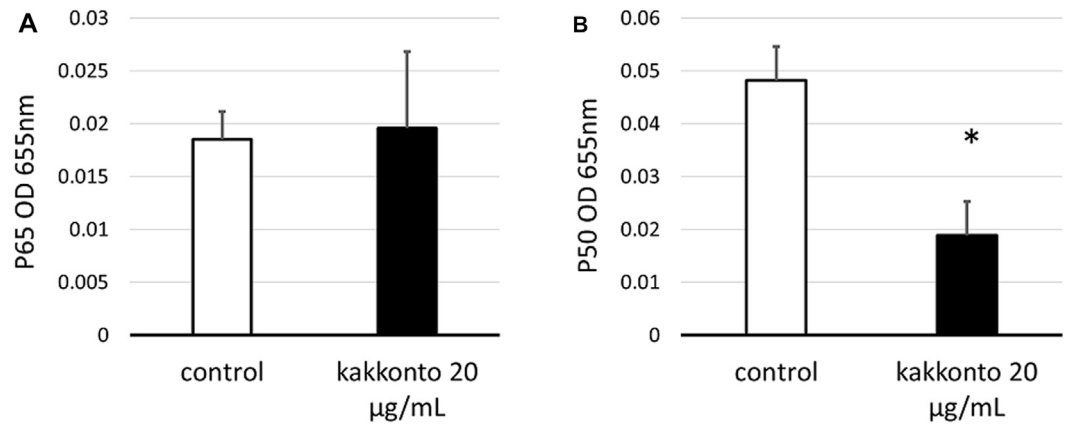

FIGURE 7 | Amount of p65 **(A)** and p50 **(B)** in the nuclear extracts of cells treated with or without 20 μg/ml kakkonto extract. *p < 0.05 significant difference compared to control cells. Results are expressed as optical density and presented as mean ± SE from five HNE cultures.

following treatment of RV-infected HNE cells with kakkonto extract, kakkonto may alleviate both neutrophilic and eosinophilic inflammation in RV-infected airways.

RV14, the virus employed in the current study, represents a major-group RV. This virus uses intercellular adhesion molecule-1 as a cell surface receptor for entry. After adhesion, viral antigen recognition occurs via pattern recognition receptors in airway epithelial cells. Toll-like receptor (TLR) 2, on the cell surface, recognizes viral capsid proteins. After entering endosomes, TLR 3 and TLR 7/8 bind to double-and single-stranded RNA, respectively. Interactions with these TLRs trigger activation of the NF-κB pathway and increase production of interferon (IFN)-β, IFN-γ, IL-6, and IL-8 (Jacobs et al., 2013; Makris and Johnston, 2018; Ganjian et al., 2020). Geng et al. reported that kakkonto treatment decreases the mRNA and protein levels of TLR7 and myeloid differentiation primary response 88 in murine lung tissues infected with H1N1 influenza

virus, indicating that kakkonto exerts an inhibitory effect on activation of the NF-κB pathway (Geng et al., 2019). Hence, kakkonto treatment, in the current study, was predicted to decrease the abundance of NF-κB subunits, considering the reduction in IL-8 and TNF-α levels in the supernatants of HNE cells. However, although significant reduction was observed in the number of p50 subunits, no effect was observed on the expression of p65. Additionally, levels of IL-6, MCP-1, and IL-1β were slightly reduced following treatment of RV-infected cells with kakkonto. However, considering that the patients providing HNE cells in this study were treated with inhaled corticosteroids (22.7%), nasal steroids (45.5%), L-carbocisteine (45.5%), ambroxol hydrochloride (9.1%), long-acting β2 agonists (22.7%), or macrolides (63.6%), the use of these drugs may have influenced the RV titer or cytokine levels, as well as the abundance of NF-κB. Indeed, these drugs were previously reported to exert antiviral and anti-inflammatory effects against RV infection (Suzuki et al., 2002; Yamaya et al., 2011; Yamaya et al., 2014; Yamaya et al., 2016). In particular, the high rate of treatment using nasal steroids may affect the levels of cytokines associated with eosinophilic inflammation, such as IL-25, TALC, and eotaxin, which were not affected by RV infection or kakkonto treatment. In contrast, the results of the present study reflected the clinical response of patients with chronic sinusitis or BA infected with RV. Hence, kakkonto may be useful in inhibiting further inflammation when these patients contract common cold.

Kakkonto is composed of seven crude drugs obtained from *Puerariae Radix, Ephedrae Herba, Zizyphi Fructus*, *Cinnamomi Cortex, Paeoniae Radix, Glycyrrhizae Radix*, and *Zingiberis Rhizoma*. The antiviral and anti-inflammatory effects of each crude drug have been investigated to some extent. For example, Chang et al. reported that ginger (*Zingiber officinale*) and *Paeonia lactiflora* inhibit human respiratory syncytial virus-induced plaque formation in the airway epithelium by blocking viral attachment and internalization (Chang et al., 2013; Lin et al., 2013). Nomura et al. showed that influenza viral release is inhibited by *Glycyrrhizae Radix* extract (Nomura et al., 2019). Meanwhile, *Cinnamomum* possesses antiviral, antibacterial, antioxidant, and anti-inflammatory effects (Kumar et al., 2019). Similarly, glycyrrhizin, the major component of *Glycyrrhizae Radix*, has been reported to exert anti-inflammatory and antiviral effects on respiratory viruses, hepatitis viruses, and human immunodeficiency virus (Fiore et al., 2008; Sun et al., 2019). Moreover, puerarin, one of the components of *Pueraria Radix*, may have an inhibitory effect on oxidative stress and apoptosis (Wei et al., 2014). We previously reported that glycyrrhizin reduces RV release in the supernatants of human tracheal epithelial cells (Yamaya et al., 2007). Meanwhile, in the current study, although kakkonto extract decreased the concentration of IL-8 in the supernatants of RV-infected HNE cells, none of the crude drugs present in kakkonto significantly impacted IL-8 levels. These results suggest that the inhibitory effects of kakkonto extracts on IL-8 production may be due to the overall effect of its constituent crude drugs. However, the antiviral and anti-inflammatory effects of these crude drugs, as well as their chemical components, and synergistic action, have not been sufficiently elucidated and require further investigation to confirm their efficacy.

Certain limitations were noted in this study. First, we did not investigate the effects of each chemical component of kakkonto (e.g., ephedrine, puerarin, glycyrrhizin, cinnamaldehyde, and paeoniflorin). Meanwhile, Wang et al. reported that puerarin inhibits the expression of TNF-α, IL-6, and IL-1β in LPS-induced murine lung tissue and reduces IL-8 production in A549 cells (Wang et al., 2018). Glycyrrhizin also inhibits IL-8 production and NF-κB activity in lung epithelial cells (Takei et al., 2008). Considering none of the crude drugs significantly decreased the IL-8 levels after RV infection, we speculated that these chemical components may also exert cumulative effects on inflammation or immunoreaction after RV infection. Second, we used 10–20 μg/ml kakkonto, as serum ephedrine and pseudoephedrine concentrations may be 40–60 ng/ml after administration of the kakkonto extract; whereas most previous *in vitro* studies used > 30–300 μg/ml kakkonto (Chang et al., 2012; Kitamura et al., 2014; Geng et al., 2019). Hence, more significant results may have been attained had we employed higher concentrations of kakkonto. Nevertheless, along with the primary culture of HNE cells, the concentrations adopted in this study are likely more reflective of the true physiological conditions of the human body. Finally, the mechanisms of the anti-inflammatory effect of kakkonto were not sufficiently investigated. A previous study indicated that production of IL-8 is also associated with NF-κB p50 activation (Das et al., 2012). Kakkonto might contribute to the inhibition of cytokine production by reducing NF-κB activation, although why it did not inhibit NF-κB p65 activation is unclear. There is a need to evaluate the expression levels of proteins relevant to the signaling pathway of NF-κB, such as p-p65, inhibitor-of-kappa-B proteins (IκB), or IκB kinase. However, the measurements of these proteins have been performed by using murine lung tissue or human cell lines thus far (Zhou et al., 2017; Geng et al., 2019; Ma et al., 2021). Further examination using primary cultures of HNE cells is needed to analyze these factors.

The severity of viral infection reflects the regulation of virus proliferation and the extent of virus-associated inflammation (Lauder et al., 2013). Until now, more than 160 RV serotypes have been identified (Basnet et al., 2019). Owing to little cross-neutralization among serotypes, no RV vaccine has yet been established, and no antiviral therapeutics for treating RV infections are available (Jacobs et al., 2013). Here, we elucidated that kakkonto may exert anti-inflammatory and immune-modulatory effects on RV-infected cells, indicating that kakkonto may be used as a treatment strategy for RV infection.

The present study revealed that kakkonto may exert an anti-inflammatory effect following RV infection via suppression of pro-inflammatory cytokine production. Specifically, kakkonto may reduce inflammation induced by RV infection, thereby reducing chronic airway inflammation. To our knowledge, this is the first study to elucidate the effects of kakkonto extract on RV infection in primary cultures of HNE cells. However, further investigation is needed to elucidate the detailed mechanism of the anti-inflammatory effects and clinical efficacy of kakkonto in chronic respiratory diseases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Tohoku University Ethics Committee (Institutional Review Board number: 2021-1-088). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NS and AK developed the study design, performed the experiments, analyzed the data, and wrote the manuscript. MY provided advice and assistance for development of the study design and experiments and writing of the manuscript. XD performed the experiments and conducted data analysis. MS recruited participants and obtained human nasal polyps and participants' data. ST, RN, and TI provided advice interpreting the data and revised the draft manuscript. All authors read and approved the final manuscript.

FUNDING

This study was funded by the Japan Society for the Promotion of Science KAKENHI (Grants-in-Aid for Scientific Research), grant number 17K09288. This study also funded by a research grant from Tsumura and Co., Tokyo, Japan. Tsumura and Co. had no role in the design, analysis, interpretation, and writing the manuscript for this study.

ACKNOWLEDGMENTS

We thank the staff at the Biomedical Research Unit of Tohoku University Hospital for providing technical support. We would also like to thank Editage (www.editage.com) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.687818/full#supplementary-material

REFERENCES

Arita, R., Ono, R., Saito, N., Takayama, S., Namiki, T., Ito, T., et al. (2020). Kakkonto, Shosaikoto, Platycodon Grandiflorum Root, and gypsum (A Japanese Original Combination Drug Known as Saikatsugekito): Pharmacological Review of its Activity against Viral Infections and Respiratory Inflammatory Conditions and a Discussion of its Applications to COVID-19. *Traditional Kampo Med.* 7, 115–127. doi:10.1002/tkm2.1258

Basnet, S., Palmenberg, A. C., and Gern, J. E. (2019). Rhinoviruses and Their Receptors. *Chest* 155, 1018–1025. doi:10.1016/j.chest.2018.12.012

Casasnovas, J. M., and Springer, T. A. (1994). Pathway of Rhinovirus Disruption by Soluble Intercellular Adhesion Molecule 1 (ICAM-1): an Intermediate in Which ICAM-1 Is Bound and RNA Is Released. *J. Virol.* 68, 5882–5889. doi:10.1128/JVI.68.9.5882-5889.1994

Chang, J. S., Wang, K. C., Shieh, D. E., Hsu, F. F., and Chiang, L. C. (2012). Ge-Gen-Tang Has Anti-viral Activity against Human Respiratory Syncytial Virus in Human Respiratory Tract Cell Lines. *J. Ethnopharmacol.* 139, 305–310. doi:10.1016/j.jep.2011.11.018

Chang, J. S., Wang, K. C., Yeh, C. F., Shieh, D. E., and Chiang, L. C. (2013). Fresh Ginger (*Zingiber Officinale*) Has Anti-viral Activity against Human Respiratory Syncytial Virus in Human Respiratory Tract Cell Lines. *J. Ethnopharmacol.* 145, 146–151. doi:10.1016/j.jep.2012.10.043

Das, T., Mukherjee, S., and Chaudhuri, K. (2012). Effect of Quercetin on *Vibrio cholerae* Induced Nuclear Factor-Kb Activation and Interleukin-8 Expression in Intestinal Epithelial Cells. *Microbes Infect.* 14, 690–695. doi:10.1016/j.micinf.2012.02.007

Dong, T., Santos, S., Yang, Z., Yang, S., and Kirkhus, N. E. (2020). Sputum and Salivary Protein Biomarkers and point-of-care Biosensors for the Management of COPD. *Analyst* 145, 1583–1604. doi:10.1039/c9an01704f

DPPN (2011). Department of Pharmacognosy, Phytochemistry and Narcotics (DPPN), National Institute of Health Sciences (NIHS) of Japan and National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN). Available from: http://mpdb.nibiohn.go.jp/stork/) providing package inserts of kakkonto: https://www.tsumura.co.jp/english/products/pi/JPR_T001.pdf (Accessed February 1, 2021).

Fiore, C., Eisenhut, M., Krausse, R., Ragazzi, E., Pellati, D., Armanini, D., et al. (2008). Antiviral Effects of *Glycyrrhiza* Species. *Phytother. Res.* 22, 141–148. doi:10.1002/ptr.2295

Ganjian, H., Rajput, C., Elzoheiry, M., and Sajjan, U. (2020). Rhinovirus and Innate Immune Function of Airway Epithelium. *Front. Cel. Infect. Microbiol.* 10, 277. doi:10.3389/fcimb.2020.00277

Geng, Z. K., Li, Y. Q., Cui, Q. H., Du, R. K., and Tian, J. Z. (2019). Exploration of the Mechanisms of Ge Gen Decoction against Influenza A Virus Infection. *Chin. J. Nat. Med.* 17, 650–662. doi:10.1016/S1875-5364(19)30079-2

Heikkinen, T., and Järvinen, A. (2003). The Common Cold. *Lancet* 361, 51–59. doi:10.1016/S0140-6736(03)12162-9

Inotsume, N., Fukushima, S., Hayakawa, T., Kishimoto, S., Yanaguimoto, H., Toda, T., et al. (2009). Pharmacokinetics of Ephedrine and Pseudoephedrine after Oral Administration of Kakkonto to Healthy Male Volunteers. *Jpn. J. Clin. Pharmacol. Ther.* 40, 79–83. doi:10.3999/jscpt.40.79

Jacobs, S. E., Lamson, D. M., St George, K. K., and Walsh, T. J. (2013). Human Rhinoviruses. *Clin. Microbiol. Rev.* 26, 135–162. doi:10.1128/CMR.00077-12

Kikuchi, I., Kikuchi, S., Kobayashi, T., Hagiwara, K., Sakamoto, Y., Kanazawa, M., et al. (2006). Eosinophil Trans-basement Membrane Migration Induced by Interleukin-8 and Neutrophils. *Am. J. Respir. Cel. Mol. Biol.* 34, 760–765. doi:10.1165/rcmb.2005-0303OC

Kitamura, H., Urano, H., and Ara, T. (2014). Preventive Effects of a Kampo Medicine, Kakkonto, on Inflammatory Responses via the Suppression of Extracellular Signal-Regulated Kinase Phosphorylation in Lipopolysaccharide-Treated Human Gingival Fibroblasts. *ISRN Pharmacol.* 2014, 784019. doi:10.1155/2014/784019

Kumar, S., Kumari, R., and Mishra, S. (2019). Pharmacological Properties and Their Medicinal Uses of Cinnamomum: a Review. *J. Pharm. Pharmacol.* 71, 1735–1761. doi:10.1111/jphp.13173

Kurokawa, M., Tsurita, M., Brown, J., Fukuda, Y., and Shiraki, K. (2002). Effect of Interleukin-12 Level Augmented by Kakkon-To, a Herbal Medicine, on the Early Stage of Influenza Infection in Mice. *Antivir. Res.* 56, 183–188. doi:10.1016/s0166-3542(02)00104-3

Lauder, S. N., Jones, E., Smart, K., Bloom, A., Williams, A. S., Hindley, J. P., et al. (2013). Interleukin-6 Limits Influenza-Induced Inflammation and Protects against Fatal Lung Pathology. *Eur. J. Immunol.* 43, 2613–2625. doi:10.1002/eji.201243018

Lin, T. J., Wang, K. C., Lin, C. C., Chiang, L. C., and Chang, J. S. (2013). Anti-viral Activity of Water Extract of *Paeonia Lactiflora Pallas* against Human Respiratory Syncytial Virus in Human Respiratory Tract Cell Lines. *Am. J. Chin. Med.* 41, 585–599. doi:10.1142/S0192415X13500419

Lusamba Kalonji, N., Nomura, K., Kawase, T., Ota, C., Kubo, H., Sato, T., et al. (2015). The Non-antibiotic Macrolide EM900 Inhibits Rhinovirus Infection and Cytokine Production in Human Airway Epithelial Cells. *Physiol. Rep.* 3, e12557. doi:10.14814/phy2.12557

Ma, Q. H., Ren, M. Y., and Luo, J. B. (2021). San Wu Huangqin Decoction Regulates Inflammation and Immune Dysfunction Induced by Influenza Virus

by Regulating the NF-Kb Signaling Pathway in H1N1-Infected Mice. *J. Ethnopharmacol.* 264, 112800. doi:10.1016/j.jep.2020.112800

Makris, S., and Johnston, S. (2018). Recent Advances in Understanding Rhinovirus Immunity. *F1000Res.* 7, F1000. Faculty Rev-1537. doi:10.12688/f1000research.15337.1

Marc-Malovrh, M., Camlek, L., Škrgat, S., Kern, I., Fležar, M., Dežman, M., et al. (2020). Elevated Eosinophils, IL5 and IL8 in Induced Sputum in Asthma Patients with Accelerated FEV1 Decline. *Respir. Med.* 162, 105875. doi:10.1016/j.rmed.2020.105875

Message, S. D., and Johnston, S. L. (2004). Host Defense Function of the Airway Epithelium in Health and Disease: Clinical Background. *J. Leukoc. Biol.* 75, 5–17. doi:10.1189/jlb.0703315

Nakagome, K., and Nagata, M. (2018). Involvement and Possible Role of Eosinophils in Asthma Exacerbation. *Front. Immunol.* 9, 2220. doi:10.3389/fimmu.2018.02220

Nomura, T., Fukushi, M., Oda, K., Higashiura, A., Irie, T., and Sakaguchi, T. (2019). Effects of Traditional Kampo Drugs and Their Constituent Crude Drugs on Influenza Virus Replication *In Vitro*: Suppression of Viral Protein Synthesis by Glycyrrhizae Radix. *Evid. Based Complement. Alternat. Med.* 2019, 3230906. doi:10.1155/2019/3230906

Numazaki, Y., Oshima, T., Ohmi, A., Tanaka, A., Oizumi, Y., Komatsu, S., et al. (1987). A Microplate Method for Isolation of Viruses from Infants and Children with Acute Respiratory Infections. *Microbiol. Immunol.* 31, 1085–1095. doi:10.1111/j.1348-0421.1987.tb01340.x

Pérez, L., and Carrasco, L. (1993). Entry of Poliovirus into Cells Does Not Require a Low-pH Step. *J. Virol.* 67, 4543–4548. doi:10.1128/JVI.67.8.4543-4548.1993

Schuler, B. A., Schreiber, M. T., Li, L., Mokry, M., Kingdon, M. L., Raugi, D. N., et al. (2014). Major and Minor Group Rhinoviruses Elicit Differential Signaling and Cytokine Responses as a Function of Receptor-Mediated Signal Transduction. *PLoS One* 9, e93897. doi:10.1371/journal.pone.0093897

Shannon, J., Ernst, P., Yamauchi, Y., Olivenstein, R., Lemiere, C., Foley, S., et al. (2008). Differences in Airway Cytokine Profile in Severe Asthma Compared to Moderate Asthma. *Chest* 133, 420–426. doi:10.1378/chest.07-1881

Shinozuka, N., Tatsumi, K., Nakamura, A., Terada, J., and Kuriyama, T. (2007). The Traditional Herbal Medicine Hochuekkito Improves Systemic Inflammation in Patients with Chronic Obstructive Pulmonary Disease. *J. Am. Geriatr. Soc.* 55, 313–314. doi:10.1111/j.1532-5415.2007.01057.x

Shirayama, R., Shoji, M., Sriwilaijaroen, N., Hiramatsu, H., Suzuki, Y., and Kuzuhara, T. (2016). Inhibition of PA Endonuclease Activity of Influenza Virus RNA Polymerase by Kampo Medicines. *Drug Discov. Ther.* 10, 109–113. doi:10.5582/ddt.2016.01010

Sun, Z. G., Zhao, T. T., Lu, N., Yang, Y. A., and Zhu, H. L. (2019). Research Progress of Glycyrrhizic Acid on Antiviral Activity. *Mini Rev. Med. Chem.* 19, 826–832. doi:10.2174/1389557519666190119111125

Suzuki, T., Yamaya, M., Sekizawa, K., Hosoda, M., Yamada, N., Ishizuka, S., et al. (2001). Bafilomycin A(1) Inhibits Rhinovirus Infection in Human Airway Epithelium: Effects on Endosome and ICAM-1. *Am. J. Physiol. Lung Cel. Mol. Physiol.* 280, L1115–L1127. doi:10.1152/ajprenal.2001.280.6.F1115

Suzuki, T., Yamaya, M., Sekizawa, K., Hosoda, M., Yamada, N., Ishizuka, S., et al. (2002). Erythromycin Inhibits Rhinovirus Infection in Cultured Human Tracheal Epithelial Cells. *Am. J. Respir. Crit. Care Med.* 165, 1113–1118. doi:10.1164/ajrccm.165.8.2103094

Takayama, S., Kikuchi, A., Makino, T., Kainuma, M., Namiki, T., and Ito, T. (2021). Basic Pharmacological Mechanisms and Clinical Evidence of the Efficacy of Hochuekkito against Infectious Diseases and its Potential for Use against COVID-19. *Traditional Kampo Med.* 8, 3–21. doi:10.1002/tkm2.1264

Takei, H., Baba, Y., Hisatsune, A., Katsuki, H., Miyata, T., Yokomizo, K., et al. (2008). Glycyrrhizin Inhibits Interleukin-8 Production and Nuclear Factor-kappaB Activity in Lung Epithelial Cells, but Not through Glucocorticoid Receptors. *J. Pharmacol. Sci.* 106, 460–468. doi:10.1254/jphs.fp0072378

Tatsumi, K., Shinozuka, N., Nakayama, K., Sekiya, N., Kuriyama, T., and Fukuchi, Y. (2009). Hochuekkito Improves Systemic Inflammation and Nutritional Status in Elderly Patients with Chronic Obstructive Pulmonary Disease. *J. Am. Geriatr. Soc.* 57, 169–170. doi:10.1111/j.1532-5415.2009.02034.x

The Ministry of Health, Labor and Welfare (2016). The Japanese Pharmacopoeia Seventeenth Edition. Available from: https://www.mhlw.go.jp/file/06-Seisakujouhou-11120000-Iyakushokuhinkyoku/JP17_REV.pdf (Accessed Feb 1, 2021).

Wang, X., Yan, J., Xu, X., Duan, C., Xie, Z., Su, Z., et al. (2018). Puerarin Prevents LPS-Induced Acute Lung Injury via Inhibiting Inflammatory Response. *Microb. Pathog.* 118, 170–176. doi:10.1016/j.micpath.2018.03.033

Wei, S. Y., Chen, Y., and Xu, X. Y. (2014). Progress on the Pharmacological Research of Puerarin: a Review. *Chin. J. Nat. Med.* 12, 407–414. doi:10.1016/S1875-5364(14)60064-9

Wu, M. S., Yen, H. R., Chang, C. W., Peng, T. Y., Hsieh, C. F., Chen, C. J., et al. (2011). Mechanism of Action of the Suppression of Influenza Virus Replication by Ko-Ken Tang through Inhibition of the Phosphatidylinositol 3-kinase/Akt Signaling Pathway and Viral RNP Nuclear export. *J. Ethnopharmacol.* 134, 614–623. doi:10.1016/j.jep.2011.01.005

Yafune, A., and Cyong, J. C. (2001). Population Pharmacokinetic Analysis of Ephedrine in Kampo Prescriptions: a Study in Healthy Volunteers and Clinical Use of the Pharmacokinetic Results. *Int. J. Clin. Pharmacol. Res.* 21, 95–102.

Yamaya, M., Nishimura, H., Hatachi, Y., Yoshida, M., Fujiwara, H., Asada, M., et al. (2011). Procaterol Inhibits Rhinovirus Infection in Primary Cultures of Human Tracheal Epithelial Cells. *Eur. J. Pharmacol.* 650, 431–444. doi:10.1016/j.ejphar.2010.09.056

Yamaya, M., Nishimura, H., Nadine, L., Kubo, H., and Nagatomi, R. (2014). Formoterol and Budesonide Inhibit Rhinovirus Infection and Cytokine Production in Primary Cultures of Human Tracheal Epithelial Cells. *Respir. Investig.* 52, 251–260. doi:10.1016/j.resinv.2014.03.004

Yamaya, M., Nomura, K., Arakawa, K., Nishimura, H., Lusamba Kalonji, N., Kubo, H., et al. (2016). Increased Rhinovirus Replication in Nasal Mucosa Cells in Allergic Subjects Is Associated with Increased ICAM-1 Levels and Endosomal Acidification and Is Inhibited by L-Carbocisteine. *Immun. Inflamm. Dis.* 4, 166–181. doi:10.1002/iid3.102

Yamaya, M., Sasaki, T., Yasuda, H., Inoue, D., Suzuki, T., Asada, M., et al. (2007). Hochu-ekki-to Inhibits Rhinovirus Infection in Human Tracheal Epithelial Cells. *Br. J. Pharmacol.* 150, 702–710. doi:10.1038/sj.bjp.0707135

Zhou, B., Yang, Z., Feng, Q., Liang, X., Li, J., Zanin, M., et al. (2017). Aurantiamide Acetate from Baphicacanthus Cusia Root Exhibits Anti-inflammatory and Anti-viral Effects via Inhibition of the NF-Kb Signaling Pathway in Influenza A Virus-Infected Cells. *J. Ethnopharmacol.* 199, 60–67. doi:10.1016/j.jep.2017.01.038

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

frontiers in Pharmacology

REVIEW
published: 25 October 2021
doi: 10.3389/fphar.2021.705077

Tradition to Pathogenesis: A Novel Hypothesis for Elucidating the Pathogenesis of Diseases Based on the Traditional Use of Medicinal Plants

Kenny Kuchta[1]* *and Silke Cameron*[2,3]*

[1]Forschungsstelle für Fernöstliche Medizin, Department of Vegetation Analysis and Phytodiversity, Albrecht von Haller Institute of Plant Sciences, Georg August University, Göttingen, Germany, [2]Clinic for Gastroenterology and Gastrointestinal Oncology, University Medicine Göttingen, Göttingen, Germany, [3]Clinic, Hann. Münden, Germany

Traditional medicines embody knowledge on medicinal plants that has been accumulated through cultural evolution over millennia. In the latter half of the 20th century, two approaches to medicinal plant research have been established: the "Bench to Bedside" and the "Bedside to Bench" approaches which serve primarily for the development of more efficient therapeutics. Here, we propose a third, novel approach: from "Tradition to Pathogenesis" which aims to understand the pathogenesis of diseases based on the cultural evolution of their respective empirical treatments. We analyse multiple examples of diseases where the acting mechanism of traditional treatments across multiple cultures points to the pathogenesis of the respective disease. E.g., many cultures traditionally treat rheumatism with anti-bacterial botanical drugs, which is at odds with our current understanding that rheumatism is an aseptic inflammation. Furthermore, gastric ailments have traditionally been treated with anti-infectious botanical drugs indicating local infections, as demonstrated by the discovery of *Helicobacter pylori* as a common cause of gastric ulcer. Understanding traditional treatments can thus help to elucidate the pathogenesis of the disease.

Keywords: traditional medicine, pharmacology, pathogenesis, mechanism of action, Evolution, Cultural evolution

OPEN ACCESS

Edited by:
Michael Heinrich,
UCL School of Pharmacy,
United Kingdom

Reviewed by:
Abhay Prakash Mishra,
University of the Free State,
South Africa
Chi-Jung Tai,
Pingtung Hospital, Taiwan

***Correspondence:**
Kenny Kuchta
kenny.kuchta@med.uni-goettingen.de
Silke Cameron
silke.cameron@med.uni-goettingen.de

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

Received: *04 May 2021*
Accepted: *27 September 2021*
Published: *25 October 2021*

Citation:
Kuchta K and Cameron S (2021) Tradition to Pathogenesis: A Novel Hypothesis for Elucidating the Pathogenesis of Diseases Based on the Traditional Use of Medicinal Plants. Front. Pharmacol. 12:705077. doi: 10.3389/fphar.2021.705077

CO-EVOLUTION OF TRADITIONAL MEDICINE AND MEDICINAL PLANTS

Recent anthropological research data have led to the astounding conclusion that traditional herbal medicine has most probably a longer history than mankind: Apes have been observed to use medicinal plants for the treatment of diseases (Huffman, 2001). Moreover, human populations that settled in the same region of Africa use the same plants with very similar indications (Huffman, 2001). One example for this transfer of medicinal knowledge from animals to humans is *Vernonia amygdalina* Del. Chimpanzees (*Pan troglodytes*) have been observed on numerous occasions to chew on the bitter pith of this plant as self-medication in case of parasitic nematode infections (Huffman, 2001). Traditional healers of the WaTongwe people of the Mahale Mountains in Tanzania, where the use of *V. amygdalina* by Chimpanzees has also been observed, use this plant for intestinal parasites, diarrhoea, and stomach upset.

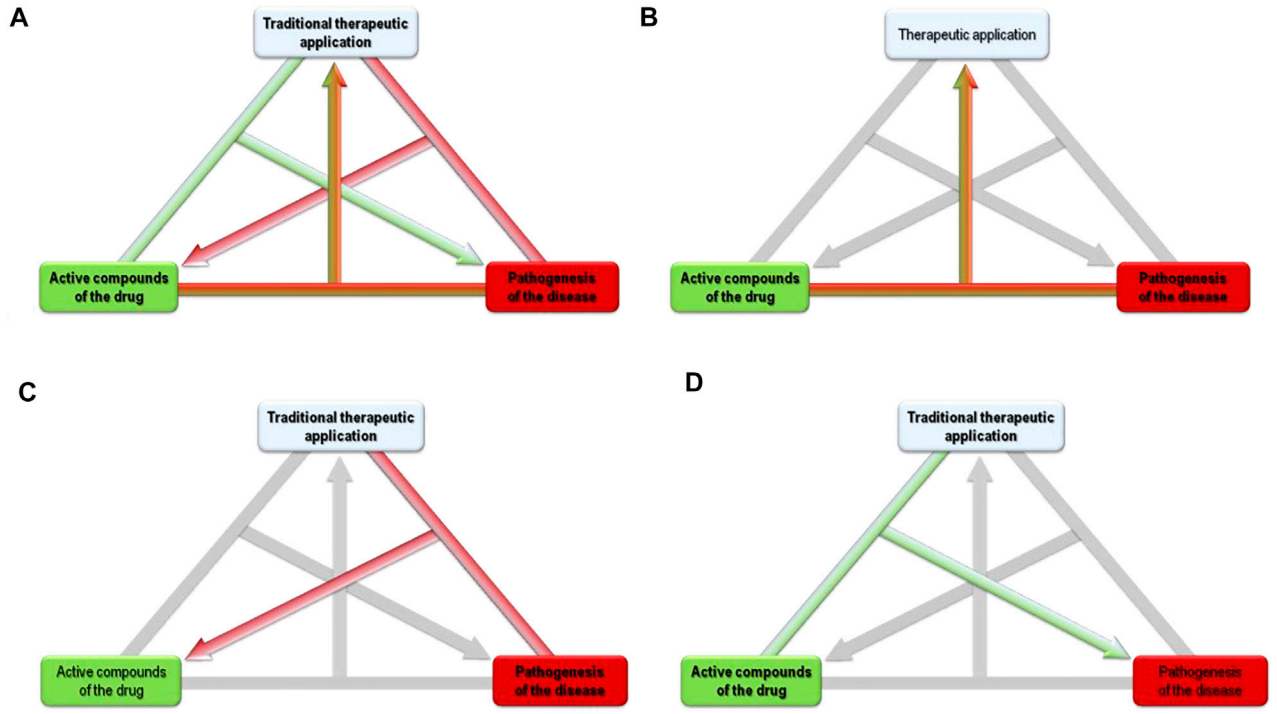

FIGURE 1 | (A) From "active compounds of the drug," to its "traditional therapeutic application," or "pathogenesis of the disease." **(B)** Bench to Bedside: Pathogenesis + Active compounds known → Therapeutic applications unknown. **(C)** Bedside to Bench: Pathogenesis + Therapeutic application known → Active compounds unknown. **(D)** Tradition to Pathogenesis: Active compounds + Therapeutic application known → Pathogenesis unknown.

Phytochemical research has demonstrated that sesquiterpene lactones in *V. amygdalina* possess anthelmintic, antiamoebic, antitumor, and antibiotic properties (Huffman, 2001).

We can thus propose a long-term co-evolution between man and his food and medicinal plants, resulting in the adaption of human pharmacology to the bioactive plant metabolites. The fact that already Neanderthals 50.000 years ago used yarrow (*Achillea millefolium*) and camomile (*Matricaria chamomilla*) - two plants still registered as medicinal plants in the European Pharmacopoeia - as well as poplar buds (*Populus spec.*) (Hardy et al., 2012; Weyrich et al., 2017) as medicine, demonstrates that contemporary phytotherapeutic practice goes back to the dawn of man. The accumulated body of knowledge (referred to as "tradition" or "culture") is transferrable from person to person as humans and their closest relatives are further able to learn successful behaviours. The process of the improvement and distribution of this knowledge can be referred to as "cultural evolution." The evolutionary pressure that drives this cultural evolution is the survival benefit for tribes with knowledge of effective treatments. Just as for the use of single herbs, their traditional combinations evolved over time. Various prescriptions include the same medicinal plants in different combinations as the individual effects i.e., anti-inflammatory, mucoprotective or microcirculation enhancing, add to the synergistic effect of the whole. This is referred to as multicomponent-multitargeted therapy. These considerations enable us to understand the pathological processes of diseases by analysing the commonalities in the pharmacological properties of traditional medicinal plant drugs used in multiple cultures to treat the disease. This approach constitutes a new possible use of pharmacognosy, a discipline that has for the past century been dominated by two approaches, the "bench to bedside" and the "bedside to bench" approach.

THE TWO ESTABLISHED HYPOTHESIS OF MEDICINAL RESEARCH

Both the "bench to bedside" and the "bedside to bench" approach are based on the application of the ideas of modern biomedicine to the medicinal plants and practices of traditional medicine. In the "bench to bedside" approach, pure chemical compounds are isolated from medicinal plants and tested by high throughput screening for their activity in various *in vitro* model systems as potential drugs.

The alternative "bedside to bench" approach uses modern biochemistry and pharmacology in order to characterize traditional medicinal plant preparations and their acting mechanisms, and to develop refined extracts. Based on this approach, bioassay guided fractionation can be applied in order to develop advanced herbal medicinal products with improved therapeutic activity (**Figure 1**).

TRADITION TO PATHOGENESIS: A THIRD, NOVEL HYPOTHESIS OF MEDICINAL RESEARCH

Here, we propose a third way of medicinal plant research: We propose that the traditional use of botanical drugs may clarify the pathogenesis of modern diseases. The known bioactivities of the plant constituents and their traditional application might thus help to understand the pathological processes of the treated disease. **Figure 1** intends to visualize that if two of the three items "traditional therapeutic application," "active compounds of the drug" and "pathogenesis of the disease" are known, the third can be researched based on the other two. E.g., in "bench to bedside," a compound and its pharmacology are known, the research aims to find a fitting therapeutic application. In "bedside to bench," the traditional therapeutic application and the pathophysiology of the treated disease are known, research aims to find the respective active components (of the plant extract). Our new proposal completes the logical triangle by using the known traditional therapeutic application of the plant - and its known compounds with known activities - for research that aims to find the pathophysiology of the treated disease. This approach can thus be called "Tradition to Pathogenesis" in line with the two previously established approaches. The knowledge of medicinal plants thus helps to understand the shared pathogenesis or association of different diseases and the association between traditional and modern biomedical based pathogenesis.

The idea of predicting the source of evolutionary pressure from an observed adaptation is not new. In 1862, Charles Darwin predicted that the 40 cm long nectary of the Madagascan orchid *Angraecum sesquipedale* Thouars indicated that there must be a pollinating insect with an equally long proboscis (Darwin, 1862). This was confirmed in 1903 when the sphinx moth *Xanthopan morganii praedicta* was discovered by W Rothschild and K Jordan. Here, we apply the same line of reasoning to the cultural evolution of medicine for the first time.

LEARNING FROM HISTORY

Helicobacter pylori as the Causative Agent of Gastric Ulcers

As one example, in the 1980s, it was found that the bacterium *Helicobacter pylori* can cause peptic ulcers - a discovery honoured with the 2005 Nobel Prize in medicine to JR Warren and BJ Marshall (Marshall et al., 1985). This result could have been anticipated as numerous systems of traditional medicine worldwide treat peptic ulcers with herbal drugs that exert pronounced anti-bacterial properties.

One amongst several such examples are the plant drugs from *Glycyrrhiza spec.* (*G. glabra* L. and *G. uralensis* Fisch.exDC.) that are used as remedies against peptic ulcers from the Atlantic to the Pacific, and the anti-bacterial activity of which is well documented in the literature (Verheijen, 1948). Recent experimental work has verified the effectiveness of *Glycyrrhiza spec.* extracts against *Helicobacter pylori* (Asha et al., 2013). Typical active constituents of *Glycyrrhiza spec.* are triterpene glycosides like saponins such as glycyrrhizic acid (**Figure 2**).

Another famous plant drug against gastric ulcers is the Mediterranean species *Cistus creticus* L. (including some other species of the same genus). In Cretan traditional medicine, small clumps of labdanum resin, which is collected from the leaves of the plant, are swallowed with Raki as a traditional treatment for gastric ulcer (oral communication, Nyktaris Dimitris, Crete). In Turkish and Italian traditional medicine, tee infusions of the flowers and leaves are used in the same indication and have been successfully tested in animal models (Attaguile et al., 1995; Yesilada et al., 1997). Direct activity of *Cistus spec.* extracts on *Helicobacter pylori* cultures *in vitro* have also been reported (Yesilada et al., 1999). However, in the ancient East Mediterranean, the plant found much wider uses as incense, anti-infective, and for wound treatment (Husemann, 1889; Zohary, 1983). Most recently, a strong activity of the volatile oil phase of the extract against *Borrelia burgdorferi in vitro*, could be demonstrated (Hutschenreuther et al., 2010; Kuchta et al., 2012; Rauwald et al., 2013). This volatile oil is mainly characterised by manoyloxides such as (manoyloxide, 3-acetoxy-manoyloxide, 3-hydroxy-manoyloxide-epimanoyloxide, 2-keto-manoyloxide), (**Figure 3**) (Kuchta et al., 2012).

In our previously published research (Rauwald et al., 2019), we were able to show that these manoyloxides are specific for labdanum from Cretan *C. creticus*. Besides monoterpenes, simple alkanes were dominant in Spanish labdanum - traditionally prepared by hot water extraction of the aerial parts of *Cistus ladanifer* L., whereas only traces of the major anti-bacterial and anti-viral manoyloxide constituents could be detected (Rauwald et al., 2019). This corresponds with the historical development of the use of labdanum in European traditional herbal medicine: After the Ottoman conquest of Crete in 1645, Western European doctors shifted from Cretan labdanum to Spanish labdanum for the treatment of infectious diseases. Shortly thereafter, labdanum largely fell out of pharmaceutical use in most areas outside of the natural range of *C. creticus* (Rauwald et al., 2019). Labdanum of *Cistus ladanifer* L. continues to be used for perfume.

Apart from marker compounds of medicinal plant drugs, we also have "markers" for judging treatment response. The aim of medical treatments has changed from "absence of symptoms" in traditional medicine to "absence of analytical marker compounds or organisms in the human body" today. Consequently, a direct comparison of antibacterial *in vitro* effects of plant extracts (based on historical documents) with that of mono-molecular antibiotics will often give confusing results. In the case of *H. pylori*, about one third of the world population test positive for its presence in the stomach, although only a small fraction of these will ever develop gastric ulcer. In the vast majority of cases, *H. pylori* remains present but inactive. As traditional healers had no means of detecting the bacteria in the human organism, they could only judge the success of their therapy based on the symptoms of their patients. Consequently, the restoration of this inactive, symptom free state was the adaptive peak in the cultural evolution of traditional medicine.

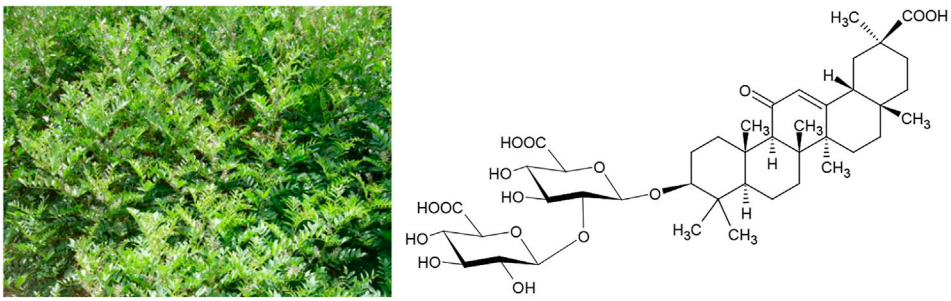

FIGURE 2 | *Glycyrrhiza uralensis* Fisch.exDC. and its active constituent glycyrrhizic acid.

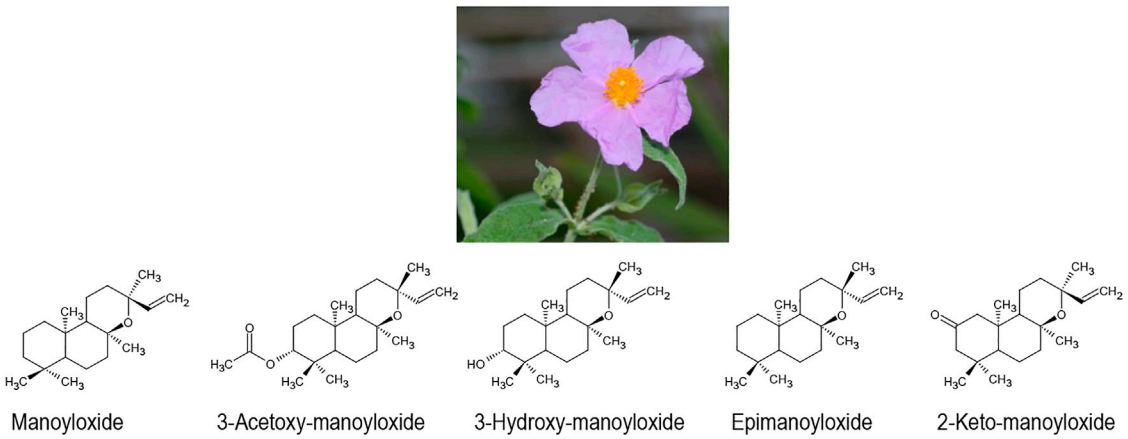

FIGURE 3 | *Cistus creticus* L. and its active manoyloxides.

Based on the above example of *H. pylori*, we have collected data on two other diseases where the apparent mechanism of action of traditional herbal medications across a multitude of cultures should help to clarify the underlying pathogenesis.

Rheumatoid Arthritis and Spirochaeta Infection

One of the most interesting and most consistent correlations between seemingly unrelated traditional indications of medicinal plants in numerous human cultures is the correlation between syphilis and rheumatoid arthritis.

For example, in Japanese Kampo medicine, the main indication for *Smilax china* L. is syphilis. The same is true for its most common formulation "Hachimitaigeho," which is further used against numerous infectious and inflammatory diseases of the female reproductive system (Otsuka et al., 2016). In addition, in the Chinese "Bencao Gangmu," the most extensive and famous compendium of classical Chinese drugs, rheumatoid arthritis is mentioned as a secondary indication (Luo, 2003). Its most significant active components are triterpenes like sarsasapogenin (**Figure 4**).

We find both indications in Western Herbal Medicine, where the Mesoamerican species of the same genus, mainly *Smilax aristolochiifolia* Mill. (syn. *S. medica* Schltdl.&Cham.) and *Smilax officinalis* Kunth are used. In the Eclectic Medicine Tradition of the United States but also in Europe, these species were used as the preferred herbal remedy for syphilis, especially in the chronic stage of the disease, and also highly recommended for the treatment of rheumatic affections (Felter and Lloyd, 1905; Pereira and Brown, 1855). It is interesting to note that the British pharmacognosist Jonathan Pereira, one of the most famous of his time, already theorised a hidden "venereal origin," i.e., syphilis infection, as a possible cause of rheumatism (Pereira and Brown, 1855) and Felter and Lloyd (Felter and Lloyd, 1905) also mention "gonorrhoeal rheumatism."

Another medicinal plant that was intensively used against syphilis is the so called "lignum vitae," the resin or alcoholic extract of the wood of the Caribbean trees *Guaiacum officinale* L. or *Guaiacum sanctum* L. After the epidemic spread of syphilis through Europe in the 15th century, these drugs were imported in large quantities and praised for their effectiveness in the treatment - or at least suppression - of the disease. One of the first patient narratives in the history of medicine were the treaties "De morbo Gallico" (1519) by the German knight and scholar

FIGURE 4 | *Smilax china* L. and its active constituent sarsasapogenin.

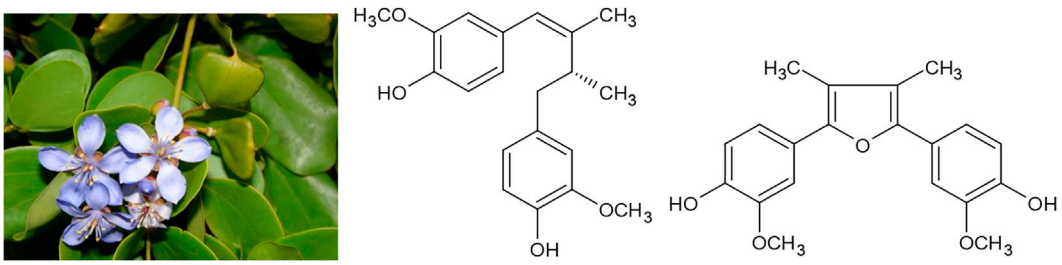

FIGURE 5 | *Guaiacum officinale* L. and its active constituents dehydroguaialignan and furoguajacin.

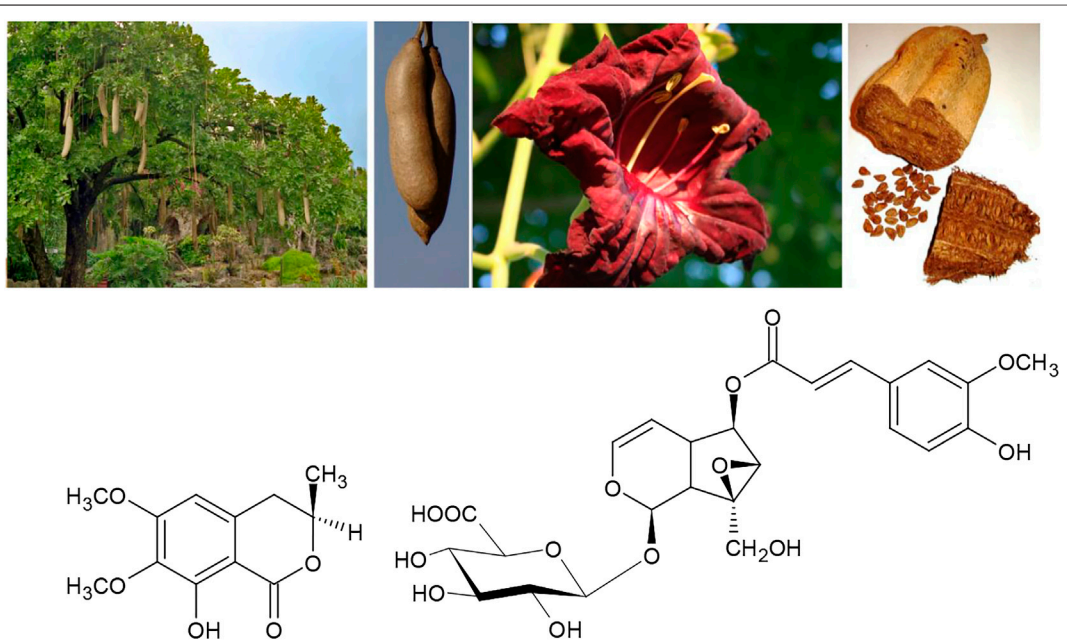

FIGURE 6 | *Kigelia africana* (Lam.)Benth. and its active constituents kigelin and minecoside.

Ulrich von Hutten, who suffered from syphilis himself and described his own treatment (von Hutten, 1533). Among the therapies von Hutten described, *Guaiacum* resin seems to have been the most effective. Soon, the same drug also found use against rheumatoid arthritis and even as late as 1907, the "British pharmaceutical codex" of the Pharmaceutical Society of Great Britain describes an alkaline solution of *Guaiacum* resin which "use is empirical in chronic rheumatism, rheumatoid arthritis, and syphilis" (Pharmaceutical Society of Great Britain and Council: University College, 1907). Major components of *Guaiacum* resin are lignans like dehydroguaialignan and furoguajacin (**Figure 5**).

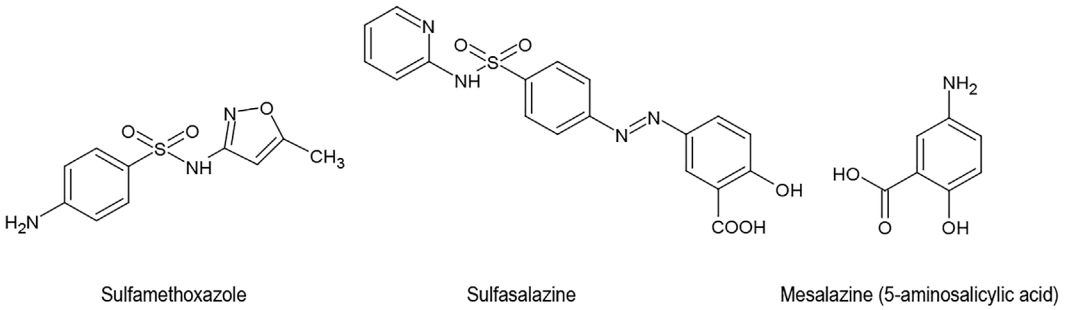

FIGURE 7 | Sulfamethoxazole Sulfasalazine Mesalazine (5-aminosalicylic acid).

Turning to African traditions, a considerable amount of medicinal knowledge has been documented during the last two centuries. Also in this tradition, an example of the dual use of the same medicinal plant against both, rheumatoid arthritis and syphilis features very prominent: "Both the unripe fruits and the bark of the sausage tree *Kigelia africana* (Lam.)Benth. are taken as a traditional remedy for syphilis and rheumatism" (Neuwinger, 1996; Orwa et al., 2009). Potentially active constituents are kigelin and minecoside (**Figure 6**). *K. africana* has also been shown to interfere with the response of bacteria to quorum sensing autoinducer compounds that inform the microbes about the density of their own population in several types of bacteria. This mechanism therefore facilitates the manipulation of bacterial growth speed making it a promising candidate for developing the ancestral knowledge of Traditional African medicine to a future in the context of integrative medicine (Kahumba et al., 2015).

Bacterial Translocation Through the "Leaky Gut" and the Pathogenesis of "Autoimmune Diseases"

The above described cultural evolution of therapeutic procedures does not only apply to the traditional use of medicinal plants but also to empirical experience in the use of modern medicine. In this context, interesting observations concerning the therapy of chronic diseases of the bone and joints in correlation with potential gastrointestinal infections have been reported.

Sulphonamides are a class of antibiotic agents that were developed in Germany during the 1930s. They suppress the enzyme dihydropteroate synthase and inhibit the incorporation of para-aminobenzoic acid into folic acid. The affinity of sulphonamides for the bacterial enzyme is about 10,000 times greater than its affinity for the corresponding mammalian enzyme. However, the sulphonamide sulfamethoxazole, which was introduced to the US in 1961 as a remedy for bacterial infections such as urinary tract infections and bronchitis, soon developed a secondary, purely empirical career in the therapy of alleged "autoimmune diseases" and especially osteoarthritis (Rozin, 2007). These observations are not limited to sulfamethoxazole. The related sulfasalazine is also capable of suppressing clinical symptoms and biochemical signs of rheumatoid arthritis (Neumann et al., 1983; Pullar et al., 1983). Sulfasalazine can further be used for joint-pain associated with inflammatory bowel disease. Its use has however declined because of side effects (Voulgari, 2011). Instead, 5-aminosalicylic acid (mesalazine) is used, which is devoid of the antibacterial sulphonamide group in the molecule (**Figure 7**).

These empirical developments of the clinical use of chemosynthetic agents - a short term "cultural evolution" if you like - have already repeatedly led to publications of theories proclaiming the bacterial origin of rheumatism (Rozin, 2007; Neumann, 1988). These theories could however not enter the clinical mainstream as bacterial products could not yet be detected in the liquid after joint puncture. In this context of inflammation and intractable bacterial infection, spontaneous bacterial peritonitis in liver cirrhosis is one of the rare cases where standard therapy consists of broad-spectrum antibiotics, even though no bacterium is found in the ascites. For diagnosis, abdominal pain and elevated leucocyte or neutrophil count within the ascites are sufficient. As for Crohn's disease, NOD2 variants are genetic risk factors for bacterial translocation (Appenrodt et al., 2010).

Bacterial translocation is also of highest interest in relation to the so called "leaky gut" syndrome, which is currently at the centre of the scientific discussion concerning the pathogenesis of diverse "autoimmune diseases." In pathologic conditions, the permeability of the gut epithelial lining can be compromised allowing the passage of toxins, antigens, and bacteria in the lumen to enter the blood stream creating a "leaky gut." Commensal bacteria from the gut lumen are able to escape from a "leaky gut" together with their products, inducing inflammation and even systemic tissue damages if translocated into peripheral circulation (Brenchley and Douek, 2012). The increased membrane permeability of the intestinal mucosal barrier appears to further correlate with a host of clinical disorders including: inflammatory and functional bowel disease, food allergies, allergic disorders, rheumatoid arthritis, celiac disease, and chronic dermatological conditions (Porras et al., 2006; Zhou et al., 2009).

All the above hints strongly to an antibacterial therapy regime as a promising treatment approach. Further treatment considerations should include modulation of the intestinal flora or mucosal protection, both of which are available in

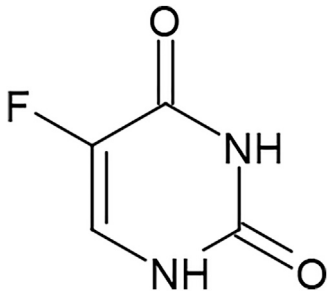

FIGURE 8 | Fluoruracil (5-FU).

herbal therapies such as *Glycyrrhiza glabra* (Asha et al., 2013), *Cistus* spec. (Attaguile et al., 1995; Yesilada et al., 1997), or the Japanese Kampo prescription Juzentaihoto (Otsuka et al., 2016).

The Cultural Evolution of "Off Label Uses" for Active Constituents

The observation already described by Darwin (Darwin, 1871) that evolutionary processes - replication, mutation, and selection of information - occur throughout all aspects of human culture poses the question whether the historical repurposing of a medicines - as proposed above i.e., for sulphonamides - can be compared with the development of traditional medicine through cultural evolution. This includes many cultural practices such as therapeutic applications and can - also in the case of synthetic drugs - lead to the development of "off label uses" *via* the described evolutionary processes. Their empirical use can evolve in the same way as for herbal remedies, a form of "short-term cultural evolution" that shall serve here as an introductory example: 5-FU is a chemotherapeutic agent that acts as a wrong base analogue based on its structural similarity with the pyrimidine base cytosine and thymidine (DNA) and uracil (RNA). It has been used as therapy for gastrointestinal cancers since the 60s (**Figure 8**). Whereas most cases show tumour regression upon 5-FU therapy, some cases react to this agent with - as of yet unexplained - complete tumour remission.

In order to understand these varying response rates, we might look at alternative uses for 5-FU, namely as a mutagenic agent for RNA viruses. The antiviral activity of 5-FU against the lymphocytic choriomeningitis virus was demonstrated in an animal model and has been interpreted to predict a potential efficacy for other arenaviruses, such as Lassa fever (Ruiz-Jarabo et al., 2003). As 5-FU boosts the rate of mutations *via* incorporation during viral RNA synthesis it is a prime candidate for research into approaches for anti-viral therapy based on the quasi-species model of RNA virus evolution. This effect has been experimentally demonstrated, e.g., for influenza viruses (Pauly and Lauring, 2015). As 5-FU is an anti-mitotic agent which also inhibits thymidylate synthase, it further prevents DNA synthesis. In this context it has been shown that for noncancerous manifestations of human papilloma virus (HPV), a group of DNA viruses of which certain strains cause common warts (verruca vulgaris), local application is effective (Kollipara et al., 2015).

The quasi-species model (Eigen, 1971; Eigen, 1996) proposes that it should be possible to cure viral infections by boosting the mutation rate of the viral genome above the selection rate of the surrounding evolutionary pressure. Above this error threshold (Biebricher and Eigen, 2005), nonsense mutants are generated faster than they can be selected against, resulting in a meltdown of the genetic information that is referred to as "error catastrophe" (Eigen, 2002).

The bench to bedside research on potential therapeutic agents that target the viral "error catastrophe" has not yet resulted in approved drugs for clinical use. However, newer research into human resistance mechanisms against HIV-AIDS indicate that the human immune system itself applies precisely this antiviral strategy in form of the APOBEC3G protein that initiates an increased mutation rate in the viral genome (Malim, 2009). This mechanism appears to be evolutionarily conserved and not just active against HIV but also hepatitis B virus, simple retroviruses, and even endogenous retroelements (Holmes et al., 2007).

Can we therefore assume, that those gastrointestinal tumours which quickly and completely recede under 5-FU might be caused by viral infection at the initial stages?

Could the viral "error catastrophe" or the APOBEC3G system be a potential target of traditional antiviral plant drugs with as of yet unknown mechanisms of action?

Similar reasoning also applies to numerous traditional medicinal plants and their use.

One of the most impressive examples is certainly *Artemisia annua* L. and its constituent artemisinine. Based on the traditional application of the fresh plant juice in the treatment of malaria as documented in the Zhou Hou Jiu Zu Fang (A Handbook of Formulas for Emergencies), written in 340 AD by the Chinese physician and Daoist philosopher Ge Hong, Tu You-you et al. (You-you, 1982a; You-you et al., 1982b) isolated the active sesquiterpene lactone artemisinine, that has since been marketed worldwide as a treatment for malaria (*Plasmodium falciparum*). This research was later honoured with the 2015 Nobel Prize in Physiology or Medicine. After their introduction however, artemisinine and its derivatives like artesunate have proven effective in a number of ailments seemingly unrelated to malaria. These "off label uses" include most prominently the use of *Artemisia annua* and artemisinin for cancer therapy (Efferth, 2017; Efferth, 2006), their activity against viral infections (Efferth et al., 2008) even including hepatitis C virus (HCV) infections (Efferth et al., 2008; Dai et al., 2016), and their ability to attenuate arthritis (Lin et al., 2016). The question, if and how these seemingly unrelated activities are connected and if e.g., viral or protozoal infections might play a role in the development of arthritis might be an interesting starting point for future research.

Galantamine, an alkaloid isolated from *Galanthus woronowii* Losinsk., has shown a similar broad therapeutic versatility. It was originally developed based on the local use in the treatment of poliomyelitis documented in an observational study in the Caucasus Mountains (Heinrich, 2010). In 1951, Mashkovsky and Kruglikova-Lvov (Mashkovsky and Kruglikova-Lvov, 1951) published the first work that establishes the acetyl-choline esterase inhibiting properties

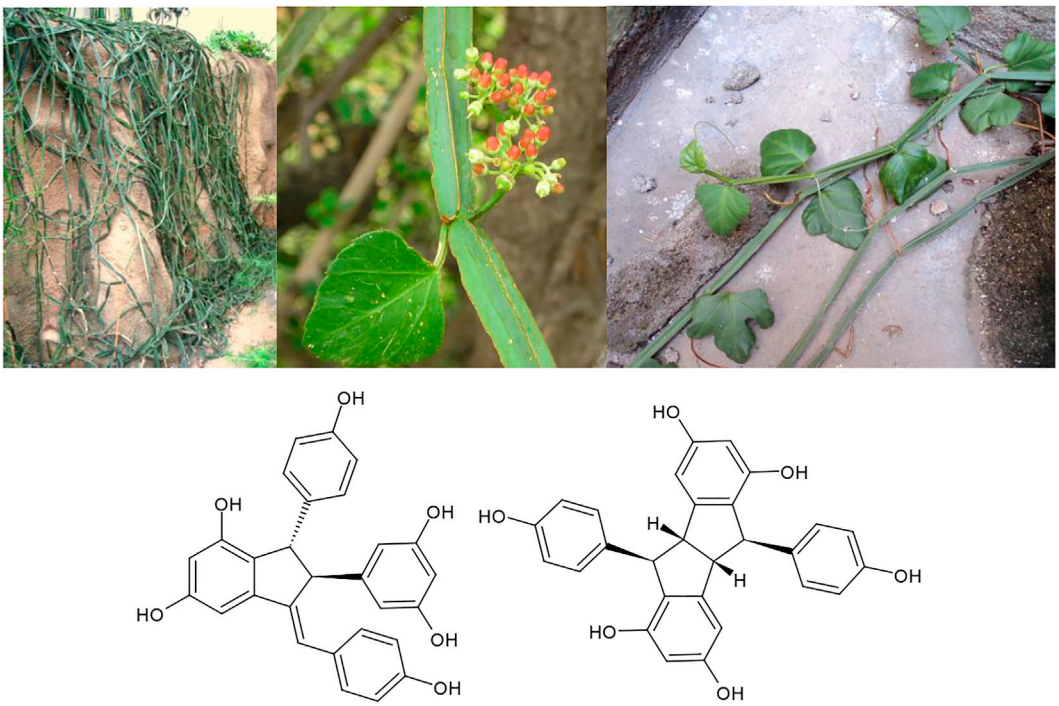

FIGURE 9 | *Cissus quadrangularis* L. and its active constituents quadrangularin A and pallidol.

of isolated galantamine. Its indication soon broadened to also include myasthenia gravis and muscular dystrophy, residual poliomyelitis paralysis symptoms, trigeminal neurologica, and other forms of neuritis. The scientific rationale for using cholinesterase inhibitors like galantamine in the management of Alzheimer's disease is based on the cholinergic hypothesis. Impairment of the central cholinergic system is typically observed in Alzheimer's patients and is accompanied by a loss of cholinergic neurons in the forebrain and a marked decrease in the activity of choline acetyltransferase. Overall, galantamine represents an example for the successful ethnobotanydriven development of a natural product into a clinically important drug (Heinrich, 2010).

Bone Turnover Related to Improved Testicular Functions

It is very striking that through numerous medicinal traditions around the world, identical plants are used both for osteological ailments like osteoporosis and for accelerating the healing of broken bones, as well as for the treatment of male sexual dysfunctions like infertility and decreasing sperm production. Whereas numerous "aphrodisiac" effects are reported from local medicine worldwide, this specific effect is most strongly correlated with the parallel use of the same plant in osteology. E.g., in Sub-Saharan Africa and in India, *Cissus quadrangularis* L. extracts are used in both of these indications (Neuwinger, 1996). Typical constituents of these extracts are stilbenoids like quadrangularin A and pallidol (**Figure 9**). Extracts of *C. quadrangularis* have been experimentally demonstrated to accelerate the healing of fractured jaw bones in an animal model (Brahmkshatriya et al., 2015), to alleviate bone deterioration in osteotomized rats *via* p38 MAPK signalling (Kanwar et al., 2015), to up-regulate the matrix mineralization of human osteoblast like SaOS-2 cells (Muthusami et al., 2011), and to enhance biomineralization through up-regulation of MAPK-dependent alkaline phosphatase activity in osteoblasts (Parisuthiman et al., 2009). The validity of the traditional use of the same plant for increasing sperm production has also been experimentally validated (Neuwinger, 1996). Most recently, extracts of *C. quadrangularis* were demonstrated to prevent quinalphos induced male reproductive toxicity in an animal model (Kokilavani et al., 2014). For the similarly used closely related species *Cissus populnea* Guill.&Perr. a proliferation effect on the sperm producing TM4 Sertoli cell line was observed (Osibote et al., 2011).

In East Asia, we find several medicinal plant drugs with the same pattern of dual traditional use. Especially for species of the genus *Epimedium* (e.g., *E. grandiflorum* C. Morren) numerous uses in both indications are known. A review of its uses in osteoporosis therapy has been published by Zhai et al. (Zhai et al., 2013). It was shown to induce bone neoformation, to reduce osteocyte and osteoclast densities (Burim et al., 2016) and to induce osteogenesis from bone marrow mesenchymal stem cells (Kim et al., 2017). These effects have mainly been attributed to its prenylated flavonol glycoside icariside II, which can enhance the osteogenic differentiation of bone marrow stromal cells (Luo et al., 2015), and icariin, which modulates the process of bone formation *via* the BMP-2/Smad4 signal transduction pathway (**Figure 10**) (Liang et al., 2012). Furthermore, in a mouse model,

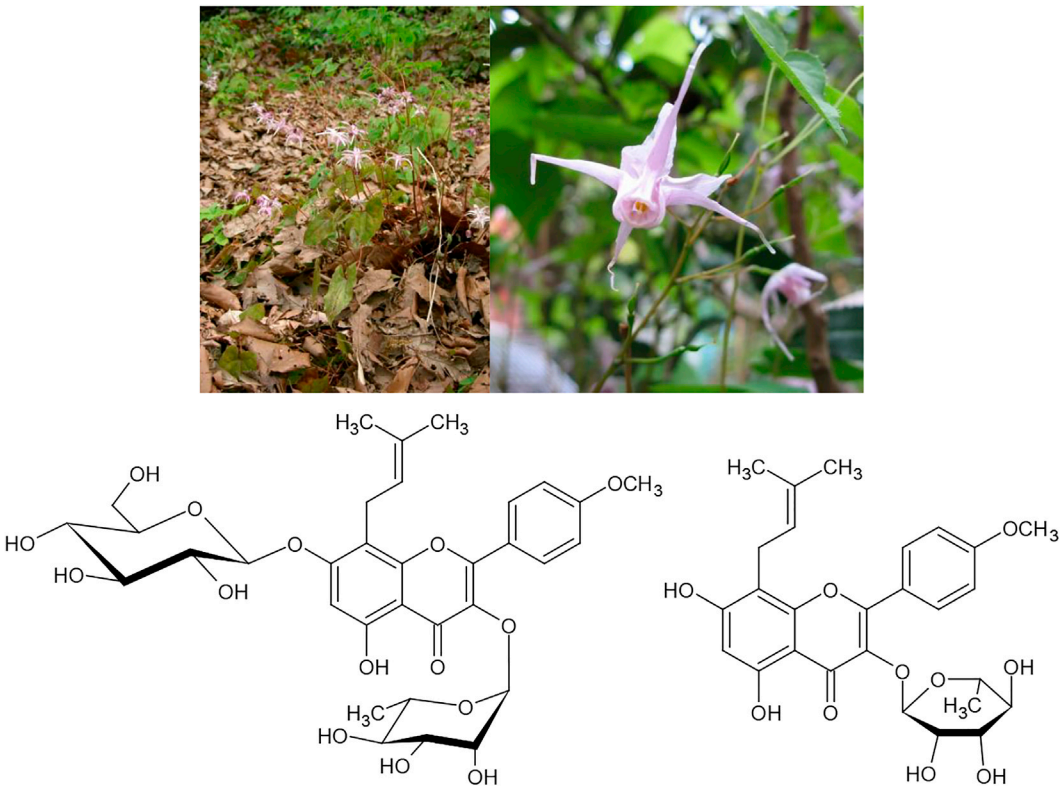

FIGURE 10 | *Epimedium grandiflorum* C.Morrenv and its active constituents icariin and icariside II.

significant increases of testicular weights, sperm counts and sperm motility were observed under treatment with the total flavonoid fraction of the drug (Yuan et al., 2014). In an *in vitro* model, isolated icariin was shown to promote the proliferation of Sertoli cells by activating the ERK1/2 signal pathway. In a parallel study the prenylflavonoid (i.e., icariin derivatives) fraction of an extract of leaves of the closely related species *E. koreaum* was shown to exert powerful protective effects on ovariectomy induced osteoporosis in rats by stimulating bone formation and inhibiting bone turnover and bone resorption, suggesting that the extract fraction could be an alternative to hormone replacement therapy for the prevention of postmenopausal osteoporosis (Zhao et al., 2016).

The above discussed case of the application of *Epimedium* spec. for both osteoporosis and sexual dysfunction reflects the symptom pattern in traditional East Asian medicine systems referred to as "decreased kidney function."

Yet another East Asian plant drug for which this dichotomy of indications can easily be observed is *Panax ginseng* C.A.Mey (**Figure 11**). In both, *in vitro* and animal models Korean Red Ginseng was shown to prevent radiation-induced bone loss (Kim et al., 2015) and to counteract glucocorticoid-induced osteoporosis (Lee et al., 2013). In a trial with elderly rats, sperm number, germ cell count, Sertoli cell count and Sertoli cell index were significantly restored (Kopalli et al., 2015). In similar experiments, a significant increase of testicle weight was observed (Kim et al., 2010) and testicular damage through 2,3,7, 8-tetrachlorodibenzo-p-dioxin was minimised (Kim et al., 1999).

One possible explanation for the described twofold activity of numerous plant drugs on both the bone and the testicular system may lie in the close ontological relation between the testicular cells and the bone marrow cells in vertebrate ontogenesis (Nayernia et al., 2006). This is especially true for bone marrow cells, in the case of which a direct effect of *P. ginseng* extracts has been experimentally observed (Kim et al., 2014).

The testicular functions are mainly regulated *via* androgenic hormones like testosterone (Kopalli et al., 2015), whereas the formation of bone tissue depends on the p38 MAPK signalling (Kanwar et al., 2015). Recent research points to a regulatory connection between the early steps of these two pathways (Jin et al., 2010; Banerjee et al., 2016; Chen et al., 2016). It therefore seems likely that the traditionally used plant extracts interact with pharmacological targets early in the regulatory pathways. However, experimental data in this regard are sparse and much further research is needed.

The presented insights into this field may not only give us a deeper understanding of male reproductive problems but also lead to "new" approaches in the treatment of common osteological diseases like osteoporosis and further to adjuvant treatments for bone fractures. Last but not least, the possibility of prevention of bone loss may be of interest for deep space travel as a pharmaceutical approach to prevent the ensuing bone loss from microgravity.

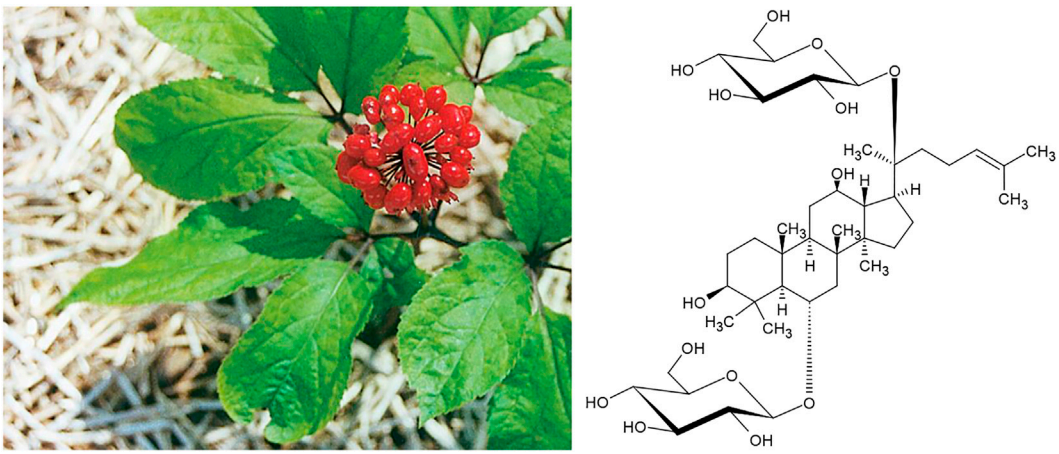

FIGURE 11 | *Panax ginseng* C.A.Mey and its representative active constituent ginsenoside Rg1.

CONCLUSION

Based on the above presented series of documented cases of dual use of the same medicinal plant for seemingly unrelated diseases from various systems of traditional medicine worldwide, as well as by theoretical considerations grounded on the principles of biological and cultural evolution, we propose "Tradition to Pathogenesis" as a completely new approach in medicinal plant research: Using the known pharmacological properties of medicinal plants and the documented empirical knowledge of their use, it is possible to gain a new understanding of the pathogenesis of the treated diseases. The study of the "disease symptom patterns" (Chin. Zheng) (Zhao et al., 2015) that are traditionally associated with certain herbal drugs and drug mixtures, may be an important guiding light for future discoveries.

In such traditional medicinal systems like in Traditional Chinese Medicine (TCM), Ayurveda, and Kampo medicine the empirical use of medicinal plants are traditionally not based on the modern knowledge of physiology. However, they contain internally consistent theories of pathogenesis. Anthropologically, these theories of pathogenesis in traditional medicinal systems seem to be based on the accumulation of empirical traditional knowledge over the centuries that was later systematised. Empirical traditional knowledge is - just as any other cultural tradition - subject to cultural evolution. In the context of medicine, this means that successful treatments are remembered and replicated whereas unsuccessful treatment attempts are forgotten or discarded if toxic. However, treatment success depends on 1) human physiology and 2) the pathophysiology of the disease. These two factors form the equivalent of Darwinian evolutionary pressure in the cultural evolution of traditional medicine. Thus - although traditional medicine is not based on the modern knowledge of human physiology, biochemistry or genetics - underlaying information is "imprinted" onto the traditional theories of pathogenesis by cultural evolution. Traditional medical knowledge therefore exhibits an *a priori* internal structure that corresponds to human pharmacology and physiology, long before these were scientifically understood.

The idea that human cultures undergo a similar evolutionary process as genetic evolution goes back at least to Darwin himself (Darwin, 1871). The first dynamic model of gene-culture co-evolution based on Darwin's principles was published in 1976 by Feldman and Cavalli-Sforza (Feldman and Cavalli-Sforza, 1976). For example, the above discussed case of the traditional application of *Epimedium* spec. for both osteoporosis and sexual dysfunction reflects the symptom pattern in traditional East Asian medicine systems referred to as "decreased kidney function". I.e., these applications of *Epimedium* spec. have a direct footing in human physiology that was "imprinted" onto the traditional medicine system by cultural evolution. This application of the previously established concept of cultural evolution to traditional medicine and pathophysiology is highlighted in our present work for the first time. It is of note that in modern pathophysiology the suprarenal glands are involved in both bone turnover (cortisol, zona fasciculata) and the production of sexual hormones (zona reticularis).

Whilst the isolation and search for single acting compounds from plants did not lead to a further boost of new chemical drugs, as was seen for salicine, codeine, morphine, digitoxin, irinotecan, vincristine, and taxol, the unlifted treasure lies in the clarification of acting mechanisms of traditional herbal extracts. How the theory of cultural evolution can be applied in order to correlate Traditional to Modern pharmacology is an interesting topic for a wide array of future research. Here, we are focusing on the modern understanding of pathological processes based on traditional views on pathogenesis as a first step towards this aim.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

Prof. Dr. med. Harald Schwörer, clinic for gastroenterology and gastrointestinal oncology, University Medicine Göttingen, is kindly acknowledged for his valuable

suggestions concerning both the historical use of sulphonamides in the therapy of rheumatism and the use of antibiotics against bacterial peritonitis. We thank Hans Rausch, Phytochem Referenzsubstanzen, Neu-Ulm, for his extremely helpful suggestions on rheumatism and discussions on *H. pylori*. We are grateful to Dr. Robert Cameron, Max-Planck-Institute for Solar System Research for his contribution concerning epistemology. The work in Göttingen is supported by the "Förderkreis der Forschungsstelle für Fernöstliche Medizin.". We acknowledge support by the Open Access Publication Funds of the Göttingen University.

REFERENCES

Appenrodt, B., Grünhage, F., Gentemann, M. G., Thyssen, L., Sauerbruch, T., and Lammert, F. (2010). Nucleotide-binding Oligomerization Domain Containing 2 (NOD2) Variants Are Genetic Risk Factors for Death and Spontaneous Bacterial Peritonitis in Liver Cirrhosis. *Hepatology* 51, 1327–1333. doi:10.1002/hep.23440

Asha, M. K., Debraj, D., Prashanth, D., Edwin, J. R., Srikanth, H. S., Muruganantham, N., et al. (2013). *In Vitro* anti-Helicobacter pylori Activity of a Flavonoid Rich Extract of Glycyrrhiza Glabra and its Probable Mechanisms of Action. *J. Ethnopharmacol* 145, 581–586. doi:10.1016/j.jep.2012.11.033

Attaguile, G., Caruso, A., Pennisi, G., and Savoca, F. (1995). Gastroprotective Effect of Aqueous Extract of Cistus Incanus L. In Rats. *Pharmacol. Res.* 31, 29–32. doi:10.1016/1043-6618(95)80043-3

Banerjee, B., Nandi, P., Chakraborty, S., Raha, S., Sen, P. C., and Jana, K. (2016). Resveratrol Ameliorates Benzo(a)pyrene-Induced Testicular Dysfunction and Apoptosis: Involvement of P38 MAPK/ATF2/iNOS Signaling. *J. Nutr. Biochem.* 34, 17–29. doi:10.1016/j.jnutbio.2016.04.003

Biebricher, C. K., and Eigen, M. (2005). The Error Threshold. *Virus. Res.* 107, 117–127. doi:10.1016/j.virusres.2004.11.002

Brahmkshatriya, H. R., Shah, K. A., Ananthkumar, G. B., and Brahmkshatriya, M. H. (2015). Clinical Evaluation of Cissus Quadrangularis as Osteogenic Agent in Maxillofacial Fracture: A Pilot Study. *Ayu* 36, 169–173. doi:10.4103/0974-8520.175542

Brenchley, J. M., and Douek, D. C. (2012). Microbial Translocation across the GI Tract. *Annu. Rev. Immunol.* 30, 149–173. doi:10.1146/annurev-immunol-020711-075001

Burim, R. A., Sendyk, D. I., Hernandes, L. S., de Souza, D. F., Correa, L., and Deboni, M. C. (2016). Repair of Critical Calvarias Defects with Systemic Epimedium Sagittatum Extract. *J. Craniofac. Surg.* 27, 799–804. doi:10.1097/SCS.0000000000002451

Chen, Y., Wu, Y., Gan, X., Liu, K., Lv, X., Shen, H., et al. (2016). Iridoid Glycoside from Cornus Officinalis Ameliorated Diabetes Mellitus-Induced Testicular Damage in Male Rats: Involvement of Suppression of the AGEs/RAGE/p38 MAPK Signaling Pathway. *J. Ethnopharmacol* 194, 850–860. doi:10.1016/j.jep.2016.10.079

Dai, R., Xiao, X., Peng, F., Li, M., and Gong, G. (2016). Artesunate, an Anti-malarial Drug, Has a Potential to Inhibit HCV Replication. *Virus Genes* 52 (1), 22–28. doi:10.1007/s11262-015-1285-7

Darwin, C. (1862). *On the Various Contrivances by Which British and Foreign Orchids Are Fertilised by Insectls*. London: John Murray, 197–203.

Darwin, C. (1871). The Descent of Man and Selection in Relation to Sex. in" *Photoreproduction of the 1871 Edition* (Princeton, New JerseyLondon: Princeton University PressJ. Murray), 60–61.

Efferth, T. (2017). From Ancient Herb to Modern Drug: Artemisia Annua and Artemisinin for Cancer Therapy. *Semin. Cancer Biol.* 46, 65–83. doi:10.1016/j.semcancer.2017.02.009

Efferth, T. (2006). Molecular Pharmacology and Pharmacogenomics of Artemisinin and its Derivatives in Cancer Cells. *Curr. Drug Targets* 7 (4), 407–421. doi:10.2174/138945006776359412

Efferth, T., Romero, M. R., Wolf, D. G., Stamminger, T., Marin, J. J., and Marschall, M. (2008). The Antiviral Activities of Artemisinin and Artesunate. *Clin. Infect. Dis.* 47 (6), 804–811. doi:10.1086/591195

Eigen, M. (2002). Error Catastrophe and Antiviral Strategy. *Proc. Natl. Acad. Sci. U S A.* 99, 13374–13376. doi:10.1073/pnas.212514799

Eigen, M. (1996). On the Nature of Virus Quasispecies. *Trends Microbiol.* 4, 216–218. doi:10.1016/0966-842X(96)20011-3

Eigen, M. (1971). Selforganization of Matter and the Evolution of Biological Macromolecules. *Naturwissenschaften* 58, 465–523. doi:10.1007/BF00623322

Feldman, M. W., and Cavalli-Sforza, L. L. (1976). Cultural and Biological Evolutionary Processes, Selection for a Trait under Complex Transmission. *Theor. Popul. Biol.* 9 (2), 238–259. doi:10.1016/0040-5809(76)90047-2

Felter, H. W., and Lloyd, J. U.*King's American Dispensatory* (1905). Cincinatti: Ohio Valley Co., 1729.

Hardy, K., Buckley, S., Collins, M. J., Estalrrich, A., Brothwell, D., Copeland, L., et al. (2012). Neanderthal Medics? Evidence for Food, Cooking, and Medicinal Plants Entrapped in Dental Calculus. *Naturwissenschaften* 99, 617–626. doi:10.1007/s00114-012-0942-0

Heinrich, M. (2010). Galanthamine from Galanthus and Other Amaryllidaceae-Cchemistry and Biology Based on Traditional Use. *Alkaloids Chem. Biol.* 68, 157–165. doi:10.1016/s1099-4831(10)06804-5

Holmes, R. K., Malim, M. H., and Bishop, K. N. (2007). APOBEC-mediated Viral Restriction: Not Simply Editing? *Trends Biochem. Sci.* 32, 118–128. doi:10.1016/j.tibs.2007.01.004

Huffman, M. A. (2001). Self-Medicative Bahavior in the African Great Apes: An Evolutionary Perspective into the Origins of Human Traditional Medicine. *BioScience*, 51, 651–661. doi:10.1641/0006-3568(2001)051[0651:smbita]2.0.co;2Self-Medicative Behavior in the

Husemann, T. (1889). Ladanum und Laudanum - Ein Beitrag zur Geschichte der Arzneimittel. *Archiv der Pharmacie* 27 (24), 1075–1132. doi:10.1002/ardp.18892272303

Hutschenreuther, A., Birkemeyer, C., Grötzinger, K., Straubinger, R. K., and Rauwald, H. W. (2010). Growth Inhibiting Activity of Volatile Oil from Cistus Creticus L. Against Borrelia Burgdorferi s.S. *In Vitro*. *Pharmazie* 65, 290–295.

Jin, H., Wang, D. Y., Mei, Y. F., Qiu, W. B., Zhou, Y., Wang, D. M., et al. (2010). Mitogen-activated Protein Kinases Pathway Is Involved in Physiological Testosterone-Induced Tissue Factor Pathway Inhibitor Expression in Endothelial Cells. *Blood Coagul. Fibrinolysis* 21, 420–424. doi:10.1097/MBC.0b013e328337b475

Kahumba, J., Rasamiravaka, T., Okusa, P. N., Bakari, A., Bizumukama, L., Kalonji, J. B., et al. (2015). Traditional African Medicine: From Ancestral Knowledge to a Modern Integrated Future. *Science* 350 (6262), S61–S63. doi:10.1126/science.350.6262.871-c

Kanwar, J. R., Samarasinghe, R. M., Kumar, K., Arya, R., Sharma, S., Zhou, S. F., et al. (2015). Cissus Quadrangularis Inhibits IL-1β Induced Inflammatory Responses on Chondrocytes and Alleviates Bone Deterioration in Osteotomized Rats *via* P38 MAPK Signaling. *Drug Des. Devel Ther.* 9, 2927–2940. doi:10.2147/DDDT.S77369

Kim, D. R., Lee, J. E., Shim, K. J., Cho, J. H., Lee, H. C., Park, S. K., et al. (2017). Effects of Herbal Epimedium on the Improvement of Bone Metabolic Disorder through the Induction of Osteogenic Differentiation from Bone Marrow-Derived Mesenchymal Stem Cells. *Mol. Med. Rep.* 15, 125–130. doi:10.3892/mmr.2016.6015

Kim, J., Lee, H., Kang, K. S., Chun, K. H., and Hwang, G. S. (2015). Protective Effect of Korean Red Ginseng against Glucocorticoid-Induced Osteoporosis *In Vitro* and *In Vivo*. *J. Ginseng Res.* 39, 46–53. doi:10.1016/j.jgr.2014.06.001

Kim, S. G., Lee, A. J., Bae, S. H., Kim, S. M., Lee, J. H., Kim, M. J., et al. (2014). Total Extract of Korean Red Ginseng Facilitates Human Bone Marrow Hematopoietic colony Formation *In Vitro*. *Blood Res.* 49, 177–181. doi:10.5045/br.2014.49.3.177

Kim, W., Hwang, S., Lee, H., Song, H., and Kim, S. (1999). Panax Ginseng Protects the Testis against 2,3,7, 8-Tetrachlorodibenzo-P-Dioxin Induced Testicular Damage in guinea Pigs. *BJU Int.* 83, 842–849. doi:10.1046/j.1464-410x.1999.00046.x

Kim, Y. H., Kim, G. H., Shin, J. H., Kim, K. S., and Lim, J. S. (2010). Effect of Korean Red Ginseng on Testicular Tissue Injury after Torsion and Detorsion. *Korean J. Urol.* 51, 794–799. doi:10.4111/kju.2010.51.11.794

Kokilavani, P., Suriyakalaa, U., Elumalai, P., Abirami, B., Ramachandran, R., Sankarganesh, A., et al. (2014). Antioxidant Mediated Ameliorative Steroidogenesis by Commelina Benghalensis L. And Cissus Quadrangularis L. against Quinalphos Induced Male Reproductive Toxicity. *Pestic. Biochem. Physiol.* 109, 18–33. doi:10.1016/j.pestbp.2014.01.002

Kollipara, R., Ekhlassi, E., Downing, C., Guidry, J., Lee, M., and Tyring, S. K. (2015). Advancements in Pharmacotherapy for Noncancerous Manifestations of HPV. *J. Clin. Med.* 4, 832–846. doi:10.3390/jcm4050832

Kopalli, S. R., Hwang, S. Y., Won, Y. J., Kim, S. W., Cha, K. M., Han, C. K., et al. (2015). Korean Red Ginseng Extract Rejuvenates Testicular Ineffectiveness and Sperm Maturation Process in Aged Rats by Regulating Redox Proteins and Oxidative Defense Mechanisms. *Exp. Gerontol.* 69, 94–102. doi:10.1016/j.exger.2015.05.004

Kuchta, K., Grötzinger, K., Birkemeyer, C., and Rauwald, H. W. (2012). *Labdanum from Mediterranean Cistus Species: GC-MS Fingerprints and Relative Quantification of Antispirochaetal Manoyloxides*, 78.

Lee, J. H., Lee, H. J., Yang, M., Moon, C., Kim, J. C., Bae, C. S., et al. (2013). Effect of Korean Red Ginseng on Radiation-Induced Bone Loss in C3H/HeN Mice. *J. Ginseng Res.* 37, 435–441. doi:10.5142/jgr.2013.37.435

Liang, W., Lin, M., Li, X., Li, C., Gao, B., Gan, H., et al. (2012). Icariin Promotes Bone Formation *via* the BMP-2/Smad4 Signal Transduction Pathway in the hFOB 1.19 Human Osteoblastic Cell Line. *Int. J. Mol. Med.* 30, 889–895. doi:10.3892/ijmm.2012.1079

Lin, Z. M., Yang, X. Q., Zhu, F. H., He, S. J., Tang, W., and Zuo, J. P. (2016). Artemisinin Analogue SM934 Attenuate Collagen-Induced Arthritis by Suppressing T Follicular Helper Cells and T Helper 17 Cells. *Sci. Rep.* 6, 38115. doi:10.1038/srep38115

Luo, G., Gu, F., Zhang, Y., Liu, T., Guo, P., and Huang, Y. (2015). Icariside II Promotes Osteogenic Differentiation of Bone Marrow Stromal Cells in Beagle Canine. *Int. J. Clin. Exp. Pathol.* 8, 4367–4377.

Luo, X. (2003). *Compendium of Materia Medica, Category of Herbs (IV): A Translation of the "Bencao Gangmu*. Beijing: Foreign Languages Press.

Malim, M. H. (2009). APOBEC Proteins and Intrinsic Resistance to HIV-1 Infection. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364, 675–687. doi:10.1098/rstb.2008.0185

Marshall, B. J., Armstrong, J. A., McGechie, D. B., and Glancy, R. J. (1985). Attempt to Fulfil Koch's Postulates for Pyloric Campylobacter. *Med. J. Aust.* 142, 436–439. doi:10.5694/j.1326-5377.1985.tb113443.x

Mashkovsky, M. D., and Kruglikova-Lvov, R. P. (1951). On the Pharmacology of the New Alklaloid Galantamine. *Farmakol. Toxicol. (Moscow)* 14, 27–30.

Muthusami, S., Senthilkumar, K., Vignesh, C., Ilangovan, R., Stanley, J., Selvamurugan, N., et al. (2011). Effects of Cissus Quadrangularis on the Proliferation, Differentiation and Matrix Mineralization of Human Osteoblast like SaOS-2 Cells. *J. Cel Biochem* 112, 1035–1045. doi:10.1002/jcb.23016

Nayernia, K., Lee, J. H., Drusenheimer, N., Nolte, J., Wulf, G., Dressel, R., et al. (2006). Derivation of Male Germ Cells from Bone Marrow Stem Cells. *Lab. Invest.* 86, 654–663. doi:10.1038/labinvest.3700429

Neumann, V. (1988). Biochemical Aspects of Infection in Rheumatoid Arthritis and Ankylosing Spondylitis. *Baillieres Clin. Rheumatol.* 2, 259–269. doi:10.1016/s0950-3579(88)80012-8

Neumann, V. C., Grindulis, K. A., Hubball, S., McConkey, B., and Wright, V. (1983). Comparison between Penicillamine and Sulphasalazine in Rheumatoid Arthritis: Leeds-Birmingham Trial. *Br. Med. J. (Clin Res. Ed.* 287, 1099–1102. doi:10.1136/bmj.287.6399.1099

Neuwinger, H. D. (1996). *African Ethnobotany: Poisons and Drugs: Chemistry, Pharmacology, Toxicology*. Weinheim, Germany: Chapman & Hall, 252–257.

Orwa, C., Mutua, A., Kindt, R., Kamnadass, R., and Anthony, S. (2009). *Agroforestry Database: A Tree Reference and Selection Guide Version 4.0*.

Osibote, E., Noah, N., Sadik, O., McGee, D., and Ogunlesi, M. (2011). Electrochemical Sensors, MTT and Immunofluorescence Assays for Monitoring the Proliferation Effects of Cissus Populnea Extracts on Sertoli Cells. *Reprod. Biol. Endocrinol.* 9, 65. doi:10.1186/1477-7827-9-65

Otsuka, K. (2016). in *Kampo: A Clinical Guide to Theory and Practice*. Editors G. T. DeSoriano and N. T. Dawes London, UK: Singing Dragon.

Parisuthiman, D., Singhatanadgit, W., Dechatiwongse, T., and Koontongkaew, S. (2009). Cissus Quadrangularis Extract Enhances Biomineralization through Up-Regulation of MAPK-dependent Alkaline Phosphatase Activity in Osteoblasts. In Vitro *Cel Dev Biol Anim* 45, 194–200. doi:10.1007/s11626-008-9158-1

Pauly, M. D., and Lauring, A. S. (2015). Effective Lethal Mutagenesis of Influenza Virus by Three Nucleoside Analogs. *J. Virol.* 89, 3584–3597. doi:10.1128/JVI.03483-14

Pereira, J., and Brown, Green. (1855). *The Elements of Materia Medica and Therapeutics*. 4th Edition. London: Longman, 290–291.

Pharmaceutical Society of Great Britain, Council: University College, L. (1907). *The British Pharmaceutical Codex: An imperial Dispensatory for the Use of Medical Practitioners and Pharmacists*. London: Pharmaceutical Society of Great Britain., 1191.

Porras, M., Martín, M. T., Yang, P. C., Jury, J., Perdue, M. H., and Vergara, P. (2006). Correlation between Cyclical Epithelial Barrier Dysfunction and Bacterial Translocation in the Relapses of Intestinal Inflammation. *Inflamm. Bowel Dis.* 12, 843–852. doi:10.1097/01.mib.0000231571.88806.62

Pullar, T., Hunter, J. A., and Capell, H. A. (1983). Sulphasalazine in Rheumatoid Arthritis: a Double Blind Comparison of Sulphasalazine with Placebo and Sodium Aurothiomalate. *Br. Med. J. (Clin Res. Ed.* 287, 1102–1104. doi:10.1136/bmj.287.6399.1102

Rauwald, H. W., Liebold, T., Grötzinger, K., Kuchta, K., and Lehmann, J. (2013). On the Antispirochaetal Activity of Manoyloxides and Carvacrol from the Oleoresin Labdanum of Cistus Creticus L. *Planta Med.* 79, PN53. doi:10.1055/s-0033-1352396

Rauwald, H. W., Liebold, T., Grötzinger, K., Lehmann, J., and Kuchta, K. (2019). Labdanum and Labdanes of Cistus Creticus and C. Ladanifer: Anti-Borrelia Activity and its Phytochemical Profiling☆. *Phytomedicine* 60, 152977. doi:10.1016/j.phymed.2019.152977

Rozin, A. (2007). Is Osteoarthritis an Infection-Associated Disease and a Target for Chemotherapy? *Chemotherapy* 53, 1–9. doi:10.1159/000098243

Ruiz-Jarabo, C. M., Ly, C., Domingo, E., and de la Torre, J. C. (2003). Lethal Mutagenesis of the Prototypic Arenavirus Lymphocytic Choriomeningitis Virus (LCMV). *Virology* 308, 37–47. doi:10.1016/s0042-6822(02)00046-6

Verheijen, H. C.De Invloed van Succus Liquiritiae op de Maagzuursecretie en zijn Werking bij Ulcus Ventriculi (1948). *Ned Tijdschr Geneeskd* 92, 2910–2912.

von Hutten, U. *Aedibus Thomae Bertheleti, MDXXXIII [printed 1533] Cum Privilegio*, De Morbo Gallico; Londini.

Voulgari, P. V. (2011). Rheumatological Manifestations in Inflammatory Bowel Disease. *Ann. Gastroenterol.* 24, 173–180.

Weyrich, L. S., Duchene, S., Soubrier, J., Arriola, L., Llamas, B., Breen, J., et al. (2017). Neanderthal Behaviour, Diet, and Disease Inferred from Ancient DNA in Dental Calculus. *Nature* 544, 357–361. doi:10.1038/nature21674

Yesilada, E., Gurbuz, I., and Ergun, E. (1997). Effects of Cistus Laurifolius L. Flowers on Gastric and Duodenal Lesions. *J. Ethnopharmacol* 55, 201–211.

Yesilada, E., Gurbuz, I., and Shibata, H. (1999). Screening of Turkish Anti-ulcerogenic Folk Remedies for Anti-Helicobacter pylori Activity. *J. Ethnopharmacol* 66, 289–293.

You-you, T., Mu-Yun, N., Yu-Rong, Z., Lan-Na, L., Shu-Lian, C., Mu-Qun, Z., et al. (1982b). Studies on the Constituents of Artemisia Annua Part II. *Planta Med.* 44 (3), 143–145. doi:10.1055/s-2007-971424

You-you, T., (1982a). Chemical Studies on Qinghaosu (Artemisinine). China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials. *J. Tradit Chin. Med.* 2 (1), 3–8.

Yuan, D., Wang, H., He, H., Jia, L., He, Y., Wang, T., et al. (2014). Protective Effects of Total Flavonoids from Epimedium on the Male Mouse Reproductive System against Cyclophosphamide-Induced Oxidative

Injury by Up-Regulating the Expressions of SOD3 and GPX1. *Phytother Res.* 28, 88–97. doi:10.1002/ptr.4956

Zhai, Y. K., Guo, X., Pan, Y. L., Niu, Y. B., Li, C. R., Wu, X. L., et al. (2013). A Systematic Review of the Efficacy and Pharmacological Profile of Herba Epimedii in Osteoporosis Therapy. *Pharmazie* 68, 713–722.

Zhao, B. J., Wang, J., Song, J., Wang, C. F., Gu, J. F., Yuan, J. R., et al. (2016). Beneficial Effects of a Flavonoid Fraction of Herba Epimedii on Bone Metabolism in Ovariectomized Rats. *Planta Med.* 82 (4), 322–329. doi:10.1055/s-0035-1558294

Zhao, X., Zheng, X., Fan, T. P., Li, Z., Zhang, Y., and Zheng, J. (2015). A Novel Drug Discovery Strategy Inspired by Traditional Medicine Philosophies. *Science* 347 (6219 Suppl. l), S38–S40. doi:10.1126/science.347.6219.337-c

Zhou, Q., Zhang, B., and Verne, G. N. (2009). Intestinal Membrane Permeability and Hypersensitivity in the Irritable Bowel Syndrome. *Pain* 146 (1-2), 41–46. doi:10.1016/j.pain.2009.06.017

Zohary, M. (1983). *Pflanzen der Bibel*. Stuttgart, Germany: Calwer Verlag, 194.

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 handling editor declared a past co-authorship with one of the authors (KK).

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fphar.2021.705376

Progress and Research Trends on *Catha edulis* (Vahl) Endl. (*Catha edulis*): A Review and Bibliometric Analysis

Shuang Ye[1†], *Jin Hu*[2†], *Zilong Liu*[1*] *and Man Liang*[1*]

[1]*Department of Forensic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China,* [2]*Department of Otolaryngology-Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

Catha edulis (Vahl) Endl., known as *Catha edulis* or Khat is a traditional and regional plant for chewing, smoking and drinking, that has posed a worldwide public health problem due to its recent emerging abused consumption. In the face of the massive use of *Catha edulis*, we reviewed related publications to analyze the progress and research trends through bibliometric methods. After screening, a total of 514 scientific publications published from 1997 to 2020 were included by systematic retrieval from the Web of Science (WoS) database. According to further scientometric analysis, the annual number of publications output kept rising in most of the years. Ethiopia and the United States of America (USA) have been devoting significant contributions to the field. Though the research emphasis had been the chemical composition and pharmacological and toxicological effects for several years, the hot spots were transferred; the mechanism investigations of *Catha edulis* have been the focus in recent years, which might be continued in the future. Furthermore, co-operations of multi-disciplinary researchers are needed to minimize abuse harms and maximize the medicinal benefits of *Catha edulis* to human beings.

Keywords: bibliometric analysis, cathinone, chemical composition, toxicology, pharmacological effect, mechanisms, *Catha edulis* (vahl) endl., *Catha edulis*

OPEN ACCESS

Edited by:
Yukihiro Shoyama, Nagasaki International University, Japan

Reviewed by:
Ambrose Okem, University of the Witwatersrand, South Africa
Ephrem Engidawork, Addis Ababa University, Ethiopia

***Correspondence:**
Zilong Liu
liuzilongfy@hust.edu.cn
Man Liang
liangman@hust.edu.cn

[†]*These authors have contributed equally to this work and share first authorship*

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

Received: *05 May 2021*
Accepted: *06 October 2021*
Published: *11 November 2021*

Citation:
Ye S, Hu J, Liu Z and Liang M (2021) Progress and Research Trends on Catha edulis *(Vahl) Endl. (*Catha edulis*): A Review and Bibliometric Analysis. Front. Pharmacol. 12:705376. doi: 10.3389/fphar.2021.705376*

INTRODUCTION

Catha edulis (Vahl) Endl., usually called *Catha edulis* or Khat, is a native plant in parts of East Africa and the Arabian Peninsula, and is commonly chewed as natural stimulants in local practice and habits (Graziani et al., 2008). It is also frequently used in other countries with different names, like "qat" in Yemen, "chat" in Ethiopia, "qaad" or "jaad" in Somalia, and "mirra" in Kenya (Kandari et al., 2014). Initially, *Catha edulis* was traditionally governed in the regions for purpose of religious, ritualistic, and medicinal use for several decades (Gebissa, 2004). In the early 1990s, *Catha edulis* spread to Europe, North America, and Australia due to emigration from the Horn of Africa (Al-Hebshi and Skaug, 2005; Gebissa, 2010). Nowadays, there are estimated to be over 20 million abusers globally, including both male and female adults, and college and middle school students (Riyaz et al., 2014; Odenwald and Al'Absi, 2017).

The main active ingredients of *Catha edulis* include phenylpropylamino alkaloids, cathine, and cathinone, which are responsible for the pharmacological and toxicological effects. Among these, the β-keto analogue of amphetamine from a structural perspective could lead to dependence via

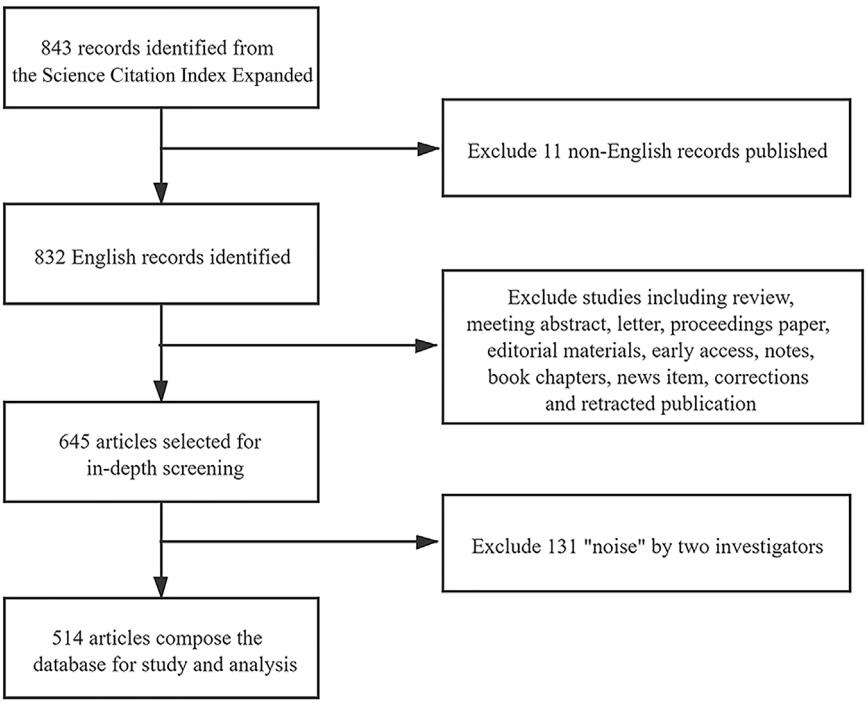

FIGURE 1 | The flow chart of database construction.

psychostimulatory effects on the nervous system (Elmai, 1983; Morghemm and Rufatm, 1983; Kalix, 1987; Giannini et al., 1992; Omar et al., 2015). Additionally, prolonged exposure to *Catha edulis* could lead to dependence, psychosis, hypertension, cardiovascular complications, sexual dysfunction, hepatotoxicity, etc (Odenwald and Al'Absi, 2017). Therefore, *Catha edulis* abuse has become one of the most serious public health concerns. However, except for such adverse outcomes, there are other reports suggesting potential medicinal benefits of *Catha edulis* such as antibacterial activity (Al-Hebshi et al., 2006), antidepressant-like activity (Alfaifi et al., 2017), adjunct treatment of obesity (Hauner et al., 2017), and neural tissue substitutes (Abdel Bary and Harmal, 2019a), which deserve comprehensive analysis.

In this study, we aimed to elucidate the progress and hotspots of *Catha edulis* research via a bibliometric analysis and aggregate the opinions of effects and mechanisms, which would help relieve the relative health concern (Zupic and Cater, 2015). This bibliometric net analysis aims to give an overview of *Catha edulis* research and reveal the directions and frontiers for future development.

METHODS AND MATERIALS

The WoS database is the most frequently used for search in various scientific fields and retrieving related literature. And the WoS core collection indexes scientific journals exerting greater impact. For accurate and representative search, we collected index topics of *Catha edulis* from Medical Subject Headings (MeSH) list in PubMed and built an initial database from the WoS core collection with the searching strategy: [(Topic = ("Khat") OR ("Khats") OR ("Catha") OR ("Catha eduli") OR ("*Catha edulis*") OR ("edulis, Catha") OR ("Qat Plant") OR ("Qat Plants") OR ("Plant, Qat") OR ("Plants, Qat") OR ("Miraa")]. Though research on *Catha edulis* started in 1952, early publications have proved to be of low impact and output, or published in journals with no official IF or low SJR like local African journals (Gaillard J, 1992). From 1997 to the present, a continuous emergence of research productivity was observed, and critical research performance related to *Catha edulis* has progressed which deserves a scientometric analysis. Based on the retrieval period between 1997 to Dec 21st, 2020, 843 records were identified. Since English served as the language of worldwide scientific communication, we excluded 11 non-English records and narrowed 832 English studies for further analysis. To detect original discoveries, we restricted document types to original articles and obtained 645 science publications. After downloading the raw data of these aforementioned articles, two investigators individually checked and eliminated noise, any divergence was reconciled, and a consensus was finally achieved through discussion. Finally, 514 publications composed the "*Catha edulis*" database (**Figure 1**). Statistical analyses of the variables of the database were conducted by CiteSpace 5.7.R1 to generate visualized graphs, including distribution of publication outputs, collaborations between countries, and co-occurrence and burst keywords for detection of research trends and frontiers (Chen, 2004; Chen, 2006; Chen et al., 2010).

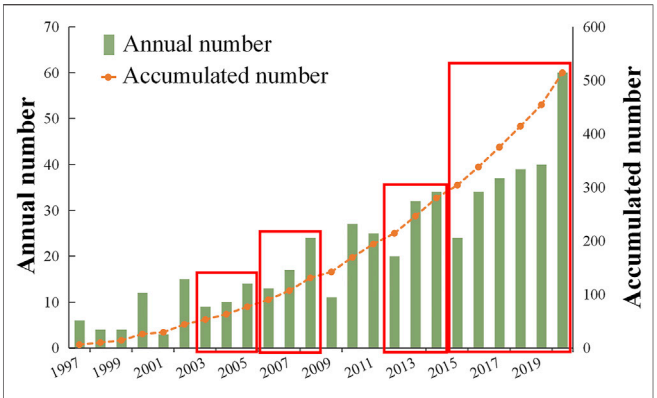

FIGURE 2 | Annual number and accumulated number of *Catha edulis* publications from 1997 to 2020.

RESULTS

Publication Outputs and Growth Trend

The annual publication outputs had a fluctuating growth rate during most times, and the fluctuation was periodic over the past 24 years according to the histogram. The number of annual publications increased dramatically in 2020, by 10-fold from 6 in 1997 to 60 in 2020 (**Figure 2**). The Mann-Kendall (MK) test was applied to the annual publication number, and the statistics results ($Z = 5.407 > 2.32 > 0$) indicated a significant increasing trend of the annual outputs related to *Catha edulis* with a 0.001 level of significance.

Contributed Countries and Institutions

According to the WoS, the retrieved publications on *Catha edulis* were indicated to be contributed by 65 countries/regions with intensive cooperation. From this sensitive indicator of reflecting attention and strength commanded by a country/region in a specific research area, the most productive countries were Ethiopia (32.9%, 169/514), followed by the USA (16.7%, 86/514), Saudi Arabia (12.6%, 65/514), and Yemen (12.3%, 63/514) (**Table 1**; **Figure 3**). Half of the top ten most productive countries were located in East Africa and the Arabian Peninsula, including Ethiopia, Saudi Arabia, Yemen, Egypt, and Kenya, which produced approximately two-thirds (67.7%) of the publications on *Catha edulis* in total. In addition, North American and European countries contributed several research achievements in recent years.

The total number of citations for publications in this field was 7978, giving an average citation per article of 15.52 (7978/514). The top three countries of citations per article were England (33.57, 1,410/42), Germany (20.37, 774/38), and Norway (19.00, 418/22). Moreover, the average H-index of the retrieved papers queried on the WoS was 42. Ethiopia with the highest H-index of 23 was the most effervescent, followed by the USA (20) and Yemen (20). Furthermore, the core institutions in the countries, such as Kenya (68.0%), Norway (63.6%), and Yemen (60.3%), completed the majority of achievements.

Figure 3 also shows the cooperation between contributing countries: Ethiopia, as the most collaborative country, cooperating with 32 countries, followed by Saudi Arabia and the USA, maintaining close cooperation with 27 and 20 countries, respectively. These collaborations with other countries and regions played a significant role in *Catha edulis* research.

Highly Cited Publications

In order to identify influential representative publications, we listed the top 10 most cited articles (**Table 2**). This high-quality scientific research was all published in authoritative journals categorized in pharmacology (No.1), toxicology (No.2, 7, 9, 10), psychiatry (No.3), analytical chemistry (No.4), and public health (No.5, 6, 8), with Impact Factor (IF, 2020), 5-Year Impact Factor (IF5) and SCImago Journal Rank (SJR) retrieved. In these interdisciplinary journals, the most-cited research involved phyto-research and social issues. Germany, Ethiopia, and Yemen contributed two of the top 10 articles, respectively; and the IF5 and SJR of the top two countries were Germany (IF5: 10.249, SJR: 3.463) and Ethiopia (IF5: 5.706, SJR: 2.849).

Hot Spots of Catha Edulis Research

In order to analyze and visualize the network of keyword co-occurrence, we excluded repeated and irrelevant keywords, and prune-sliced and merged the networks using CiteSpace

TABLE 1 | The top 10 most productive countries and institutions for *Catha edulis* publications.

Country or region	Articles (%[a])	Citations	H-Index	Citations per article	Top Institutions	Articles by top Institutions (%[b])
Ethiopia	169 (32.9%)	1759	23	10.41	Addis Ababa University	62 (36.7%)
USA	86 (16.7%)	1,200	20	13.95	University of Minnesota System	23 (26.7%)
Saudi Arabia	65 (12.6%)	514	14	7.91	Jazan University	33 (50.8%)
Yemen	63 (12.3%)	1,033	20	16.40	Sana'a University	38 (60.3%)
England	42 (8.2%)	1,410	16	33.57	University of London	21 (50.0%)
Germany	38 (7.4%)	774	13	20.37	Goethe University Frankfurt	6 (15.8%)
Egypt	26 (5.1%)	176	8	6.77	Assiut University	5 (19.2%)
Kenya	25 (4.9%)	337	11	13.48	University of Nairobi	17 (68.0%)
Norway	22 (4.3%)	418	14	19.00	University of Bergen	14 (63.6%)
Malaysia	20 (3.9%)	100	6	5.00	University Malaya	10 (50.0%)
East Africa and Arabian Peninsula	348 (67.7%)	3,819	25	10.97	Addis Ababa University	62 (17.8%)

[a]*Percentage indicates the ratio of the number of articles on* Catha edulis *published in this country or region to that in the "*Catha edulis*" database (514).*
[b]*Percentage indicates the ratio of the number of articles on* Catha edulis *published by top institutions to that by their country or region.*

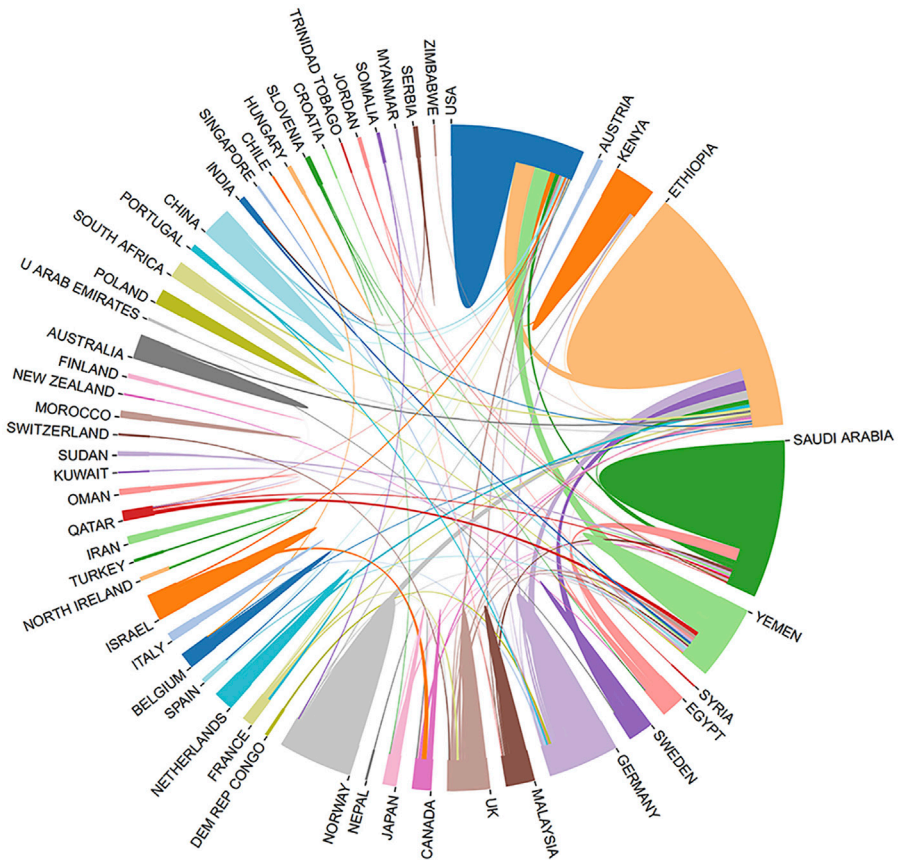

FIGURE 3 | Cooperation between contributed countries.

(threshold of occurrences frequency at 5, **Figure 4**). This map illustrated the frequency and relevance of keywords according to cross size and link. High frequency keywords included "catha eduli" (frequency: 362, centrality: 0.13), "cathinone" (frequency: 141, centrality: 0.12), "risk factor" (frequency: 58, centrality: 0.09), "abuse" (frequency: 57, centrality: 0.34), and "Ethiopia" (frequency: 55, centrality: 0.17), represented by larger crosses. Other keywords such as "prevalence" (frequency: 48, centrality: 0.05), "animal model" (frequency: 42, centrality: 0.20), "student" (frequency: 36, centrality: 0.01), "psychosis" (frequency: 29, centrality: 0.16), and "apoptosis" (frequency: 28, centrality: 0.13) showed a moderate frequency.

Scientific Landscapes of Research Trends

The co-cited references were clustered and identified *via* a timeline map (**Figure 5**), which can help identify development trends of the specific research field (Niazi and Hussain, 2011), indicate the shift of research concerns, and shape the specialty structure of knowledge. According to the timeline map of co-cited references, 10 clusters were analyzed in depth, most of which were concentrated in the period from 2003 to 2016. The earlier studies were mainly devoted to oral fibroblasts (#4) and s-(-)-cathinone (#7). Then the research focus shifted to regulation (#3), neurotoxicity (#1), cortisol (#5), norephedrine (#8), social consequences (#2), Ethiopia (#0), and hepatotoxicity (#9) which were involved in chemical composition, toxicology and global prevalence of *Catha edulis*. MDPV (#6), as one of the synthetic cathinones with a similar structure of cathinone, a main ingredient of *Catha edulis*, was focused on as well.

Keywords could summarize the subject and content of the publication, which play an essential role in tracing hotspot transfers in scientific research. A keyword burst map was obtained for visualization of keywords with the strongest bursts in scientific articles by CiteSpace algorithm-dependent analytical tool and summarization of focus transfer according to their time of appearance (**Figure 6**). Besides, burst keywords can indicate prominent research topics studied over different periods by reflecting the intensity and duration of hot spot issues, while the latest burst keywords represent up-to-date transfers of research focus (Kleinberg, 2003; Chen et al., 2014). Based on the top 20 keywords with the strongest citation bursts, the early research focus was to explore the chemical composition of *Catha edulis* (cathinone), identification (high-performance liquid chromatography), and prevalence. Then, the burst keywords were transferred to 1) health harms (psychosis, carcinoma, cardiovascular disease, and risk factor), 2) toxicology (apoptosis) and pharmacology (alkaloid), and 3) mechanism (antioxidant and dopamine). Besides, extract and animal

TABLE 2 | The characteristics of highly cited articles.

Rank	Total citations	Article title	Journal	Published year	Country	IF 2020	IF 5-years	SJR 2020
1	147	Pharmacokinetics of cathinone, cathine and norephedrine after the chewing of khat leaves	British Journal of Clinical Pharmacology	2003	Germany	4.335	4.902	1.216
2	128	Clinical characteristics of mephedrone toxicity reported to the UK National Poisons Information Service	Emergency Medicine Journal	2011	England	2.740	3.135	0.708
3	122	Khat use as risk factor for psychotic disorders: A cross-sectional and case-control study in Somalia	BMC Medicine	2005	Germany	8.775	10.249	3.463
4	107	Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography-electrospray tandem mass spectrometry	Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences	2011	Denmark	3.205	3.068	0.729
5	87	Khat and alcohol use and risky sex behaviour among in-school and out-of-school youth in Ethiopia	BMC Public Health	2005	Ethiopia	3.295	4.003	1.230
6	87	The prevalence and socio-demographic correlates of khat chewing in Butajira, Ethiopia	Acta Psychiatrica Scandinavica	1999	Ethiopia	6.392	5.706	2.849
7	82	Investigation into the toxicological effects of *Catha edulis* leaves: a short term study in animals	Phytotherapy Research	2002	Yemen	5.878	5.286	1.019
8	79	Khat chewing is a risk factor for acute myocardial infarction: a case-control study	British Journal of Clinical Pharmacology	2005	Wales	4.335	4.902	1.216
9	78	Khat (*Catha edulis*) consumption causes genotoxic effects in humans	International Journal of Cancer	2001	Austria	5.145		
10	75	Toxicological evaluation of *Catha edulis* leaves: a long term feeding experiment in animals	J Ethnopharmacol	2002	Yemen	3.690		

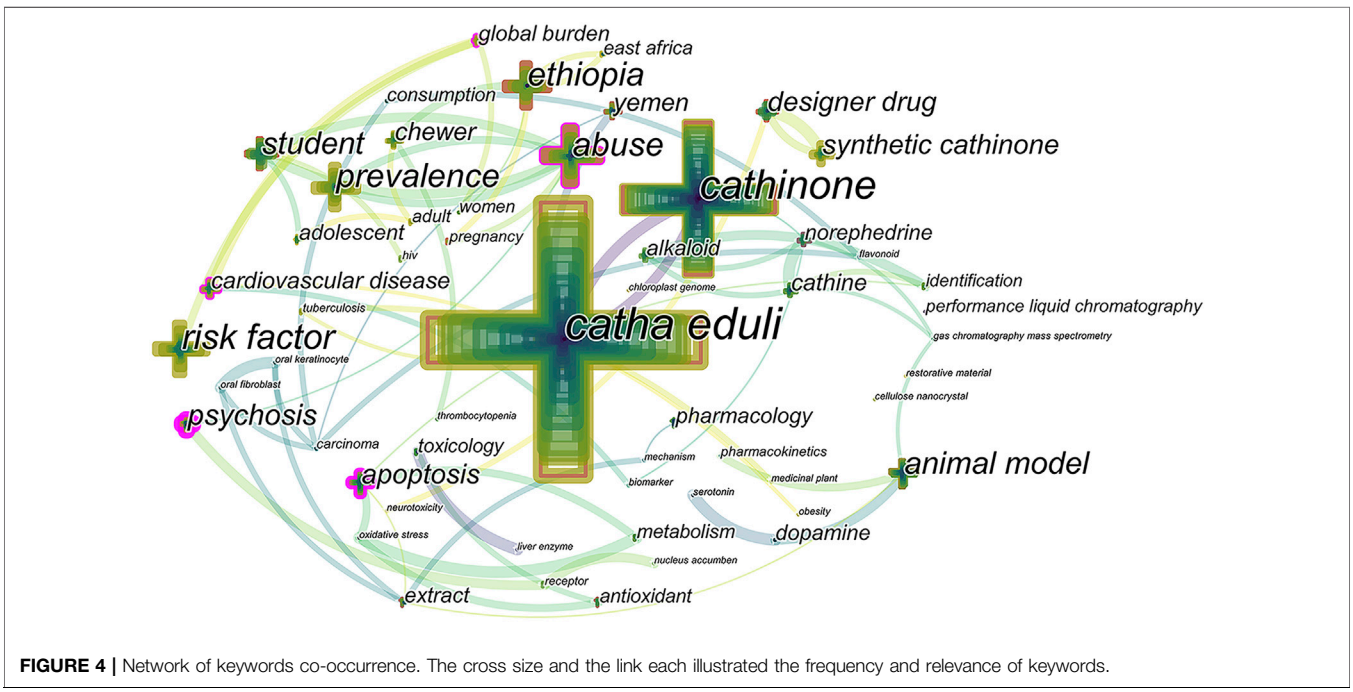

FIGURE 4 | Network of keywords co-occurrence. The cross size and the link each illustrated the frequency and relevance of keywords.

model(s) played an essential role in scientific phyto-research. In the last decade, the new designer drug started to be popular. Students have become an emerging part of new abusers of this medical plant.

DISCUSSION

This study conducts a visualized bibliometric analysis of the literature regarding *Catha edulis* from 1997 to 2020. During

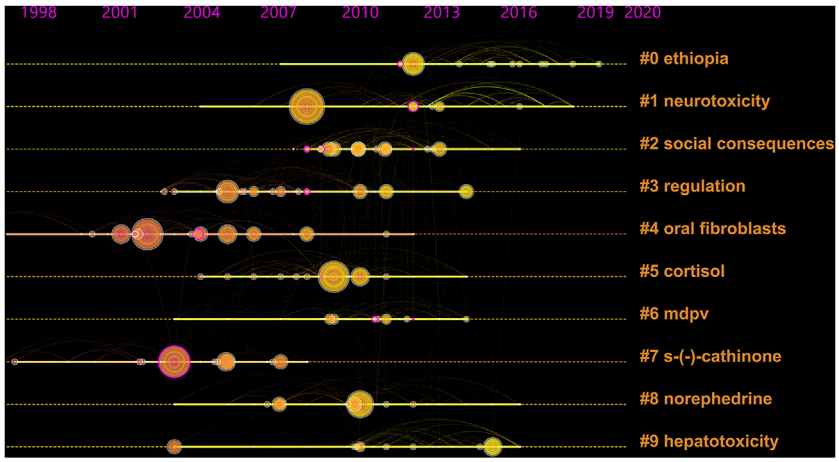

FIGURE 5 | Co-cited references timeline map of *Catha edulis*. Nodes on the map represent referenced documents. The size of nodes represents the frequencies of cited references, and the location reflects the present time. And years are arranged horizontally at the top. The clusters are performed based on the themes of co-cited references and the label of each cluster is shown at the end of the timeline.

Keywords	Year	Strength	Begin	End	1997 - 2020
cathinone	1997	10.9858	1997	2011	
khat (catha edulis)	1997	9.6078	1997	2006	
performance liquid chromatography	1997	3.7962	1997	2003	
prevalence	1997	6.3232	1998	2004	
psychosis	1997	4.5346	1999	2005	
risk factor	1997	3.3121	2003	2009	
toxicology	1997	2.1134	2004	2011	
pharmacology	1997	2.014	2005	2008	
dopamine	1997	3.1754	2006	2012	
animal model	1997	2.5491	2006	2009	
carcinoma	1997	2.4728	2007	2012	
apoptosis	1997	3.3776	2008	2009	
alkaloid	1997	3.1309	2010	2016	
cardiovascular disease	1997	3.0842	2011	2012	
designer drug	1997	3.6853	2012	2016	
student	1997	3.4147	2013	2018	
antioxidant	1997	3.0653	2013	2015	
medical plant	1997	3.0431	2014	2015	
extract	1997	1.9933	2016	2018	
global burden	1997	3.6376	2017	2020	

FIGURE 6 | Top 20 keywords with the strongest citation bursts. Each blue or red short line represents a year, and a red line stands for a burst detected year.

TABLE 3 | The estimated percentage of users in different countries/regions of East Africa and southwestern Arabian Peninsula.

Countries/regions	Percentage of *Catha edulis* users	
Saudi Arabia Jazan Region	43% males	11% females
Yemen	80–90% males	35–60% females 12–15% under 12 years
Djibouti	90%	
Ethiopia Harari (Hararghe) District	30–50%	
Somalia	18–55% males	10–25% females
Kenya	36.8%	
Kenya Coast & North Eastern District	88%	
Uganda	32%	

the past 24 years, *Catha edulis* publications from Ethiopia and Yemen increased due to the local prevailing major cash crops, legal use, and commercial trading. Consumed for socialization and leisure activities in East Africa and Arabian Peninsula, adults, adolescent students, and even pregnant women use *Catha edulis* daily; the estimated percentage of users in different countries and regions of East Africa and the southwestern Arabian Peninsula are extraordinarily high (**Table 3**) (Dimba et al., 2004; Al-Mugahed, 2008; Feyissa and Kelly, 2008; Odenwald and Al'Absi, 2017; Wondemagegn et al., 2017; Horn of Africa map, 2018). Gradually, *Catha edulis* has become prevalent in other parts of the world, such as Australia, Europe, North American countries, China, and Malaysia, owing to immigrant communities (Gebissa, 2010; Klein and Metaal, 2010), development of air transport (Kalix, 1992; Al-Hebshi and Skaug, 2005), and internet communication (Feyissa and Kelly, 2008). From the perspective of legislation, it is banned in the USA but is available and legal in some countries in Europe, including the UK, the Middle East, and Africa (Anderson and Carrier, 2009; Lemieux et al., 2015). So far, there are over 20 million *Catha edulis* abusers all over the world (Riyaz et al., 2014; Odenwald and Al'Absi, 2017), which causes emerging health concerns, such as psychiatric disorders, oral and cardiovascular disease, as well as corresponding research attention (Nichols et al., 2015). Theoretically, a legal restriction as an illicit drug would partly protect against the *Catha edulis* hazards in the USA (ACMD, 2013) and UK (Jones et al., 2014) compared to the producing regions or countries with the impact of cultural and traditional use (Gebissa, 2010). However, policies regarding the cultivation, transfer, trafficking, and use of *Catha edulis* are needed to be implemented. Public awareness should be launched to limit *Catha edulis* use and improve individual primary prevention activities.

For Ethiopia, the USA, Saudi Arabia, and Yemen, as leading countries of the research output, the reasons could be possibly different. Except for the large abuse population in these countries, the USA, usually offering abundant research budgets to support high-level research activities, acted as the main driving force with a high academic reputation in scientific research and characteristic H-index value, while the legal cultivation, trade, and consumption of *Catha edulis* in Ethiopia and Yemen facilitate scientific research productivity (Gebissa, 2010).

The productive countries' cooperation and research output are positive, especially among Ethiopia, the USA, and Saudi Arabia (Bozeman and Corley, 2004; Wagner, 2005; Huamani, 2015). Among these countries, Ethiopia and Saudi Arabia, also called the *Catha edulis* belt countries as main areas of commercial cultivation and consumption, could innately offer more information and collaborative opportunities, and the USA could be regarded as a financial grants and research collaboration provider to achieve higher productivity (Adams et al., 2014). With the prevalence of *Catha edulis* use, global cooperation should be promoted to improve research quality, and research outcomes should help recognize the necessity of legal restrictions and control of addiction at national levels (Douglas et al., 2011; Rahim et al., 2012).

The indicators, including the number of publications, citation per article, and H-index by a country, can be regarded as the quality of research activity in a specific field (Joshi, 2014; Luo et al., 2015). Our results show that the average citation per article on *Catha edulis* was 15.52, which was steadily increasing compared with the previous bibliometric results (Zyoud, 2015). Such vital information resources indicated that more attention was paid to the research related to *Catha edulis*. Though the average citations per article on tobacco and cannabis (25.27 and 27.23, retrieved by the WoS) were much higher than on *Catha edulis*, the citation growth on tobacco or cannabis were correspondingly declined compared to *Catha edulis* during the same period. This indicated that the amount of research focus on these two uncontrolled or divergent-legalization substances was steady, however, the yields of research attention poured into *Catha edulis* grew faster (Clermont et al., 2021).

Generally, the most cited articles are published in influential journals with high SJR or IF (Falagas et al., 2008; Kulkarni et al., 2009; Santa et al., 2010; de Granda-Orive et al., 2013). Our results show that more than half of the highly cited articles on *Catha edulis* focused on the adverse effects, and others related to mental health and harms to users and society were published in journals with IF 2020 < 10 and SJR 2020 < 4. Moreover, these top 10 highly cited articles were all published during 1999–2011. To some degree, this could be interpreted as these research performances gaining increasing attention in these years, which might help develop effective policies to gain public attention and prohibition measurements.

According to highly cited publications and keywords co-occurrence network, the active research area, including "prevalence", "risk factor", "chemical composition", "toxicology", and "pharmacology" over the past 24 years, are scoping and critical. According to these keywords, important research was retrieved. Similarly to amphetamine, a powerful psychotomimetic stimulant, *Catha edulis* produces various mental distress and psychotic symptoms such as irritability, insomnia, depression, reduced appetite, strange experiences, and hallucinations (Lemieux et al., 2015; Widmann, 2017; Abbay et al., 2018; Ongeri et al., 2019). The prolonged anorexia leads to low body weight in *Catha edulis* chewers and low birth weight of newborns in maternal users (Tesfay et al., 2019). The main chemical compounds of *Catha edulis*, cathinone, cathine, and norephedrine, account for all the psychostimulatory effects (Szendri, 1980). Cathinone, the natural amphetamine-like composition in *Catha edulis* with the highest levels in stems and young leaves, was proved to play a major role in this euphorising plant (Kalix, 1996; Alsanosy et al., 2020; Dhabbah, 2020). Some studies identified mechanisms of cathinone on the central nervous system in changing presynaptic striatal dopamine system and interfering with pituitary cell integrity in vervet monkeys (Nyongesa et al., 2014; Nyongesa et al., 2015), altering levels of dopamine and its metabolites and accelerating oxidative stress in limbic areas of swiss albino mice (Sathi et al., 2014; Safhi et al., 2018), inhibiting monoamine (dopamine, norepinephrine, etc) reuptake in human nerve cells (Bredholt et al., 2013), inducing striatal c-fos

expression in Siberian hamster (Jones et al., 2014), and other complex mechanisms of psychosis caused by *Catha edulis* use (Odenwald et al., 2005; Bredholt et al., 2013). The network, combined with the co-occurring keywords, demonstrated the progress and correlation of the original research of *Catha edulis* at a global level *via* bibliometric mapping.

Besides, the highly cited article showed that researchers were interested in the potential genotoxic effects of *Catha edulis* by micronucleus assay with exfoliated cells in humans. In light of the pronounced increase in micronucleated buccal mucosa cells of volunteers who chewed *Catha edulis* regularly, it suggests that *Catha edulis* chewing may cause genetic damage and further lead to cancer in the oral cavity or other parts of the upper digestive tract (Kassie et al., 2001). About 50% of *Catha edulis* chewers develop keratosis of buccal mucosa, a pre-cancerous lesion, and 2–12% of individuals with such lesions develop oral cancer (Ahmed et al., 2011). On the other side, *Catha edulis* extracts have been shown to induce oral fibroblasts and keratinocytes apoptosis and arrest in G1-phase *in vitro*, which further adds to speculations about anti-carcinogenicity of *Catha edulis* (Al-Maweri et al., 2018). Moreover, *Catha edulis* has been implicated in causing other symptoms such as periodontitis, caries, gastritis, hypertension, and acute myocardial infarction (Al-Hebshi and Skaug, 2005). Other studies also proved that genetic factors had an important role to greatly deepen the understanding of toxicity. In all, such research focused on the complex adverse effects to health over the past years, however, these may provide potential targets for treatment in the future.

The co-cited clusters include "s-(-)-cathinone" and "norephedrine" from 2003 to 2016. S-(-)-cathinone, one of the enantiomers of cathinone, is more psychoactive than its R antipode and is detected only in *Catha edulis* (Alsanosy et al., 2020). Unstable cathinone in *Catha edulis* would mainly degrade to cathine and norephedrine within 48 h after harvest (Alsenedi and Morrison, 2018). Research revealed that norephedrine extracted from *Catha edulis* could induce T-lymphocyte proliferation in Swiss albino mice, which may result in liver and kidney injury and immune-stimulation (Ketema et al., 2015). Cathine and norephedrine can directly affect mammalian sperm function, such as accelerating capacitation, inhibiting spontaneous acrosome loss, and enhancing natural fertility at appropriate doses (Adeoya-Osiguwa et al., 2005). Furthermore, other constituents of *Catha edulis* such as tannic acid may be mutagenic and carcinogenic in human buccal mucosa cells, which might account for combining effects as well as other chemical compounds like flavonoid and polyphenolics (Kassie et al., 2001). Generally, this bibliometric result confirmed that more research was performed to investigate the main components of *Catha edulis*, like cathine and norephedrine, and quite a few noticed others, including tannic acid, flavonoid, and polyphenolics.

According to the co-cited timeline of *Catha edulis* research evolution, cortisol (#5) has been a focus for a long time (Liu et al., 2016). Interestingly, the retrieved studies indicated contradictory results that cortisol was reduced in male olive baboons with oral administration of *Catha edulis* oil to regulate hormones (Mwenda et al., 2006); by contrast, dose-dependent increase of cortisol in male SD rats and male NZW rabbits was associated with *Catha edulis* extract treatment (Nyongesa et al., 2008; Mohammed and Engidawork, 2011). However, the elevation of cortisol in humans was more obvious among chewers in the early evening than non-chewers in a cross-sectional study (Al'Absi et al., 2013). Additionally, Catha edulis-induced high serotonin levels in the human brain were associated with decreased testosterone, which can inhibit the release of cortisol (Muniyappa et al., 2010; Montoya et al., 2012; Lovallo, 2013; Lovallo et al., 2015). All the studies mentioned above on *Catha edulis*-induced cortisol changes are inconclusive, which may be due to differences in chemical components between the original plant and its extracts, or different administration methods, frequencies, and doses, or interaction between different drugs abused by addicts, or stress induced by its use (Al'Absi et al., 2013). Based on these, further research was recommended on cortisol regulation, like *Catha edulis* caused sympathetic excitation (Alshagga et al., 2016).

Among the keywords with the strongest burst citation, the "designer drug" burst since 2012 indicates another option of synthetic cathinones compared with traditional *Catha edulis* (Karila et al., 2015; Wang et al., 2020). Compared with mild stimulants used for better concentration and performance during trading, farming, socialization, and leisure activities, heavy use and/or concomitant abuse of substances (poly-drug use behavior, such as *Catha edulis* and a designer drug) due to stronger psychostimulatory desire is rapidly growing (Ali, 2018). Except for *Catha edulis*, the natural structural basis of emerging designer drugs, synthetic cathinones, as a preferred constitution and/or replacement, became increasingly abused (Anderson and Carrier, 2009; Risca et al., 2020). In face of such overwhelming social problems induced by a complex combination of original plant and structure-modified analogs, the research frontiers would be transferred to pharmacological and toxicological effects and abuse risks of synthetic cathinones such as methcathinone (MC), mephedrone (MEPH), 4-methylmethcathinone (4-MMC), 3-fluoromethcathinone (3-FMC), and methylenedioxypyrovalerone (MDPV) (Carlsson et al., 2018; Risca et al., 2020), or polydrug abuse.

Considering the keywords shared by **Figures 4–6**, researchers have been exploring the therapeutic value of *Catha edulis* for decades. In the past, *Catha edulis* was traditionally perceived to treat headaches, common cold, and respiratory diseases (Al-Hebshi and Skaug, 2005). In recent years, many medicinal values of Catha edulis have been discovered gradually. Callus of Catha edulis have "HIV-1" reverse transcriptase inhibition effects and exhibit high antibacterial properties against both gram-positive and gram-negative bacteria compared to plant leaves (Kumari et al., 2015). Besides, the "medical plant" induces apoptosis in human breast cancer MDA-MB-231 cells *via* sustainable activation of C-Jun NH2-terminal kinase (JNK) and MAPK, and mitochondrial-mediated apoptosis pathway, which suggests that *Catha edulis* has substantial potential as a source of anticancer agents (Bredholt et al., 2009; Lu et al., 2017). Accordingly, the different effects of *Catha edulis* on different types of cancer cells might be a future research direction for medicinal potentials.

Moreover, the bibliometric results noticed other properties of Catha edulis. High dose *Catha edulis* extract, cathinone, blocked

the body weight gain of male mice on an obesity genic diet through upregulating lipolytic genes activity in white adipose tissue (Alshagga et al., 2020). Cathine in *Catha edulis* acted as an effective weight loss agent for adjunct treatment of obesity with significant weight loss in overweight and obese patients (Hauner et al., 2017). Poly (vinyl alcohol) hydrogels by cellulose nanofibers (CNFs) originated from *Catha edulis* have been prepared in the field of tissue engineering applications. The hydrogels, with favorable mechanical, thermal properties, biodegradation nature, and antimicrobial activities against pathogenic bacteria, are suitable for neural substitutes (Abdel Bary and Harmal, 2019a; Abdel Bary and Harmal, 2019b). More similar scientific studies could be future focus areas for more comprehensive medicinal plant research.

CONCLUSION

We made a systematic bibliometric assessment of the literature on *Catha edulis* from 1997 to 2020 and constructed a series of visualized graphs to elucidate the progress and emerging trends of the research. As an increasingly popular natural stimulant, the research trend was to explore not only phytochemical constituents and biological activities, but also toxicological and pharmacological effects and abuse threats, as well as the clinical therapeutic potential. This study is essential to raise public awareness of limited use and primary prevention activities of *Catha edulis*; furthermore, multidisciplinary efforts will still be needed to understand the further mechanisms of carcinogenic or antitumor effects, pathogenicity, and other medical values in the future. Getajiu and Krikoriaand, 1983, Kamdem et al., 2019, Miro et al., 2009, Regunath et al., 2012, Zyoud et al., 2017.

LIMITATIONS

There were inevitable limitations to our study. First, the science literature database (WoS) keeps constantly publishing from time to time, and the time lag between the publication and the retrieval of publications might affect the time-sensitivity of the research. Second, in order to use the co-occurrence and co-citation analysis methods by CiteSpace, only English studies were analyzed due to the incompatibility of multiple languages in the software. Last but not least, the full taxonomic name "*Catha edulis* (Vahl) Endl." was seldom used in previous publications; instead of "*Catha edulis*", it is not sufficient for a scientific name but suitable for bibliometric research.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ML and JH contributed to the conception and design of the study. SY organized the database and performed the statistical analysis. SY and JH wrote the first draft of the manuscript. ML and ZL reviewed and edited the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

FUNDING

This study was financially supported by the "National Engineering Laboratory for Forensic Science" (Grant No. 2019NELKFKT11) and the "Key Laboratory of Forensic Toxicology, Ministry of Public Security, People's Republic of China (Beijing Municipal Public Security Bureau)" (Grant No. 2019FTDWFX06).

ACKNOWLEDGMENTS

We would like to thank the reviewers for their insightful comments on the manuscript, as their remarks led to an improvement of the work. This study was supported by grants from the National Engineering Laboratory for Forensic Science and the Key Laboratory of Forensic Toxicology, Ministry of Public Security, People's Republic of China (Beijing Municipal Public Security Bureau).

REFERENCES

Abdel Bary, E. M., and Harmal, A. N. (2019a). A Novel Method to Prepare Microporous and Nanofibrous Hydrogel Scaffolds as Neural Tissue Engineering. *J. Macromolecular Sci. A* 56 (7), 648–657. doi:10.1080/10601325.2019.1593792

Abdel Bary, E. M., and Harmal, A. N. (2019b). A Novel Method to Prepare Three-Component Hydrogels as Neural Tissue Engineering. *Polym. Bull.* 76 (9), 4451–4468. doi:10.1007/s00289-018-2617-2

ACMD (2013). Khat: a Review of its Potential Harms to the Individual and Communities in the UK. Available at: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/144120/report-2013.pdf (Accessed March 9, 2021.

Adams, J., Gurney, K., Hook, D., and Leydesdorff, L. (2014). International Collaboration Clusters in Africa. *Scientometrics* 98 (1), 547–556. doi:10.1007/s11192-013-1060-2

Adeoya-Osiguwa, S. A., and Fraser, L. R. (2005). Cathine and Norephedrine, Both Phenylpropanolamines, Accelerate Capacitation and Then Inhibit Spontaneous Acrosome Loss. *Hum. Reprod.* 20 (1), 198–207. doi:10.1093/humrep/deh566

Ahmed, H. G., Omer, A. S., and Abd Algaffar, S. A. (2011). Cytological Study of Exfoliative Buccal Mucosal Cells of Qat Chewers in Yemen. *Diagn. Cytopathol* 39 (11), 796–800. doi:10.1002/dc.21462

al'Absi, M., Khalil, N. S., Al Habori, M., Hoffman, R., Fujiwara, K., and Wittmers, L. (2013). Effects of Chronic Khat Use on Cardiovascular, Adrenocortical, and Psychological Responses to Stress in Men and Women. *Am. J. Addict.* 22 (2), 99–107. doi:10.1111/j.1521-0391.2013.00302.x

Al-Hebshi, N., Al-haroni, M., and Skaug, N. (2006). *In Vitro* antimicrobial and Resistance-Modifying Activities of Aqueous Crude Khat Extracts against Oral Microorganisms. *Arch. Oral Biol.* 51 (3), 183–188. doi:10.1016/j.archoralbio.2005.08.001

Al-Hebshi, N. N., and Skaug, N. (2005). Khat (Catha Edulis)-An Updated Review. *Addict. Biol.* 10 (4), 299–307. doi:10.1080/13556210500353020

Al-Maweri, S. A., Warnakulasuriya, S., and Samran, A. (2018). Khat (Catha Edulis) and its Oral Health Effects: An Updated Review. *J. Investig. Clin. Dent* 9 (1), e12288. doi:10.1111/jicd.12288

Al-Mugahed, L. (2008). Khat Chewing in Yemen: Turning over a New Leaf. *Bull. World Health Organ.* 86 (10), 741–742. doi:10.2471/BLT.08.011008

Alfaifi, H., Abdelwahab, S. I., Mohan, S., Elhassan Taha, M. M., Syame, S. M., Shaala, L. A., et al. (2017). Catha Edulis Forsk. (Khat): Evaluation of its Antidepressant-like Activity. *Pharmacogn Mag.* 13 (502), S354–S358. doi:10.4103/pm.pm442_1610.4103/pm.pm_442_16

Ali, S. S. (2018). Substance Abuse in the Ethiopian Afar Pastoral Community. *J. Addict. Dis.* 37 (3-4), 245–251. doi:10.1080/10550887.2019.168048710.1080/10550887.2019.1668742

Alsanosy, R., Alhazmi, H. A., Sultana, S., Abdalla, A. N., Ibrahim, Y., Al Bratty, M., et al. (2020). Phytochemical Screening and Cytotoxic Properties of Ethanolic Extract of Young and Mature Khat Leaves. *J. Chem.* 2020, 1–9. doi:10.1155/2020/7897435

Alsenedi, K. A., and Morrison, C. (2018). Determination and Long-Term Stability of Twenty-Nine Cathinones and Amphetamine-type Stimulants (ATS) in Urine Using Gas Chromatography-Mass Spectrometry. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* 1076, 91–102. doi:10.1016/j.jchromb.2018.01.027

Alshagga, M. A., Alshawsh, M. A., Seyedan, A., Alsalahi, A., Pan, Y., Mohankumar, S. K., et al. (2016). Khat (Catha Edulis) and Obesity: A Scoping Review of Animal and Human Studies. *Ann. Nutr. Metab.* 69 (3-4), 200–211. doi:10.1159/000452895

Alshagga, M. A., Mohamed, Z., Seyedan, A., Ebling, F. J. P., and Alshawsh, M. A. (2020). Khat (Catha Edulis) Upregulates Lipolytic Genes in white Adipose Tissue of Male Obese Mice (C57BL/6J). *J. Ethnopharmacol* 262, 113187. doi:10.1016/j.jep.2020.113187

Anderson, D., and Carrier, N. (2009). Khat in Colonial kenya: A History of Prohibition and Control. *J. Afr. Hist.* 50 (3), 377–397. doi:10.1017/S0021853709990752

Bozeman, B., and Corley, E. (2004). Scientists' Collaboration Strategies: Implications for Scientific and Technical Human Capital. *Res. Pol.* 33 (4), 599–616. doi:10.1016/j.respol.2004.01.008

Bredholt, T., Dimba, E. A., Hagland, H. R., Wergeland, L., Skavland, J., Fossan, K. O., et al. (2009). Camptothecin and Khat (Catha Edulis Forsk.) Induced Distinct Cell Death Phenotypes Involving Modulation of C-FLIPL, Mcl-1, Procaspase-8 and Mitochondrial Function in Acute Myeloid Leukemia Cell Lines. *Mol. Cancer* 8, 101. doi:10.1186/1476-4598-8-101

Bredholt, T., Ersvær, E., Erikstein, B. S., Sulen, A., Reikvam, H., Aarstad, H. J., et al. (2013). Distinct Single Cell Signal Transduction Signatures in Leukocyte Subsets Stimulated with Khat Extract, Amphetamine-like Cathinone, Cathine or Norephedrine. *BMC Pharmacol. Toxicol.* 14, 35. doi:10.1186/2050-6511-14-35

Carlsson, A., Sandgren, V., Svensson, S., Konradsson, P., Dunne, S., Josefsson, M., et al. (2018). Prediction of Designer Drugs: Synthesis and Spectroscopic Analysis of Synthetic Cathinone Analogs that May Appear on the Swedish Drug Market. *Drug Test. Anal.* 10 (7), 1076–1098. doi:10.1002/dta.2366

Chen, C. (2004). Searching for Intellectual Turning Points: Progressive Knowledge Domain Visualization. *Proc. Natl. Acad. Sci. U S A.* 101 Suppl 1 (Suppl. 1), 5303–5310. doi:10.1073/pnas.0307513100

Chen, C. (2006). CiteSpace II: Detecting and Visualizing Emerging Trends and Transient Patterns in Scientific Literature. *J. Am. Soc. Inf. Sci.* 57 (3), 359–377. doi:10.1002/asi.20317

Chen, C., Dubin, R., and Kim, M. C. (2014). Orphan Drugs and Rare Diseases: a Scientometric Review (2000 - 2014). *Expert Opin. Orphan Drugs* 2 (7), 709–724. doi:10.1517/21678707.2014.920251

Chen, C., Ibekwe-SanJuan, F., and Hou, J. (2010). The Structure and Dynamics of Cocitation Clusters: A Multiple-Perspective Cocitation Analysis. *J. Am. Soc. Inf. Sci.* 61 (7), 1386–1409. doi:10.1002/asi.21309

Clermont, M., Krolak, J., and Tunger, D. (2021). Does the Citation Period Have Any Effect on the Informative Value of Selected Citation Indicators in Research Evaluations? *Scientometrics* 126, 1019–1047. doi:10.1007/s11192-020-03782-1

Dhabbah, A. M. (2020). Determination of Chiral Cathinone in Fresh Samples of Catha Edulis. *Forensic Sci. Int.* 307, 110105. doi:10.1016/j.forsciint.2019.110105

Dimba, E. A., Gjertsen, B. T., Bredholt, T., Fossan, K. O., Costea, D. E., Francis, G. W., et al. (2004). Khat (Catha Edulis)-Induced Apoptosis Is Inhibited by Antagonists of Caspase-1 and -8 in Human Leukaemia Cells. *Br. J. Cancer* 91 (9), 1726–1734. doi:10.1038/sj.bjc.6602197

Douglas, H., Boyle, M., and Lintzeris, N. (2011). The Health Impacts of Khat: a Qualitative Study Among Somali-Australians. *Med. J. Aust.* 195 (11-12), 666–669. doi:10.5694/mja11.10166

Elmai (1983). *Khat Consumption and Problems in somalia*. Antananarivo, Madagascar: International Conference on Khat.

Feyissa, A. M., and Kelly, J. P. (2008). A Review of the Neuropharmacological Properties of Khat. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 32 (5), 1147–1166. doi:10.1016/j.pnpbp.2007.12.033

Gaillard, J. (1992). Science Policies and Cooperation in Africa. *Knowledge* 14 (2), 212–233. doi:10.1177/107554709201400212

Gebissa, E. (2010). Khat in the Horn of Africa: Historical Perspectives and Current Trends. *J. Ethnopharmacol* 132 (3), 607–614. doi:10.1016/j.jep.2010.01.063

Gebissa, E. (2004). *Leaf of Allah: Khat and Agricultural Transformation in Harerge, Ethiopia, 1875 - 1991*. Oxford: James curry Ltd.

Gebrekidan Abbay, A., Tibebe Mulatu, A., and Azadi, H. (2018). Community Knowledge, Perceived Beliefs and Associated Factors of Mental Distress: A Case Study from Northern Ethiopia. *Int. J. Environ. Res. Public Health* 15, 2423. doi:10.3390/ijerph15112423

Getajiu, A., and Krikoriaand, N. (1983). The Economic and Social Importance of Khat and Suggested Research and Services. Antananarivo, Madagascar: 1983 Proceedings of the International Conference on Khat.

Giannini, A. J., Miller, N. S., and Turner, C. E. (1992). Treatment of Khat Addiction. *J. Subst. Abuse Treat.* 9 (4), 379–382. doi:10.1016/0740-5472(92)90034-L

Graziani, M., Milella, M. S., and Nencini, P. (2008). Khat Chewing from the Pharmacological point of View: an Update. *Subst. Use Misuse* 43 (6), 762–783. doi:10.1080/10826080701738992

Hauner, H., Hastreiter, L., Werdier, D., Chen-Stute, A., Scholze, J., and Blüher, M. (2017). Efficacy and Safety of Cathine (Nor-Pseudoephedrine) in the Treatment of Obesity: A Randomized Dose-Finding Study. *Obes. Facts* 10 (4), 407–419. doi:10.1159/000478098

Horn of Africa map (Cartographer) (2018). Horn of Africa - States [map]. Available at: https://d-maps.com/carte.php?num_car=34260andlang=en (Accessed March 9, 2021.

Huamaní, C., Rey de Castro, J., González-Alcaide, G., Polesel, D. N., Tufik, S., and Andersen, M. L. (2015). Scientific Research in Obstructive Sleep Apnea Syndrome: Bibliometric Analysis in SCOPUS, 1991-2012. *Sleep Breath* 19 (1), 109–114. doi:10.1007/s11325-014-0969-x

Jones, S., Fileccia, E. L., Murphy, M., Fowler, M. J., King, M. V., Shortall, S. E., et al. (2014). Cathinone Increases Body Temperature, Enhances Locomotor Activity, and Induces Striatal C-Fos Expression in the Siberian Hamster. *Neurosci. Lett.* 559, 34–38. doi:10.1016/j.neulet.2013.11.032

Joshi, M. A. (2014). Bibliometric Indicators for Evaluating the Quality of Scientifc Publications. *J. Contemp. Dent Pract.* 15 (2), 258–262. doi:10.5005/jp-journals-10024-1525

Kalix, P. (1996). Catha Edulis, a Plant that Has Amphetamine Effects. *Pharm. World Sci.* 18 (2), 69–73. doi:10.1007/BF00579708

Kalix, P. (1992). Cathinone, a Natural Amphetamine. *Pharmacol. Toxicol.* 70, 77–86. doi:10.1111/j.1600-0773.1992.tb00434.x

Kalix, P. (1987). Khat: Scientific Knowledge and Policy Issues. *Br. J. Addict.* 82 (1), 47–53. doi:10.1111/j.1360-0443.1987.tb01436.x

Kamdem, J. P., Duarte, A. E., Lima, K. R. R., Rocha, J. B. T., Hassan, W., Barros, L. M., et al. (2019). Research Trends in Food Chemistry: A Bibliometric Review of its 40 years Anniversary (1976-2016). *Food Chem.* 294, 448–457. doi:10.1016/j.foodchem.2019.05.021

Kandari, L. S., Yadav, H. R., Thakur, A. K., and Kandari, T. (2014). Chat (Catha Edulis): a Socio Economic Crop in Harar Region, Eastern Ethiopia. *SpringerPlus* 3, 579. doi:10.1186/2193-1801-3-579

Karila, L., Megarbane, B., Cottencin, O., and Lejoyeux, M. (2015). Synthetic Cathinones: A New Public Health Problem. *Curr. Neuropharmacol* 13 (1), 12–20. doi:10.2174/1570159X13666141210224137

Kassie, F., Darroudi, F., Kundi, M., Schulte-Hermann, R., and Knasmüller, S. (2001). Khat (Catha Edulis) Consumption Causes Genotoxic Effects in Humans. *Int. J. Cancer* 92 (3), 329–332. doi:10.1002/ijc.1195

Ketema, T., Yohannes, M., Alemayehu, E., and Ambelu, A. (2015). Evaluation of Immunomodulatory Activities of Methanolic Extract of Khat (Catha Edulis, Forsk) and Cathinone in Swiss Albino Mice. *BMC Immunol.* 16, 9. doi:10.1186/s12865-015-0072-5

Klein, A., and Metaal, P. (2010). A Good Chew or Good Riddance-Hhow to Move Forward in the Regulation of Khat Consumption. *J. Ethnopharmacol* 132 (3), 584–589. doi:10.1016/j.jep.2010.07.005

Kleinberg, J. (2003). Bursty and Hierarchical Structure in Streams. *Data Min Knowl Discov.* 7 (4), 373–397. doi:10.1038/s41598-020-80059-w10.1023/a:1024940629314

Kumari, A., Baskaran, P., and Van Staden, J. (2015). Enhanced HIV-1 Reverse Transcriptase Inhibitory and Antibacterial Properties in Callus of Catha Edulis Forsk. *Phytother Res.* 29 (6), 840–843. doi:10.1002/ptr.5318

Lemieux, A. M., Li, B., and al'Absi, M. (2015). Khat Use and Appetite: An Overview and Comparison of Amphetamine, Khat and Cathinone. *J. Ethnopharmacol* 160, 78–85. doi:10.1016/j.jep.2014.11.002

Liu, X. H., Xie, B. W., Wang, Z. J., Jin, L., and Zhang, Y. G. (2016). The Secretion, Synthesis, and Metabolism of Cortisol and its Downstream Genes in the H-P-I axis of Rare Minnows (Gobiocypris Rarus) Are Disrupted by Acute Waterborne Cadmium Exposure. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 185-186, 112–121. doi:10.1016/j.cbpc.2016.03.009

Lovallo, W. R. (2013). Early Life Adversity Reduces Stress Reactivity and Enhances Impulsive Behavior: Implications for Health Behaviors. *Int. J. Psychophysiol* 90(1), 8–16. doi: doi:doi:10.1016/j.ijpsycho.2012.10.006

Lovallo, W. R., Enoch, M. A., Acheson, A., Cohoon, A. J., Sorocco, K. H., Hodgkinson, C. A., et al. (2015). Cortisol Stress Response in Men and Women Modulated Differentially by the Mu-Opioid Receptor Gene Polymorphism OPRM1 A118G. *Neuropsychopharmacology* 40 (11), 2546–2554. doi:10.1038/npp.2015.101

Lu, Y., Li, Y., Xiang, M., Zhou, J., and Chen, J. (2017). Khat Promotes Human Breast Cancer MDA-MB-231 Cell Apoptosis via Mitochondria and MAPK-Associated Pathways. *Oncol. Lett.* 14 (4), 3947–3952. doi:10.3892/ol.2017.6708

Luo, X., Liang, Z., Gong, F., Bao, H., Huang, L., and Jia, Z. (2015). Worldwide Productivity in the Field of Foot and Ankle Research from 2009-2013: a Bibliometric Analysis of Highly Cited Journals. *J. Foot Ankle Res.* 8, 12. doi:10.3892/ol.2017.670810.1186/s13047-015-0070-0

Miró, O., Montori, E., Ramos, X., Galicia, M., and Nogué, S. (2009). Trends in Research Activity in Toxicology and by Toxicologists in Seven European Countries. *Toxicol. Lett.* 189 (1), 1–4. doi:10.1016/j.toxlet.2009.04.029

Mohammed, A., and Engidawork, E. (2011). Reproductive Parameters Are Differentially Altered Following Subchronic Administration of Catha Edulis F. (Khat) Extract and Cathinone in Male Rats. *J. Ethnopharmacol* 134 (3), 977–983. doi:10.1016/j.jep.2011.02.006

Montoya, E. R., Terburg, D., Bos, P. A., and van Honk, J. (2012). Testosterone, Cortisol, and Serotonin as Key Regulators of Social Aggression: A Review and Theoretical Perspective. *Motiv. Emot.* 36 (1), 65–73. doi:10.1007/s11031-011-9264-3

Morghemm, M., and Rufatm, I. (1983). Cultivation and Chewing of Khat in the yemen arab republic. Antananarivo, Madagascar: International Conference on Khat.

Muniyappa, R., Veldhuis, J. D., Harman, S. M., Sorkin, J. D., and Blackman, M. R. (2010). Effects of Testosterone Administration on Nocturnal Cortisol Secretion in Healthy Older Men. *J. Gerontol. A. Biol. Sci. Med. Sci.* 65 (11), 1185–1192. doi:10.1093/gerona/glq128

Mwenda, J. M., Owuor, R. A., Kyama, C. M., Wango, E. O., M'Arimi, M., and Langat, D. K. (2006). Khat (Catha Edulis) Up-Regulates Testosterone and Decreases Prolactin and Cortisol Levels in the Baboon. *J. Ethnopharmacol* 103 (3), 379–384. doi:10.1016/j.jep.2005.08.016

Niazi, M., and Hussain, A. (2011). Agent-based Computing from Multi-Agent Systems to Agent-Based Models: A Visual Survey. *Scientometrics* 89 (2), 479–499. doi:10.1007/s11192-011-0468-9

Nichols, T., Khondkar, P., and Gibbons, S. (2015). The Psychostimulant Drug Khat (Catha Edulis): A Mini-Review. *Phytochemistry Lett.* 13, 127–133. doi:10.1016/j.phytol.2015.05.016

Nyongesa, A., Oduma, J., Al'Absi, M., and Chirwa, S. (2015). Immunohistochemical Localization of Anterior Pituitary Cell Types of Vervet Monkey (Chlorocebus Aethiops) Following Sub-chronic Cathinone Exposure. *J. Ethnopharmacol* 174, 168–177. doi:10.1016/j.jep.2015.08.007

Nyongesa, A. W., Oduma, J. A., Nakajima, M., Odongo, H. O., Adoyo, P. A., and al'Absi, M. (2014). Acute and Sub-chronic Effects of Purified Cathinone from Khat (Catha Edulis) on Behavioural Profiles in Vervet Monkeys (Chlorocebus Aethiops). *Metab. Brain Dis.* 29 (2), 441–449. doi:10.1007/s11011-013-9441-z

Nyongesa, A. W., Patel, N. B., Onyango, D. W., Odongo, H. O., and Wango, E. O. (2008). Khat (Catha Edulis) Lowers Plasma Luteinizing Hormone (LH) and Testosterone Secretion, but Increases Cortisol Levels in Male Rabbits. *J. Ethnopharmacol* 116 (2), 245–250. doi:10.1016/j.jep.2007.11.022

Odenwald, M., and Al'Absi, M. (2017). Khat Use and Related Addiction, Mental Health and Physical Disorders: the Need to Address a Growing Risk. *East. Mediterr. Health J.* 23 (3), 236–244. doi:10.26719/2017.23.3.236

Odenwald, M., Neuner, F., Schauer, M., Elbert, T., Catani, C., Lingenfelder, B., et al. (2005). Khat Use as Risk Factor for Psychotic Disorders: a Cross-Sectional and Case-Control Study in Somalia. *BMC Med.* 3 (1), 5–9. doi:10.1186/1741-7015-3-5

Omar, Y. S., Jenkins, A., Altena, Mv., Tuck, H., Hynan, C., Tohow, A., et al. (2015). Khat Use: what Is the Problem and what Can Be Done? *Biomed. Res. Int.* 2015, 472302. doi:10.1155/2015/472302

Ongeri, L., Kirui, F., Muniu, E., Manduku, V., Kirumbi, L., Atwoli, L., et al. (2019). Khat Use and Psychotic Symptoms in a Rural Khat Growing Population in Kenya: a Household Survey. *BMC Psychiatry* 19 (1), 137. doi:10.1186/s12888-019-2118-3

Rahim, B. E. A., Yagoub, U., Mahfouz, M. S., Solan, Y. M. H., and Alsanosi, R. (2012). Abuse of Selected Psychoactive Stimulants: Overview and Future Research Trends. *Life Sci. J.* 9 (4), 2295–2308.

Regunath, H., Ariyamuthu, V. K., Dalal, P., and Misra, M. (2012). Bath Salt Intoxication Causing Acute Kidney Injury Requiring Hemodialysis. *Hemodial Int.* 16 Suppl 1, S47–S49. doi:10.1111/j.1542-4758.2012.00750.x

Risca, H. I., Zuarth-Gonzalez, J. D., and Baker, L. E. (2020). Conditioned Place Preference Following Concurrent Treatment with 3, 4-methylenedioxypyrovalerone (MDPV) and Methamphetamine in Male and Female Sprague-Dawley Rats. *Pharmacol. Biochem. Behav.* 198, 173032. doi:10.1016/j.pbb.2020.173032

Riyaz, S., Imran, M., Gleeson, D., and Karajeh, M. A. (2014). Khat (Catha Edulis) as a Possible Cause of Autoimmune Hepatitis. *World J. Hepatol.* 6 (3), 150–154. doi:10.4254/wjh.v6.i3.150

Safhi, M. M., Alam, M. F., Khuwaja, G., Hussain, S., Siddiqui, M. H., Islam, F., et al. (2018). Catha Edulis Active Principle, Cathinone, Suppresses Motor Coordination, Accelerates Anxiety and Alters the Levels of Dopamine and its Metabolites in the Limbic Areas of Male Swiss Albino Mice. *Acta Pharm.* 68 (4), 485–495. doi:10.2478/acph-2018-0038

Safhi, M. M., Alam, M. F., Hussain, S., Hakeem Siddiqui, M. A., Khuwaja, G., Jubran Khardali, I. A., et al. (2014). Cathinone, an Active Principle of Catha Edulis, Accelerates Oxidative Stress in the Limbic Area of Swiss Albino Mice. *J. Ethnopharmacology* 156, 102–106. doi:10.1016/j.jep.2014.08.004

Szendri, K. (1980). The Chemistry of Khat. *Bull. Narcotics* 32 (3), 5–35.

Tesfay, K., Abera, M., Wondafrash, M., and Tesfaye, M. (2019). Effect of Khat Use during Pregnancy on the Birth Weight of Newborn in Jimma, Ethiopia. *Int. J. Ment. Health Addict.* 17 (6), 1432–1441. doi:10.1007/s11469-018-9888-6

Wagner, C. S. (2005). Six Case Studies of International Collaboration in Science. *Scientometrics* 62 (1), 3–26. doi:10.1007/s11192-005-0001-0

Wang, K., Duan, W., Duan, Y., Yu, Y., Chen, X., Xu, Y., et al. (2020). A Bibliometric Insight of Genetic Factors in ASD: Emerging Trends and New Developments. *Brain Sci.* 11 (1), 33. doi:10.3390/brainsci11010033

Widmann, M., Apondi, B., Musau, A., Warsame, A. H., Isse, M., Mutiso, V., et al. (2017). Comorbid Psychopathology and Everyday Functioning in a Brief Intervention Study to Reduce Khat Use Among Somalis Living in Kenya: Description of Baseline Multimorbidity, its Effects of Intervention and its

Moderation Effects on Substance Use. *Soc. Psychiatry Psychiatr. Epidemiol.* 52 (11), 1425–1434. doi:10.1007/s00127-017-1368-y

Wondemagegn, A. T., Cheme, M. C., and Kibret, K. T. (2017). Perceived Psychological, Economic, and Social Impact of Khat Chewing Among Adolescents and Adults in Nekemte Town, East Welega Zone, West Ethiopia. *Biomed. Res. Int.* 2017, 7427892. doi:10.1155/2017/7427892

Zupic, I., and Čater, T. (2015). Bibliometric Methods in Management and Organization. *Organizational Res. Methods* 18 (3), 429–472. doi:10.1177/1094428114562629

Zyoud, S. H. (2015). Bibliometric Analysis on Global Catha Edulis (Khat) Research Production during the Period of 1952-2014. *Glob. Health* 11, 39. doi:10.1186/s12992-015-0124-x

Zyoud, S. H., Waring, W. S., Al-Jabi, S. W., and Sweileh, W. M. (2017). Global Cocaine Intoxication Research Trends during 1975-2015: A Bibliometric Analysis of Web of Science Publications. *Subst. Abuse Treat. Prev. Pol.* 12 (1), 6. doi:10.1186/s13011-017-0090-9

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 18 November 2021
doi: 10.3389/fphar.2021.766402

Immunological and Preventive Effects of Hochuekkito and Kakkonto Against Coronavirus Disease in Healthcare Workers: A Retrospective Observational Study

Keiko Ogawa-Ochiai[1], Hideki Ishikawa[2], Hongyang Li[1], Lam Vu Quang[3], Izumi Kimoto[4], Mitsuyuki Takamura[5], Tetsuya Hongawa[6], Yasuyuki Hane[7], Susumu Suzuki[8,9], Masaki Okajima[10], Keita Mori[11], Masanori Ito[12] and Akiyoshi Takami[3]*

[1]Kampo Clinical Center, Department of General Medicine, Hiroshima University Hospital, Hiroshima, Japan, [2]Department of Molecular-Targeting Cancer Prevention, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Osaka, Japan, [3]Department of Internal Medicine, Division of Hematology, Aichi Medical University School of Medicine, Nagakute, Japan, [4]Hirose Clinic, Kariya, Japan, [5]Kampo Medicine Outpatient Clinic, Mie University Hospital, Tsu, Japan, [6]Shizuoka Prefectural Government Office Clinic, Shizuoka, Japan, [7]Hane Pediatric Clinic, Toba, Japan, [8]Department of Tumor Immunology, Aichi Medical University School of Medicine, Nagakute, Japan, [9]Research Creation Support Center, Aichi Medical University, Nagakute, Japan, [10]Department of Emergency and Disaster Medicine, Faculty of Medicine Institute of Medical, Pharmaceutical and Health Sciences Kanazawa University, Kanazawa, Japan, [11]Clinical Research Support Center, Shizuoka Cancer Center, Shizuoka, Japan, [12]Department of General Medicine, Hiroshima University Hospital, Hiroshima, Japan

OPEN ACCESS

Edited by:
Silke Cameron, University Medical Center Göttingen, Germany

Reviewed by:
Denichiro Yamaoka, Ehime University, Japan
Nqobile Masondo, Agricultural Research Council of South Africa (ARC-SA), South Africa

***Correspondence:**
Keiko Ogawa-Ochiai
ikkandoo@gmail.com

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

Received: *29 August 2021*
Accepted: *19 October 2021*
Published: *18 November 2021*

Citation:
Ogawa-Ochiai K, Ishikawa H, Li H, Vu Quang L, Kimoto I, Takamura M, Hongawa T, Hane Y, Suzuki S, Okajima M, Mori K, Ito M and Takami A (2021) Immunological and Preventive Effects of Hochuekkito and Kakkonto Against Coronavirus Disease in Healthcare Workers: A Retrospective Observational Study. Front. Pharmacol. 12:766402. doi: 10.3389/fphar.2021.766402

Amid the global outbreak of coronavirus disease 2019 (COVID-19), it may be expected that low-toxicity natural compounds, such as Kampo formulas, will have a preventive effect on COVID-19. Although the biological properties and safety of the representative Kampo compounds, hochuekkito (HET) and kakkonto (KKT), have been confirmed in various animal model experiments, clinical studies, and a few human studies to induce biological effects on various infectious diseases without significant toxicity, it is unclear whether HET and KKT are safe and effective for COVID-19 prevention. The study population included healthcare workers (HCWs), as they are at a higher risk of infection than the other populations. We retrospectively investigated the immunological and preventive effects of HET and KTT against COVID-19. We included 27 HCWs (aged 21–72 years, F:M = 18:9) from hospitals and clinics of the Hokuriku-Tokai region. The HCWs received HET and KKT for general fatigue and myalgia during this period for 28 days. We obtained patient clinical data from electronic medical records. We analyzed the changes in immunomodulation before and after the administration of the formulas from residual specimens based on the expression of relevant surface markers. The specimens were also tested for the presence of antibodies against severe acute respiratory syndrome coronavirus 2. The following side effects were reported: abdominal discomfort in five patients, diarrhea in two, and loose or soft stool in three. All 27 HCWs tested negative for COVID-19 antibodies. HET and KKT administration significantly increased the absolute number of circulating lymphocytes expressing the activating receptors NKp46, NKp30, and suppressing receptor NKG2A. There was also a significant increase in the absolute number of circulating lymphocytes expressing the receptors TLR4, OX40, 4–1BB, GITR, PD-1, and ICOS.

These data indicate that HET and KKT can enhance and modulate NK activity in circulating human immune cells. The immunomodulatory effects, such as activation and regulation of T cells, are consistent with a putative improvement in infectious immunosurveillance. An increase in the number of T cells and CD4/CD8-positive cells indicates an enhanced ability to protect against infection. HET and KKT may prevent the onset or worsening of COVID-19 through their immunomodulatory effects.

Keywords: Kampo medicine, immunomodulation, COVID-19, SARS-CoV-2, healthcare workers, Kampo formulas, hochuekkito, kakkonto

INTRODUCTION

Healthcare workers (HCWs) may experience an increased risk of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection because of close contact with infected patients. HCWs have emerged as a critical population during the current coronavirus disease (COVID-19) pandemic. During the pandemic, 138 patients, including 40 HCWs (29%), were admitted to Zhongnan Hospital in Wuhan (Wang et al., 2020). Another retrospective analysis of 1099 patients with confirmed COVID-19 in 552 hospitals from 31 provinces found that the proportion of HCWs was 2.09%. It is very important to safely protect frontline HCWs from acquiring severe SARS-CoV-2 infections. The plight of HCWs during the pandemic has been widely noted (Gao et al., 2020; Kluytmans-van den Bergh et al., 2020). Moreover, infection control requires a lot of effort and time, and it is presumed that most HCWs become exhausted and reach an immunocompromised state. Between February and July 2020, hochuekkito (HET) and kakkonto (KKT) were prescribed to patients who wanted the HCWs to alleviate fatigue.

Hochuekkito (HET, Bu-Zhong-Yi-Qi-Tang in Chinese) is an herbal formula of Kampo medicine that is widely used for the treatment of severe weakness, loss of appetite, and indigestion in elderly patients and for the prevention of opportunistic infections. HET contains the following 10 crude drugs: Astragali radix (*Astragalus membranaceus* var. *mongholicus* (Bunge)), Atractylodis lanceae rhizoma (*Atractylodes lancea* (Thunb.) DC.), Ginseng radix (*Panax ginseng* C. A. Mey.), Angelicase radix (*Angelica acutiloba* (Siebold and Zucc.) Kitag.), Bupleuri radix (*Bupleurum falcatum* L.), Zizyphi fructus (*Ziziphus jujuba* var. inermis (Bunge) Rehder), Citri Unshiu Pericarpium (*Citrus unshiu* (Yu.Tanaka ex Swingle) Marcow.), Glycyrrhizae radix (*Glycyrrhiza uralensis* Fisch.), Cimicifugae rhizoma (*Betula dahurica* var. maximowiczii (Rupr.) Trautv.), and Zingiberis rhizome (*Zingiber officinale Roscoe*) The therapeutic effects of HET have been reported in terms of improving weakness in the elderly (Kuroiwa et al., 2004; Satoh et al., 2005) and preventing chronic obstructive pulmonary disease in the elderly (Tatsumi et al., 2009). HET has demonstrated improvements in fungal infection (Abe et al., 1999), protective action against *Listeria monocytogenes* infection (Yamaoka et al., 2001), and antiviral action through the activation of natural killer (NK) T cells (Yamaya M, 2007). HET is expected to exert effectiveness for preventing infectious diseases including COVID-19. HET promotes the production of interferon and inhibits the production of interleukin (IL)-1α and IL-6 (Tokura et al., 1998; Mori et al., 1999). In this study, HET was used in the form of spray-dried decoction extracts of a mixture of ten medicinal plants (manufactured by Tsumura and Co., Tokyo, Japan).

Kakkonto (KKT), a traditional Japanese herbal medicine, is commonly used in Japan. KKT contains the following seven crude drugs: Cinnamomi cortex (*Cinnamomum cassia* (L.) J. Presl), Zingiberis rhizome (*Zingiber officinale Roscoe*), Paeonia lactiflora Radix (*Paeonia lactiflora Pallas*), Zizyphi fructus (*Ziziphus jujuba* var. inermis (Bunge) Rehder), Glycyrrhizae radix (*Glycyrrhiza uralensis* Fisch.), Ephedrae Herba (*Ephedra sinica* Stapf) and Puerariae Radix (*Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep). KKT is considered to be a highly effective and safe medicine for the treatment of the common cold or influenza in Japan; the Ephedra herb in KKT contains tannins, and they inhibit endosome acidification and influenza A virus fusion to the cell membrane (Mantani et al., 1999). Glycyrrhizin, an active component of glycyrrhiza, exerts its effects by reducing the number of cells infected with the influenza A virus and by inhibiting viral uptake through the cell membrane during the early phase of infection (Wolkerstorfer et al., 2009). Cinnamaldehyde, which is derived from cinnamon bark, inhibits viral protein synthesis (influenza A virus) at the post-transcriptional level. In one research carried out in mice, inhalation and nasal inoculation of cinnamaldehyde after viral infection increased the survival rate (Hayashi et al., 2007). Puerarin, one of the bioactive compounds from Puerariae Radix, is an isoflavonoid that exerts many pharmacological effects such as anti-inflammatory, vasodilation, neuroprotective, antioxidant, and anticancer effects (Zhou et al., 2013). Kampo formulas including HET have been approved by the Ministry of Health, Labor, and Welfare in Japan as a prescription covered under the National Health Insurance; therefore, HET can be easily used for the treatment of patients.

In medical literature, there is accumulating evidence for the biological properties and safety of HET and KKT, but it is unclear whether HET and KKT are safe and effective for COVID-19 prevention. In this study, we intended to study the effectiveness of the HET/KKT combination in improving immunity, which explains the empirical use of these formulas based on Kampo

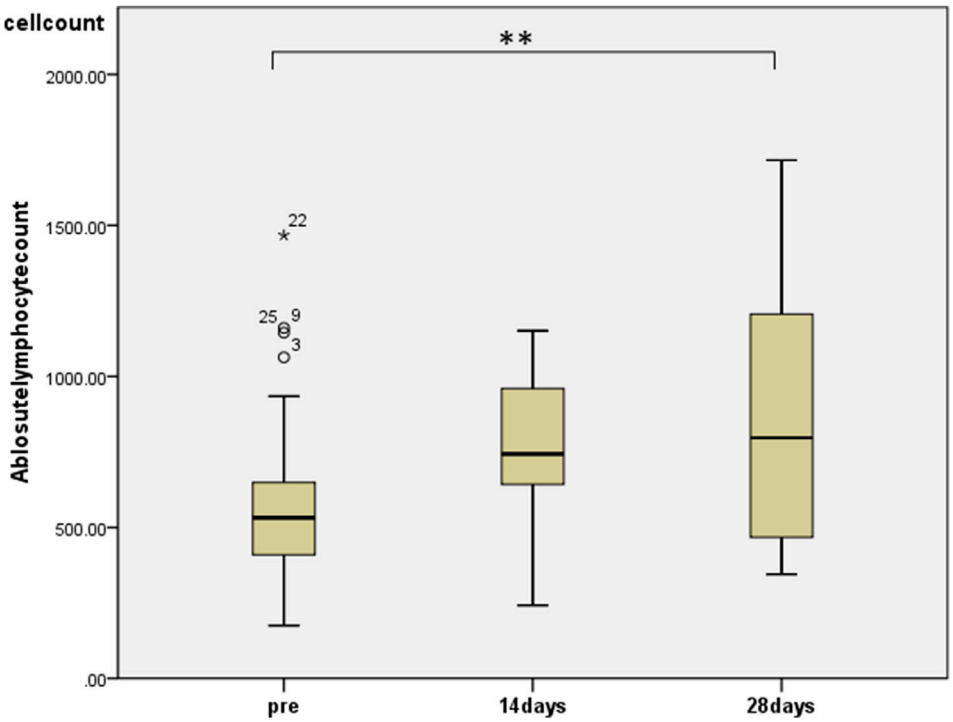

FIGURE 1 | The effects of HET and KKT on whole blood cell counts. HET and KKT administration resulted in a significant increase in the absolute lymphocyte count in the blood.

diagnosis to clarify the clinical indications of HET for the prevention of infectious diseases.

Therefore, we aimed to retrospectively determine the risk and preventive effects of HET and KKT on COVID-19 and identify immune response features through immunological investigation. We conducted this study in HCWs who received HET and KKT.

MATERIALS AND METHODS

Study Design

A total of 27 HCWs (male, n = 9; female, n = 18; median age, 48 years (range, 21–72 years)] were enrolled in this retrospective observational study, which was approved by the Kanazawa University Review Board (approval no. 2020-015) and conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent to participate in this study. The inclusion criteria were as follows: HCWs receiving HET and KKT daily (5–7.5 g/ day, twice or three times a day before meals, Tsumura, Tokyo, Japan) with standard insurance coverage as patients, those whose blood samples were collected before and approximately 14 or 28 days after taking the Kampo formulas. In clinical practice, HET is often used for fatigue. On the other hand, KKT is used for musculoskeletal pains that HCWs often complain of, such as stiff shoulders, and is often used in combination with HET in actual clinical practice. In this study, many HCWs were prescribed these two prescriptions together because the number of HCWs complaining of fatigue due to the increased workload in the medical field following the COVID-19 pandemic.

The primary outcome of this study was to assess the potential effects of HET and KKT on circulating lymphocytes. The secondary outcome was to evaluate the safety of HET and KKT. In Kampo Clinic, patients are usually evaluated around 2 weeks to 1 month after the first administration of Kampo formulas to investigate their drug compliance and the incidence of adverse events; therefore, we retrospectively evaluated the patients using their charts and laboratory data.

Since there were no cases in the group of cases evaluated that developed symptoms during the course of the disease, no PCR or other tests were performed, and antibody measurement was performed on the serum after 1 month by HISCLTM SARS-CoV-2 N-immunoglobulin G (N-IgG), N-IgM, S-IgG, and S-IgM (Sysmecs, Kobe, Japan).

Sample Preparation and Pharmacokinetic Evaluations

Residual blood samples with EDTA-2Na were centrifuged under refrigeration (for 8 min, 3,000 × g) to isolate serum. Peripheral blood mononuclear cells (PBMCs) were isolated from the remaining blood by gradient centrifugation. A fraction of the isolated PBMCs from each sample was cryopreserved for further use.

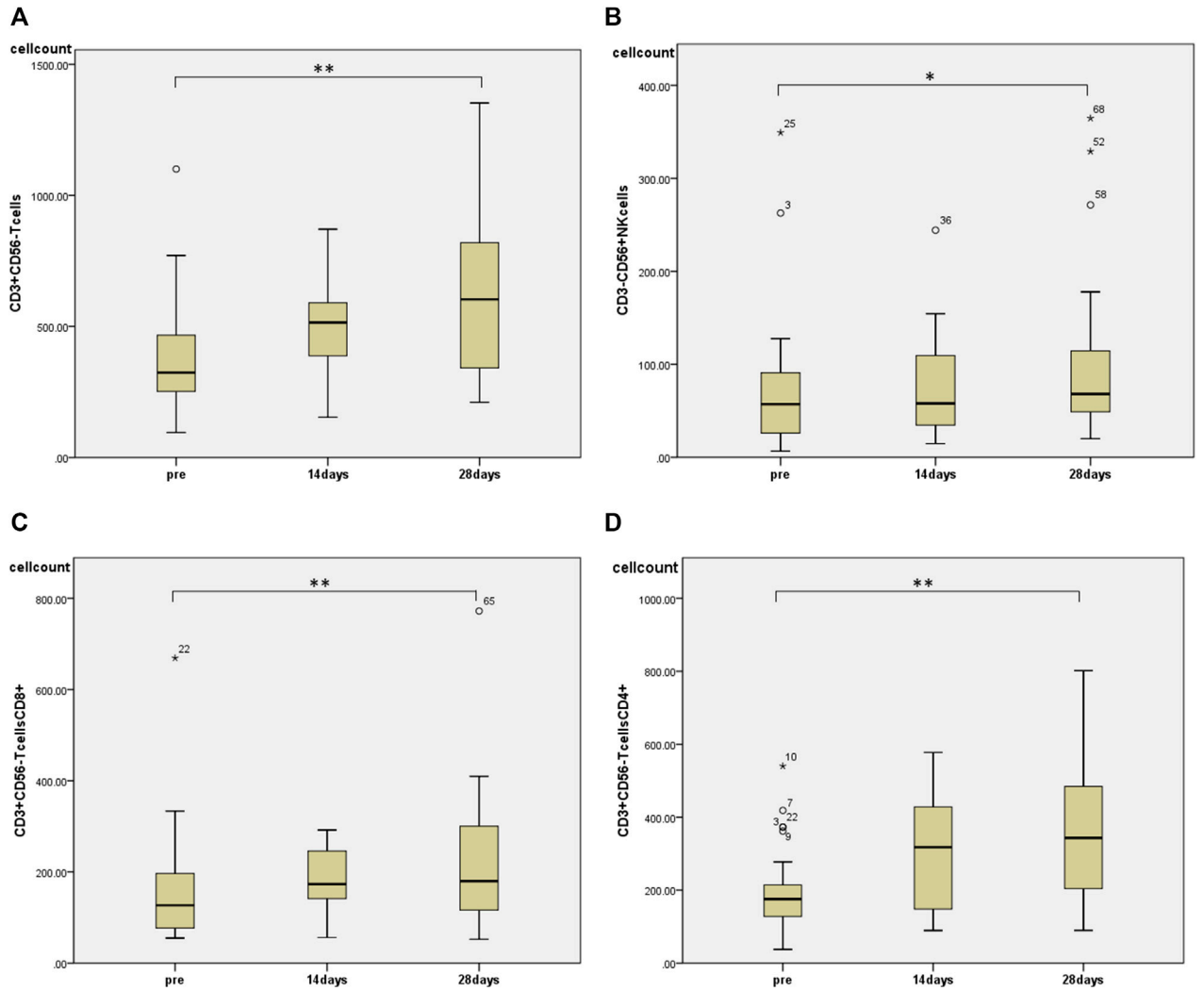

FIGURE 2 | The effects of HET and KKT on circulating peripheral blood mononuclear cells (PBMCs). The administration of HET and KKT resulted in a significant increase in the **(A)** T cell population; **(B)** NK cells; **(C)** CD_8^+T cells, and **(D)** CD_4^+ T cells. * $p < 0.05$, ** $p < 0.01$.

Whole-Blood Cell Counts

Whole-blood cell counts and individual leukocyte fractions were analyzed individually at each hospital or clinic. Absolute cell counts were calculated by multiplying the leukocyte count.

Flow Cytometry

Isolated PBMCs were stained with antibodies specific to the cell surface markers of NK lymphocytes, T cells, and B cells, including anti-CD3, CD19, CD20, CD4, CD8, CD16, CD56, NKp46, NKG2D, NKp30, TLR4, DNAM-1, NKG2A, 4-1BB, OX40, ICOS, PD-1, CTLA4, GITR, LAG3, TIGIT, and TIM3 (BioLegend, San Diego, CA, United States). The stained cells were analyzed using FACSCant II (BD Biosciences, San Jose, CA, United States), and the data were analyzed using the FlowJo software package (ver. 10; Tree Star, Ashland, OR, United States). In the PBMC gating, $CD3^+CD56^-$cells were defined as T cells, $CD3^-CD56^+$ cells were defined as NK cells, CD19 + $CD20^+$ cells were defined as B cells, and T cells were further divided into $CD4^+$ T cells and $CD8^+$ cells.

Statistical Analyses

Data are reported as median ± minimum and maximum values. When comparisons were made between two different groups, statistical significance was determined using Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$.

RESULTS

Safety and Compliance of the Kampo Formulas

Twenty-seven subjects who were enrolled in this study received 2-3 packs of HET and KKT daily (5–7.5 g/ day, Tsumura, Tokyo, Japan) for more than 28 consecutive days. All subjects had more than 70% adherence rate for Kampo formulas.

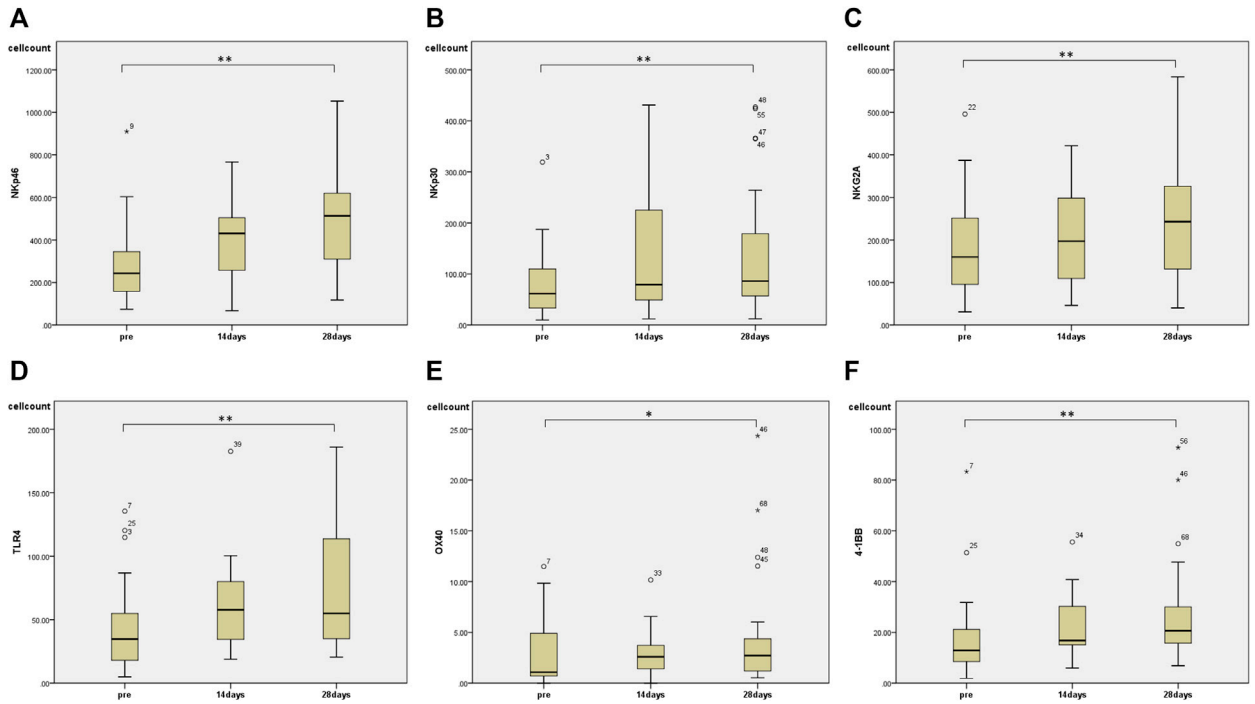

FIGURE 3 | The effects of HET and KKT on PBMC subsets. The 28 days administration of HET and KKT resulted in a significant increase in the **(A)** activator receptors NKp46 **(B)** NKp30, and **(C)** suppressing receptor NKG2A. Expression of other cell surface markers including **(D)** TLR4; **(E)** OX40, and **(F)** 4-1BB also significantly increased. * $p < 0.05$; ** $p < 0.01$.

Abdominal discomfort was found in five (3 females, 2 males), diarrhea in two (1 female and 1 male), and loose or soft stool in three (all of them are females) patients. No other serious side effects were observed.

Effects of HET and KKT on Complete-Blood Cell Counts

To assess the effects of the administration of KKT and HET in HCWs, we first performed whole-blood cell counts at baseline and after the administration of KKT and HET using a blood cell counter. We observed significant changes in the number of lymphocytes (median 532, range 175–1,145 before administration; median 797, range 345–1716 at day 28, $p < 0.01$) in the blood (**Figure 1**).

Effects of HET and KKT on Circulating PBMCs

Regarding changes in PBMC subsets, a significant increase in the number of cells was observed in all fractions (total T cells, $CD4^+$ T cells, $CD8^+$ T cells, and NK cells) except for the number of B cells 28 days after the start of HET ad KKT (**Figure 2**). There was no significant change in the CD4/CD8 ratio. Of note, there was a significant increase in the number of lymphocytes expressing activating receptors such as NKp46 (median 243, range 74.3–910 before administration; median 513, range 117–1,052 at day 28, $p < 0.01$; **Figure 3A**), NKp30 (median 61.6, range 9.69–319 before administration; median 85.9, range 12.2–427 at day 28, $p = 0.001$; **Figure 3B**), and suppressing receptor NKG2A (median 160, range 30.8–496 before administration; median 243, range 40.0–584 at day 28, $p < 0.01$; **Figure 3C**). There was also an upregulation of T-cell activation markers such as TLR4 (median 34.7, range 4.95–135 before administration; median 55.0, range 20.5–186 at day 28, $p < 0.01$; **Figure 3D**), OX40 (median 1.07, range 0–11.5 before administration; median 2.73, range 0.526–17.0 at day 28, $p < 0.05$; **Figures 3E**, **4**-1BB (median 12.9, range 1.86–83.3 before administration; median 20.7, range 6.84–92.8 at day 28, $p < 0.01$; **Figure 3F**) in T cells, which may suggest that naive T cells increased after the start of HET and KKT treatment. Notably, the administration of HET and KKT resulted in changes in GITR (median 10.1, range 2.27–84.3 before administration;; median24.6, range 3.57–457 at day 14, $p < 0.05$; median 15.3, range 3.68–544 at day 28, $p < 0.01$; **Figure 4A**), ICOS(median 4.79, range 0–82.6 before administration;; median 11.9, range 3.13–191 at day 14, $p < 0.05$; median11.9, range 4.04–198 at day 28, $p < 0.01$; **Figure 4B**), and PD-1 (median 1.58, range 0–6.75 before administration;; median1.87, range 0–4.27 at day 14, $p < 0.05$; median 2.47, range 0–12.6 at day 28, $p < 0.05$; **Figure 4C**) on days 14 and 28.

DISCUSSION

In this study, we found immunological modulation, which has some advantages regarding the prevention of infectious diseases in HCWs who were treated with a combination of HET and KKT,

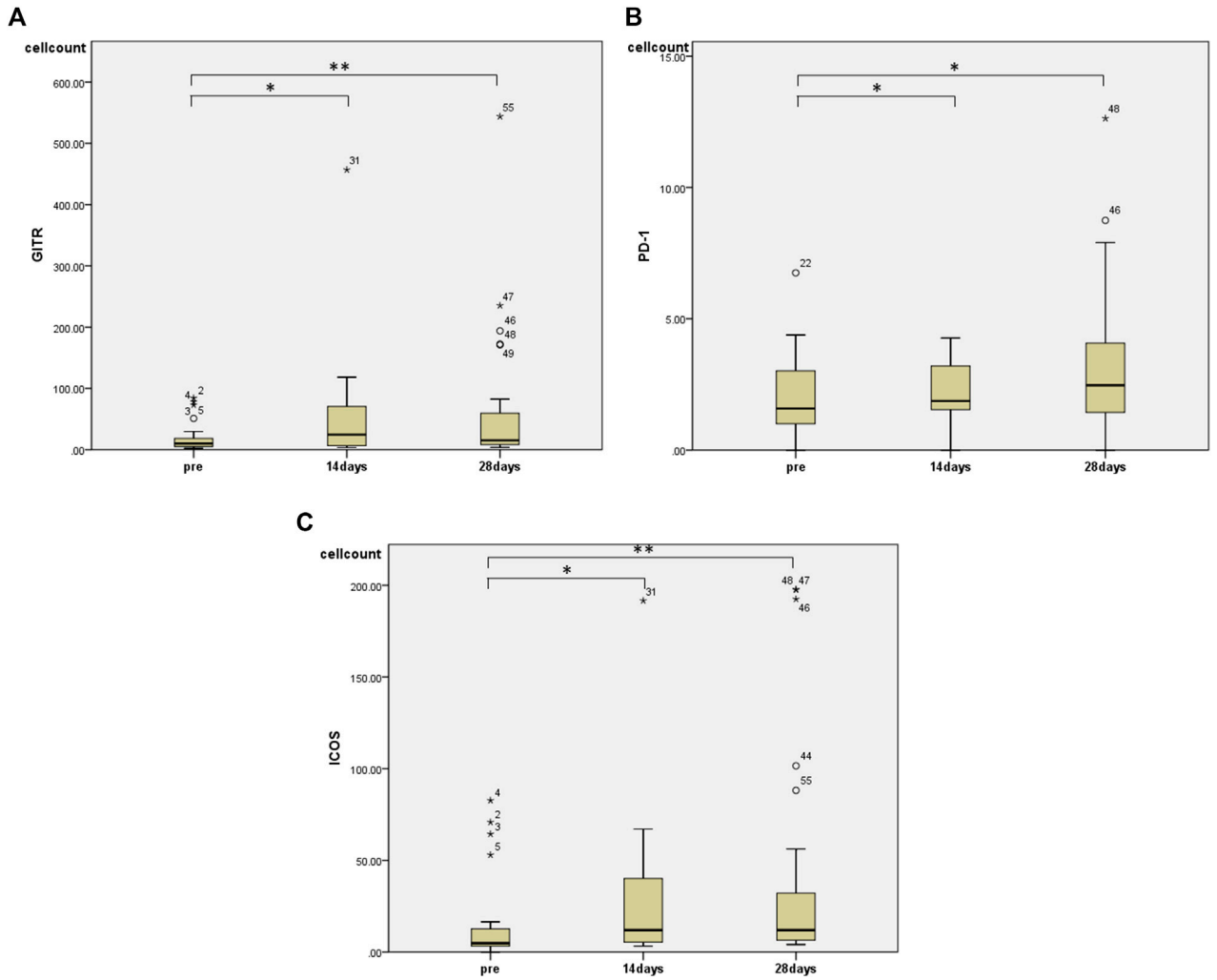

FIGURE 4 | The effects of HET and KKT on PBMC subsets. The significant increase was observed in **(A)** GITR, **(B)**PD-1, and **(C)** ICOS at both 14 and 28 days after administration. * $p < 0.05$; ** $p < 0.01$.

adjusting for comorbidities. The finding that HET and KKT modulate immune cells *in vivo* provides further evidence that Kampo medicines directly or indirectly modulate the immune system. This finding is consistent with the immunomodulatory effects proposed for some of these compounds (Mantani et al., 1999; Hayashi et al., 2007; Wolkerstorfer et al., 2009; Zhou et al., 2013).

Vaccination is certainly a powerful protective approach against infection. However, because the effect of vaccination against mutated viruses is still unknown, and it will take time to produce vaccines in case of the spread of infections caused by other viruses, Kampo medicine may be another option to consider. HET and KKT may be able to prevent infection and serious illness when vaccines cannot be supplied based on the results of this study. Kampo medicine was invented thousands of years ago when infectious diseases were rampant and had gained experience over thousands of years. If we can make use of these Kampo formulas in terms of prevention, then it may be one of the best strategies for treating and managing infectious diseases.

Moreover, "Cytokine storm," inflammation-mediated severe lung damage, and defective hemostasis are the main underlying causes for morbidity and mortality in COVID-19 patients (Huang et al., 2020). Therefore, infections must be controlled to prevent cytokine storms caused by excessive inflammation. We have already shown that the administration of another Kampo formula, juzentaihoto (JTT), modulates NK cell function. In other words, the administration of JTT activated NK cells, but, at the same time, it suppressed NK cells, thereby regulating NK cell function (Ogawa-Ochiai et al., 2021).

This study also showed that the administration of the HET–KKT combination increased the number of NK cells and T cells, the cytotoxic activity of NK cells, the total number of T cells, and CD_4/CD_8-positive cells, indicating that the infection protection ability was enhanced.

NK cells are an essential component of the innate immune system and play a major role in the elimination of virus-infected cells. NK cells express multiple activation receptors such as NKp46 and NKp30 on their cell surface, which are required to recognize specific ligands on potential target cells. By contrast, NKG2A plays an important role in the prevention of excessive inflammation. Of particular interest is the finding of this study that NK cells, mainly the NK subset, which expresses not only NKp46 and NKp30 but also NKG2A receptors on their surface, were significantly increased after the administration of HET and KKT, which may suggest immunomodulation of NK cytotoxicity following treatment with HET and KKT during the COVID-19 pandemic. Recent reports (Liao et al., 2020; Maucourant et al., 2020; Zheng et al., 2020; Bjorkstrom et al., 2021) have showed that NK cells exerted antiviral effects primarily in the lungs early after SARS-CoV-2 infection, that decrease in NK cells was associated with severity of COVID-19, and that expression of activating receptors such as NKp46 and NKp30 on NK cells was associated with antiviral effects, thus suggesting that an increase in the number of circulating NK cells positive for NKp46 and NKp30 may have a protective effect on viral infections such as SARS-CoV-2 in anti-COVID-19 antibody-negative health care workers. However, unexpectedly, in this study, the increase in the number of NKp46 and NKp30-positive circulating lymphocytes after the use of HET / KKT was mainly caused by the increase in the number of NKp46-positive T cells and NKp30-positive T cells. The functions of human T cells that express such activated NK cell receptors, also called a NK-like T cells, are not well understood as well as their roles in COVID-19. Nevertheless, previous studies (Brenchley et al., 2006; Tang et al., 2008; Cupedo et al., 2009; Hudspeth et al., 2012; Hudspeth et al., 2013) have demonstrated that such NK-like T cells are increased by activation stimuli, are endogenous to tonsil tissue, and exert antiviral activity against HIV, thus leading to a hypothesis that an increase in NK-like T cells after the use with HET / KKT in health care workers results in anti-SARS-CoV-2. This hypothesis may be supported by the fact that the function of NKp46 and NKp30 is speculated to be independent of their expressing cells (Tang et al., 2008; Correia et al., 2009; Hudspeth et al., 2013). In addition, in this study, the number of lymphocytes positive for the inhibitory NK receptor NKG2A was also increased in health care workers after using the HET / KKT. A recent report (Zheng et al., 2020) has showed an increase in NKG2A-positive lymphocytes in COVID-19 patients, suggesting that increased NKG2A expression as seen in this study may lead to decreased lymphocyte function in terms of anti-COVID-19. In contrast, it has been reported that upregulation of NKG2A expression in lymphocytes suppresses the excessive immune response of T cells and leads to the maintenance of immunological homeostasis (Jabri et al., 2002). Therefore it remains unclear whether the increase in NKG2A-positive lymphocytes in health care workers after using HET / KKT could act beneficial in preventing COVID-19. These should be clarified in future studies.

There was also an upregulation of T-cell activation markers such as TLR4, OX40, 4-1BB, and TIGIT in T cells, which may suggest that naive T cells increased after the start of HET and KKT treatment. The expression of T-cell activation markers was increased, suggesting an increase in activated T cells as well.

The administration of HET and KKT also promotes T cell-independent activation and interferon production in B cells. This combination of Kampo medicines may be useful for infection defense. On the contrary, along with the promotion of T-cell activation and T-cell differentiation, markers involved in regulation were also significantly increased, indicating immunomodulate effect. Because this was an investigative observational study, we did not have a control group of non-treated or placebo-treated subjects, but a previous study (Nakagami et al., 2019) has shown that there is little inter-individual variation in these markers in placebo-treated subjects.

Since coronaviruses can enter cells in a short time, phagocytes or antibodies alone cannot eliminate the virus. $CD8^+$ killer T cells (cytotoxic T cells) are important, as are NK cells, which destroy virus-infected cells and deprive the virus of a place to multiply. $CD4^+$ helper T cells are also essential for differentiation of IgG antibody-producing B cells and memory cells (Lucas et al., 2020).

Because the subjects were not infected, the significance of the immunomodulatory functions of HET and KKT during actual infection is not clear. However, the results suggest that they may be effective in preventing infection or severe disease through immunomodulatory changes.

This study had the following limitations. First, there is no evidence that the drug actually increased T cell or NK cell function as only a numerical increase in these immune cells was observed without functional analysis. Second, there is no direct evidence of efficacy of HET and KKT on SARS-CoV-2. Finally, there is no theoretical reason for the increase in T cells or activated NK cells following HET and KKT treatment as no patient was actually infected with coronavirus infection. It is not clear which components contained in these formulas have immune activity, which must be analyzed in the future.

CONCLUSION

In conclusion, our findings showed that HET and KKT may prevent the onset of COVID-19 through their immunomodulatory effects. In the future, we would like to clarify the preventive effects of HET and KKT by conducting prospective studies on infected patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Kanazawa University Review Board (approval no. 2020-015). Written informed consent for participation was

not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KO, KM, and HI designed the study protocol and drafted the manuscript; MT, IK, TH, and YH collected the data; MO and MI reviewed the study protocol and drafted the manuscript; VL performed staining and analyzed the FACS results; KM, SS, HL, and KO analyzed the data; KM was responsible for the statistical design and analysis. All authors have carefully read and approved the final version of the manuscript.

FUNDING

This study was supported by the Fund of Kanazawa University for Clinical Studies and Tsumura Co. The funders have no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Ms. Kumiko Matsuo and Ms. Ayako Kimura for their assistance.

REFERENCES

Abe, S., Tansho, S., Ishibashi, H., Akagawa, G., Komatsu, Y., and Yamaguchi, H. (1999). Protection of Immunosuppressed Mice from Lethal Candida Infection by Oral Administration of a Kampo Medicine, Hochu-Ekki-To. *Immunopharmacol Immunotoxicol* 21, 331–342. doi:10.3109/08923979909052766

Bjorkstrom, N. K., Strunz, B., and Ljunggren, H. G. (2021). Natural Killer Cells in Antiviral Immunity. *Nat. Rev. Immunol.* doi:10.1038/s41577-021-00558-3

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., et al. (2006). Microbial Translocation Is a Cause of Systemic Immune Activation in Chronic HIV Infection. *Nat. Med.* 12, 1365–1371. doi:10.1038/nm1511

Correia, M. P., Cardoso, E. M., Pereira, C. F., Neves, R., Uhrberg, M., and Arosa, F. A. (2009). Hepatocytes and IL-15: a Favorable Microenvironment for T Cell Survival and CD8+ T Cell Differentiation. *J. Immunol.* 182, 6149–6159. doi:10.4049/jimmunol.0802470

Cupedo, T., Crellin, N. K., Papazian, N., Rombouts, E. J., Weijer, K., Grogan, J. L., et al. (2009). Human Fetal Lymphoid Tissue-Inducer Cells Are Interleukin 17-producing Precursors to RORC+ CD127+ Natural Killer-like Cells. *Nat. Immunol.* 10, 66–74. doi:10.1038/ni.1668

Gao, W., Sanna, M., Tsai, M. K., and Wen, C. P. (2020). Geo-temporal Distribution of 1,688 Chinese Healthcare Workers Infected with COVID-19 in Severe Conditions-A Secondary Data Analysis. *PLoS One* 15, e0233255. doi:10.1371/journal.pone.0233255

Hayashi, K., Imanishi, N., Kashiwayama, Y., Kawano, A., Terasawa, K., Shimada, Y., et al. (2007). Inhibitory Effect of Cinnamaldehyde, Derived from Cinnamomi Cortex, on the Growth of Influenza A/PR/8 Virus *In Vitro* and *In Vivo*. *Antivir. Res* 74, 1–8. doi:10.1016/j.antiviral.2007.01.003

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi:10.1016/S0140-6736(20)30183-5

Hudspeth, K., Fogli, M., Correia, D. V., Mikulak, J., Roberto, A., Della Bella, S., et al. (2012). Engagement of NKp30 on Vδ1 T Cells Induces the Production of CCL3, CCL4, and CCL5 and Suppresses HIV-1 Replication. *Blood* 119, 4013–4016. doi:10.1182/blood-2011-11-390153

Hudspeth, K., Silva-Santos, B., and Mavilio, D. (2013). Natural Cytotoxicity Receptors: Broader Expression Patterns and Functions in Innate and Adaptive Immune Cells. *Front. Immunol.* 4, 69. doi:10.3389/fimmu.2013.00069

Jabri, B., Selby, J. M., Negulescu, H., Lee, L., Roberts, A. I., Beavis, A., et al. (2002). TCR Specificity Dictates CD94/NKG2A Expression by Human CTL. *Immunity* 17, 487–499. doi:10.1016/s1074-7613(02)00427-2

Kluytmans-van den Bergh, M. F. Q., Buiting, A. G. M., Pas, S. D., Bentvelsen, R. G., van den Bijllaardt, W., van Oudheusden, A. J. G., et al. (2020). Prevalence and Clinical Presentation of Health Care Workers with Symptoms of Coronavirus Disease 2019 in 2 Dutch Hospitals during an Early Phase of the Pandemic. *JAMA Netw. Open.* 3, e209673. doi:10.1001/jamanetworkopen.2020.9673

Kuroiwa, A., Liou, S., Yan, H., Eshita, A., Naitoh, S., and Nagayama, A. (2004). Effect of a Traditional Japanese Herbal Medicine, Hochu-Ekki-To (Bu-Zhong-Yi-Qi Tang), on Immunity in Elderly Persons. *Int. Immunopharmacol.* 4, 317–324. doi:10.1016/j.intimp.2003.12.004

Liao, M., Liu, Y., Yuan, J., Wen, Y., Xu, G., Zhao, J., et al. (2020). Single-cell Landscape of Bronchoalveolar Immune Cells in Patients with COVID-19. *Nat. Med.* 26, 842–844. doi:10.1038/s41591-020-0901-9

Lucas, C., Wong, P., Klein, J., Castro, T. B. R., Silva, J., Sundaram, M., et al. (2020). Longitudinal Analyses Reveal Immunological Misfiring in Severe COVID-19. *Nature* 584, 463–469. doi:10.1038/s41586-020-2588-y

Mantani, N., Andoh, T., Kawamata, H., Terasawa, K., and Ochiai, H. (1999). Inhibitory Effect of Ephedrae Herba, an oriental Traditional Medicine, on the Growth of Influenza A/PR/8 Virus in MDCK Cells. *Antivir. Res* 44, 193–200. doi:10.1016/S0166-3542(99)00067-4

Maucourant, C., Filipovic, I., Ponzetta, A., Aleman, S., Cornillet, M., Hertwig, L., et al. (2020). Natural Killer Cell Immunotypes Related to COVID-19 Disease Severity. *Sci. Immunol.* 5. doi:10.1126/sciimmunol.abd6832

Mori, K., Kido, T., Daikuhara, H., Sakakibara, I., Sakata, T., Shimizu, K., et al. (1999). Effect of Hochu-Ekki-To (TJ-41), a Japanese Herbal Medicine, on the Survival of Mice Infected with Influenza Virus. *Antivir. Res* 44, 103–111. doi:10.1016/S0166-3542(99)00048-0

Nakagami, Y., Suzuki, S., Espinoza, J. L., Vu Quang, L., Enomoto, M., Takasugi, S., et al. (2019). Immunomodulatory and Metabolic Changes after Gnetin-C Supplementation in Humans. *Nutrients* 11, 1403. doi:10.3390/nu11061403

Ogawa-Ochiai, K., Katagiri, T., Sato, Y., Shirai, A., Ishiyama, K., Takami, A., et al. (2021). Natural Killer Cell Function Changes by the Japanese Kampo Medicine Juzentaihoto in General Fatigue Patients. *Adv. Integr. Med.* 8, 33–43. doi:10.1016/j.aimed.2019.12.003

Satoh, N., Sakai, S., Kogure, T., Tahara, E., Origasa, H., Shimada, Y., et al. (2005). A Randomized Double Blind Placebo-Controlled Clinical Trial of Hochuekkito, a Traditional Herbal Medicine, in the Treatment of Elderly Patients with Weakness N of One and Responder Restricted Design. *Phytomedicine* 12, 549–554. doi:10.1016/j.phymed.2004.06.014

Tang, Q., Grzywacz, B., Wang, H., Kataria, N., Cao, Q., Wagner, J. E., et al. (2008). Umbilical Cord Blood T Cells Express Multiple Natural Cytotoxicity Receptors after IL-15 Stimulation, but Only NKp30 Is Functional. *J. Immunol.* 181, 4507–4515. doi:10.4049/jimmunol.181.7.4507

Tatsumi, K., Shinozuka, N., Nakayama, K., Sekiya, N., Kuriyama, T., Fukuchi, Y., et al. (2009). Hochuekkito Improves Systemic Inflammation and Nutritional Status in Elderly Patients with Chronic Obstructive Pulmonary Disease. *J. Am. Geriatr. Soc.* 57, 169–170. doi:10.1111/j.1532-5415.2009.02034.x

Tokura, Y., Sakurai, M., Yagi, H., Furukawa, F., and Takigawa, M. (1998). Systemic Administration of Hochu-Ekki-To (Bu-zhong-yi-qi-tang), a Japanese-Chinese Herbal Medicine, Maintains Interferon-Gamma Production by Peripheral Blood Mononuclear Cells in Patients with Mycosis Fungoides. *J. Dermatol.* 25, 131–133. doi:10.1111/j.1346-8138.1998.tb02365.x

Wang, D., Hu, B., Hu, C., Zhu, F., Liu, X., Zhang, J., et al. (2020). Clinical Characteristics of 138 Hospitalized Patients with 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. *JAMA* 323, 1061–1069. doi:10.1001/jama.2020.1585

Wolkerstorfer, A., Kurz, H., Bachhofner, N., and Szolar, O. H. (2009). Glycyrrhizin Inhibits Influenza A Virus Uptake into the Cell. *Antivir. Res* 83, 171–178. doi:10.1016/j.antiviral.2009.04.012

Yamaoka, Y., Kawakita, T., and Nomoto, K. (2001). Protective Effect of a Traditional Japanese Medicine Hochu-Ekki-To (Chinese Name: Bu-Zhong-Yi-Qi-Tang), on the Susceptibility against Listeria Monocytogenes in Infant Mice. *Int. Immunopharmacol.* 1, 1669–1677. doi:10.1016/S1567-5769(01)00076-5

Zheng, M., Gao, Y., Wang, G., Song, G., Liu, S., Sun, D., et al. (2020). Functional Exhaustion of Antiviral Lymphocytes in COVID-19 Patients. *Cell Mol Immunol* 17, 533–535. doi:10.1038/s41423-020-0402-2

Zhou, Y. X., Zhang, H., and Peng, C. (2013). Puerarin: a Review of Pharmacological Effects. *Phytother. Res.* 28, 961–975. doi:10.1002/ptr.5083

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

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 20 December 2021
doi: 10.3389/fphar.2021.751403

Relationship Between Conventional Medicine Chapters in ICD-10 and Kampo Pattern Diagnosis: A Cross-Sectional Study

Xuefeng Wu[1†], *Thomas K. Le*[2†], *Ayako Maeda-Minami*[3†], *Tetsuhiro Yoshino*[1†], *Yuko Horiba*[1†], *Masaru Mimura*[1†] *and Kenji Watanabe*[*†1]

[1]*Center for Kampo Medicine, Keio University School of Medicine, Tokyo, Japan,* [2]*School of Medicine, Johns Hopkins University, Baltimore, MD, United States,* [3]*Division of Pharmaceutical Care Sciences, Graduate School of Pharmacy, Keio University, Minato-ku, Japan*

Objectives: The newest revision to the International Classification of Diseases, the 11th edition (ICD-11) includes disease classifications from East Asian medicine, including traditional Japanese medicine (Kampo medicine). These disease classifications allow for comparisons between disease classifications from conventional medicine and Kampo medicine.

Design/Location/Subjects/Interventions: This is an exploratory, cross-sectional study exploring the relationship between conventional medicine diagnoses and Kampo medicine diagnoses at a large Kampo clinic in Japan. Patients were seen from October 1st, 2014 to June 30th, 2019 and were 20 years of age or older.

Outcome measures: Patients presented with one or more conventional medicine ICD-10 codes into the clinic and were given one descriptor from the ICD-11 within the heat-cold module, excess-deficiency module, and an optional body constituents module. The distribution of these Kampo medicine codes was examined in relation to conventional medicine chapters.

Results: 1,209 patients were included in our final analysis. Patient number, ages, sex ratio, and BMI varied within conventional medicine ICD-10 chapters and Kampo medicine descriptor codes. Certain conventional medicine chapters are related to specific Kampo medicine descriptor codes, such as chapter IV (endocrine, nutritional, and metabolic diseases) with excess, heat, and kidney qi deficiency.

Conclusion: The advent of the ICD-11 allows for systematic, standardized comparisons between Kampo medicine, and contemporary medicine. In this exploratory study, our findings support the independence of Kampo medicine pattern descriptors with ICD-10 conventional medicine chapters. Code overrepresentations in relation to conventional medicine diseases and by age and sex should be an area of future investigation to best understand how to synergize and improve patient care.

Keywords: kampo, pattern diagnosis, international classification of diseases, traditional Japanese medicine, conventional medicine, ICD-10, ICD-11

OPEN ACCESS

Edited by:
Rong-Rong He,
Jinan University, China

Reviewed by:
Kenny Kuchta,
University Medical Center Göttingen, Germany
Takao Namiki,
Chiba University, Japan
Liqun Jia,
China-Japan Friendship Hospital, China

****Correspondence:***
Kenji Watanabe
watanabekenji@keio.jp

†ORCID:
Xuefeng Wu
orcid.org/0000-0003-0437-2904;
Thomas Le
orcid.org/0000-0003-0899-4671;
Ayako Maeda-Minami
orcid.org/0000-0003-1361-4514;
Tetsuhiro Yoshino
orcid.org/0000-0001-6172-1926;
Yuko Horiba
orcid.org/0000-0003-3260-6755;
Masaru Mimura
orcid.org/0000-0002-3874-5273;
Kenji Watanabe
orcid.org/0000-0002-5823-6441

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

Received: *01 August 2021*
Accepted: *22 November 2021*
Published: *20 December 2021*

Citation:
Wu X, Le TK, Maeda-Minami A, Yoshino T, Horiba Y, Mimura M and Watanabe K (2021) Relationship Between Conventional Medicine Chapters in ICD-10 and Kampo Pattern Diagnosis: A Cross-Sectional Study. Front. Pharmacol. 12:751403. doi: 10.3389/fphar.2021.751403

INTRODUCTION

Traditional Japanese medicine (Kampo medicine) is widely used in modern Japanese society. Physicians, regardless of specialty, prescribe Kampo in daily practice as standalone treatment or alongside conventional medicine (CM) (Moschik et al., 2012). In particular, Kampo formulas have been found helpful in specific diseases, such as pediatric emotional and behavioral disorders and *hiesho* (cold disorder) (Watanabe et al., 2014). Research on Kampo medicine has been increasing in recent years (Hyun et al., 2019). One important concept in Kampo methodology is *hosho sotai* (formula versus pattern). In other words, Kampo medicine does not employ the treatment based on a disease name but rather uses treatment on a set number of presenting symptoms, or a "pattern" (Yakubo et al., 2014).

In the International Classification of Diseases (ICD-11) traditional medicine (TM) chapter, patterns are defined as "the complete clinical presentation of the patient at a given moment in time including all findings" (WHO., 2019). Different Asian traditional medicine modalities use their own subset codes in their respective practices. In Kampo, the principle pattern used to describe a patient's presentation includes descriptors from three modules: deficiency-excess, heat-cold, and optional body constituents (Yakubo et al., 2014). Deficiency-excess and heat-cold are essential and typically sufficient to describe most patient disease states. However, body constituents modules can be added for complicated health conditions and chronic diseases (detailed explanation in **Supplementary Figure S1**).

Pattern diagnosis and disease diagnosis are made simultaneously in Kampo clinic. Usually, a patient who visits a modern Kampo clinic will have one or more CM diagnoses when referred. After a Kampo-specific history and physical examination a pattern diagnosis will be given. The treating Kampo physician can also add several additional CM diagnoses if specific unique Kampo symptoms or physical exam signs are not covered within a patient's previous CM diagnoses. Thus, patients who visit a Kampo clinic in Japan can end up with CM diseases diagnoses and a pattern diagnosis together. This would include one or more CM diseases from the ICD-10 and one Kampo pattern composed of one descriptor from deficiency-excess, one from heat-cold, and one or more optional descriptors from body constituents.

It has not been possible to compare standardized codes between TM and CM before the advent of the ICD-11. Historically, TM and CM are completely different medical systems, and TM considers the holistic state of a patient presentation rather than specific organ systems in CM. Furthermore, TM and CM have differing theoretical foundations, which suggests that there is no correlative overlap between the two systems. When TM pattern diagnoses are given, they do not include elements of a CM diagnosis.

However, modern-day Kampo clinical practice has evolved in a way that utilizes some knowledge of CM, and especially since physicians who specialize in Kampo medicine are required to be licensed CM practitioners. Thus, we hypothesized there were potential undiscovered relationships between the two medical systems. To this end, we conducted an exploratory cross-sectional study at the Kampo clinic of Keio University Hospital to understand and compare the characteristics and relationships between CM ICD-10 chapters and TM pattern descriptors.

METHODS

Study Design

This is an exploratory cross-sectional study conducted at Keio University Hospital in Tokyo, Japan. Keio University Hospital is one of the largest and well-known teaching hospitals in Tokyo and houses a large and active Kampo clinic, making it an ideal location to obtain a wide range of different patient presentations. The Keio University School of Medicine Institutional Review Board approved this study (Approval No. 20100144), and the protocol is available at the UMIN clinical trials registry (unique ID: UMIN000020478).

Participants

To avoid information bias from repeatedly collecting diagnostic information in the medical record, we set our inclusion criteria as first-visit patients aged 20 or older presenting to the Kampo clinic at Keio University Hospital from October 1st, 2014 to June 30th, 2019. Exclusion criteria included records without CM diagnoses or incomplete documentation of deficiency-excess or heat-cold. Both deficiency-excess and heat-cold modules are essential in the practice of Kampo medicine. There were 456 (37.7%) patients with a single CM diagnosis, 428 (35.4%) with two, and 325 (26.9%) with more than three in our database (detailed diseases and ICD-10 chapters distribution in **Supplementary Tables S1,S2**). To reflect the real-world distribution of pattern diagnose and obtain sufficient statistical power, we decided to include data from patients with multiple CM diagnoses. Written informed consent was obtained from participants. There was a total of 10 physicians included in this study, who were the practicing Kampo physicians at our institution. All the participating physicians are board-certified specialists in Kampo medicine who also have active conventional medicine licenses. Inter-rater reliability of some of these physicians has been shown previously (Maeda-Minami et al., 2021).

Procedure

Before seeing a Kampo physician, all patients completed a standardized questionnaire, which assessed subjective symptoms and collected demographic information at their first visit. Based on the questionnaire, one Kampo physician would ask additional clarifying questions and conduct a series of Kampo physical examinations, such as tongue inspection, pulse diagnosis, and abdominal palpation. The physicians would then give their pattern diagnosis with three modules: deficiency-excess, heat-cold, and optional body constituents. In Kampo medicine, within the TM ICD-11 pattern chapter, the deficiency-excess module included deficiency, medium, and excess descriptors; the heat-cold module included heat, tangled, moderate, and cold descriptors; and the body constituents module included qi deficiency, qi stagnation, qi counterflow, blood deficiency, blood stasis, fluid disturbance,

fluid deficiency, and kidney qi deficiency descriptors (**Supplementary Figure S1**). This pattern diagnosis was given without regard to the CM diagnosis and was made independently using the TM evaluation. We did consider pattern diagnosis interrater reliability when considering our study's wider internal validity, however a study done previously by our group indicated this was not a major concern (Maeda-Minami et al., 2021).

CM diagnoses for patients visiting the Kampo clinic were coded using the ICD-10, released in 2013 by the Ministry of Health, Labor and Welfare in Japan (Ministry of Health Labor and Welfare., 2020). The number of conventional medicine diagnoses in ICD-10 is over 14,000, with no classifications for disease severity. Due to the difficulty in analyzing all >14,000 diseases in our analysis, CM diagnostic codes were grouped by ICD-10 chapters.

Information abstracted for analysis includes answers from the patient's questionnaire, findings from Kampo physical examination, and the final pattern diagnosis. Chart review was also conducted to confirm the CM diagnosis and to record demographic characteristics such as age and gender.

Statistical Analysis

All statistical calculations and analyses were performed using R software (version 3.6.3, 2020-02-29). Descriptive statistics were used in the patient's demographic characteristics. The proportion of pattern code distribution by each CM ICD-10 chapter was illustrated by a bar plot. A test for equal proportions with continuity correction was used to compare the participant number distribution and sex distribution characteristics. Age and body mass index (BMI) characteristics were summarized as an interquartile range (IQR). Wilcoxon's rank-sum test with continuity correction was used to compare the age and BMI characteristics due to non-normal distributions, comparing groups with a specific code diagnosis against groups without that code diagnosis. A p-value < 0.05 as statistically significant.

RESULTS

There were 1,568 potential study patients identified at the Keio University Hospital Kampo clinic between October 1st, 2014, and June 30th, 2019 (**Supplementary Figure S2**). Out of those 1,568, 1,319 patients (84.1%) agreed to participate in the study. There were 110 patients (8.3%) excluded due to incomplete diagnosis information, with 24 patients without deficiency-excess or heat-cold module, 12 without CM diagnoses, and 74 without both. There were 1,209 patients included in our final analysis.

Characteristics of Kampo Clinic Patients

Sex ratio of the 1,209 patients was 1:2.7 (327 males: 882 females). Age of patients ranged from 20 to 92, and BMI from 12.8 to 52.5 kg/m^2.

We report the distribution by each CM ICD-10 chapter (**Supplementary Table S3**; with top three most frequent CM diagnoses). The most common chapter was XVIII (n = 474, 39.2%), for symptoms, signs, and abnormal clinical and laboratory findings. CM ICD-10 chapters showed different distribution dependent on sex, age, and BMI. The proportion of males was higher in diseases of the circulatory system (IX, 40.7 vs 25.6%, and $p < 0.01$), and lower in diseases of the genitourinary system (XIV, 13.2 vs 30.54%, and $p < 0.01$). Age was more likely to be older in chapter IX (median 67 vs 51, $p < 0.01$), and more likely to be younger in chapter XII (diseases of the skin and subcutaneous tissue, median 41 vs 54, and $p < 0.01$). BMI was also more likely to be larger in chapter IX (median 23.1 vs 20.8, $p < 0.01$).

For pattern diagnoses (**Supplementary Table S4**), the most common descriptor within the deficiency-excess module was deficiency (n = 485, 40.1%). Within heat-cold, cold was the most common (n = 489, 40.4%). Within body constituents, qi stagnation was the most common (n = 369, 30.5%).

Pattern descriptor had different distributions depending on sex, age, and BMI (**Supplementary Table S4**). Within excess-deficiency, deficiency was more likely to be coded in elderly patients (median 57 vs 50, $p < 0.01$) and smaller BMI (median 19.2 vs 22.1, $p < 0.01$). Excess was greater in males (37.9 vs 24.1%, $p < 0.01$) and larger BMI (median 24 vs 20.2, $p < 0.01$).

Within heat-cold, cold was less likely to be in male patients (22.5 vs 30.1%, $p < 0.01$). Furthermore, patients with larger BMI were more likely to be given a heat descriptor (median 23.9 vs 20.8, $p < 0.01$) and younger patients given a tangled descriptor (median 49 vs 53, $p < 0.01$).

Within body constituents, qi deficiency was more likely to be with smaller BMI (median 19.6 vs 21.3, $p < 0.01$). Qi stagnation was more likely to be in younger patients (median 46 vs 55, $p < 0.01$). Blood stasis was more likely to be in younger patients (median 45 vs 55, $p < 0.01$) and had fewer male patients (12.2 vs 31.8%, $p < 0.01$). Fluid disturbance was also less likely in males (11.0 vs 30.1%, $p < 0.01$). Kidney qi deficiency tended to be in older (median 70 vs 48, $p < 0.01$) and male patients (50.2 vs 21.2%, $p < 0.01$).

Relationship Between CM ICD-10 Chapters and TM ICD-11 Pattern Codes

In general, while each CM ICD-10 chapter (minus those that are not seen commonly in Kampo practice, such as chapter XX External causes of morbidity) had a general distribution of all deficiency-excess (**Figure 1**), heat-cold (**Figure 2**), and body constituents descriptors (**Figure 3**), our analysis found certain overrepresentations of specific Kampo pattern codes within each chapter.

Figure 1 shows the proportion of the deficiency-excess by each CM ICD-10 chapter, where scattered lines represent the average value in each descriptor. The proportion of deficiency in chapter XVIII (Symptoms, signs and abnormal clinical and laboratory findings, and not elsewhere classified) exceeded the other chapters (46.6 vs 35.9%, $p < 0.01$), in which cold hypersensitivity, headache, dizziness, and giddiness represent the majority of CM ICD-10 codes (**Supplemental Table S3**). The proportion of excess in chapter IV (Endocrine, nutritional, and metabolic diseases) and chapter IX (Diseases of the circulatory system) exceeded the other chapters (34.4 vs 20.1%, $p < 0.01$ and 39.8 vs 19.3%, $p < 0.01$ respectively), in

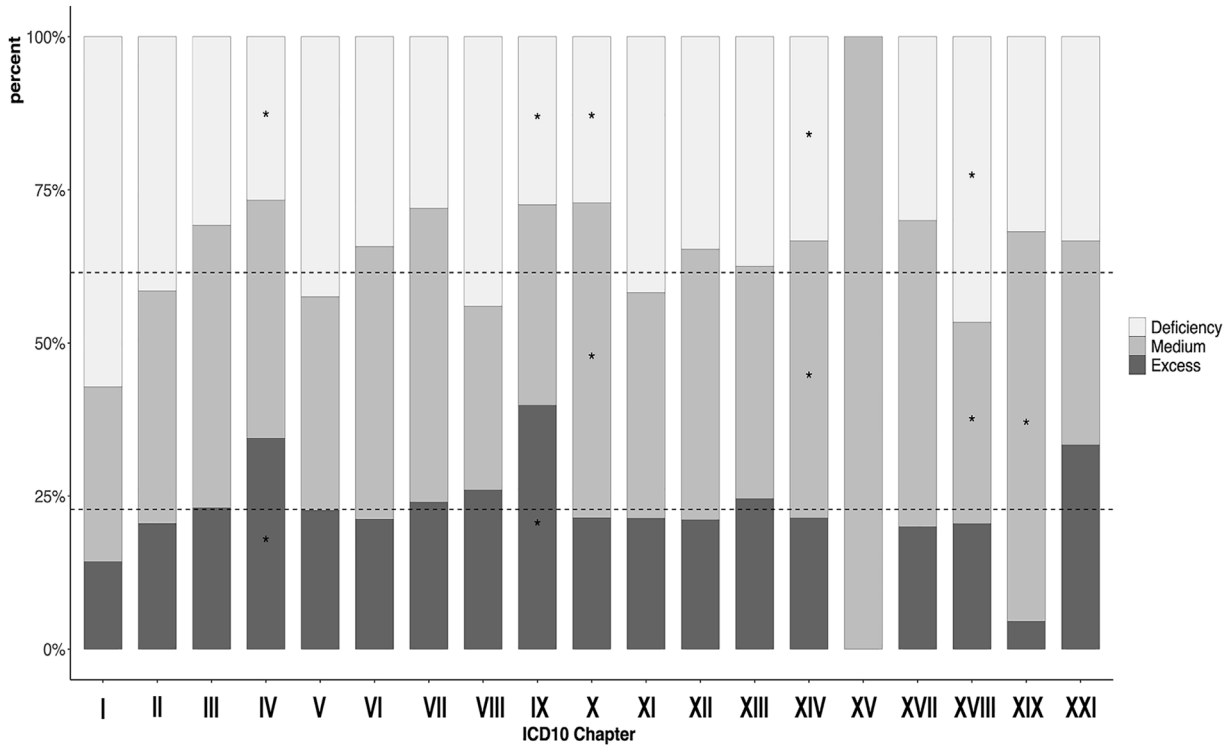

FIGURE 1 | Proportion of participants with deficiency-excess in each the 10th version of the International Classification of Diseases chapters. *$p < 0.05$ ICD = International Classification of Diseases. I: Infection (35); II: Neoplasm (200); III: Hemotology/Immunology (13); IV: Endocrine/metabolism (90); V: Psychiatry (106); VI: Neurology (146); VII: Ophthalmology (25); VIII:Audiology (50); IX: Circulatology (113); X: Pulmonology (70); XI: Gastroenterology (206); XII: Dermatology (147); XIII: Musculoskeletal (224); XIV: Genitourology (243); XV: Obstetrics (3); XVII: Congenital (10); XVIII: Symptoms/signs (474); XIX: Injury/poisoning (22); XXI: Health state (3).

which diabetes and hyperlipidemia represent the majority of codes in chapter IV and hypertension in chapter IX.

Figure 2 shows the proportion of the heat-cold by each CM ICD-10 chapter. Compared to other chapters, chapter XVIII had an excess of cold (50.6 vs 33.9%, $p < 0.01$). Chapter IV had an excess of heat (18.9 vs 7.1%, $p < 0.01$).

We analyzed body constituents by CM ICD-10 chapter and stratified each descriptor (**Figure 3**). There were three chapters that were overrepresented with qi deficiency: chapter I (Certain infectious and parasitic diseases, 51.4 vs 21.7%, $p < 0.01$), chapter II (Neoplasms, 32.5 vs 20.6%, $p < 0.01$, and chapter XI (Diseases of the digestive system, 33.5 vs 20.3%, $p < 0.01$). Infectious gastroenteritis and colitis were the most common ICD-10 codes in chapter II. Malignant breast tumor, leiomyoma of the uterus, and gastric tumor were the most common codes in chapter II. Constipation, chronic gastritis, and gastroesophageal reflux disease with esophagitis were the most common codes in chapter XI.

There were five chapters that were overrepresented with kidney qi deficiency: chapter IV (38.8 vs 18.8%, $p < 0.5$), chapter IX (42.5 vs 18.0%, $p < 0.01$), chapter VII (Diseases of the eye and adnexa, 52 vs 19.6%, $p < 0.01$), chapter VIII (Diseases of the ear and mastoid process, 38 vs 19.5%, $p < 0.01$), and chapter XIII (Diseases of the musculoskeletal system and connective tissue, 34.4 vs 17.1%, $p < 0.01$). Glaucoma was the most common CM ICD-10 code in chapter VII, tinnitus the most common ICD-10 code in chapter VIII, and lower back pain, shoulder stiffness and sicca syndrome the most common in chapter XIII. Chapter XII (Diseases of the skin and subcutaneous tissue) and XIV (Diseases of the genitourinary system) were less associated with kidney qi deficiency (9.5 vs 21.8%, $p < 0.01$ and 14.0 vs 21.8% respectively, both $p < 0.01$). In chapter XIV (menopausal and female climacteric states), unspecified female infertility, and unspecified dysmenorrhea were the most common ICD-10 codes represented.

There were two chapters that were overrepresented with qi stagnation, chapter V (Mental and behavioral disorders, 67.9 vs 26.9%, $p < 0.01$), and chapter VI (Diseases of the nervous system, 42.5 vs 28.8%, $p < 0.01$). Depressive disorder and anxiety were the most common CM ICD-10 codes represented in chapter V, and insomnias, disorder of the autonomic nervous system, and polyneuropathy in chapter VI.

There were two chapters overrepresented with qi counter flow, chapter V (17.9 vs 7.3%, $p < 0.01$) and chapter XIV (14.4 vs 6.7%, $p < 0.01$). Chapter XIII was less likely to be associated with qi counter flow pattern (17.0 vs 33.6%, $p < 0.01$).

Chapter XIV diagnoses were more likely to be associated with blood stasis (39.1 vs 20.6%, $p < 0.01$). Chapter XII diagnoses were more likely to be associated with blood deficiency (27.2 vs 15.2%, $p < 0.01$). Chapter V diagnoses

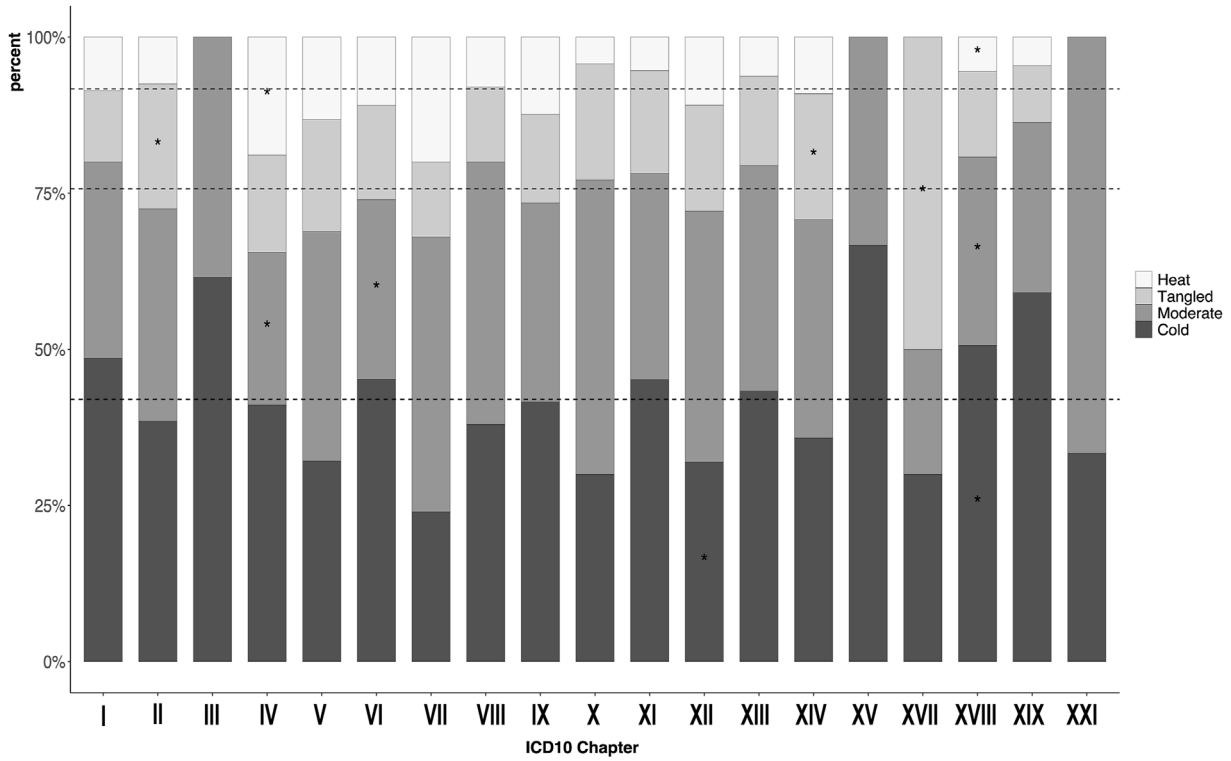

FIGURE 2 | Proportion of participants with heat-cold pattern in each the 10th version of the International Classification of Diseases chapters. *$p < 0.05$ ICD = International Classification of Diseases. I: Infection (35); II: Neoplasm (200); III: Hemotology/Immunology (13); IV: Endocrine/metabolism (90); V: Psychiatry (106); VI: Neurology (146); VII: Ophthalmology (25); VIII:Audiology (50); IX: Circulatology (113); X: Pulmonology (70); XI: Gastroenterology (206); XII: Dermatology (147); XIII: Musculoskeletal (224); XIV: Genitourology (243); XV: Obstetrics (3); XVII: Congenital (10); XVIII: Symptoms/signs (474); XIX: Injury/poisoning (22); XXI: Health state (3).

were less likely to be associated with fluid disturbance (6.6 vs 16.7%, $p < 0.01$).

DISCUSSION

This is the first study in the literature to systematically examine the relationship between CM ICD-10 diagnoses and TM pattern diagnoses using a standard classification scheme in real-world clinical practice. Our results show that all pattern descriptors were represented within the spectrum of CM ICD-10 chapters. We report certain specific overrepresentations of pattern descriptors within each ICD-10 chapter and when stratified by age, sex, and BMI.

We found diseases relating to the eye or ear (ICD-10 chapter VII or VIII) were more likely to be associated with kidney qi deficiency. This relationship follows the Kampo theory that kidney qi nourishes the eye and ear function at baseline, and an absence of kidney qi is seen in eye and ear pathology. Another trend noted was that the population of chapter XIV (Diseases of the genitourinary system) was composed of two different groups: older males in whom the prevalent disease was prostate hypertrophy, and younger females in whom menopausal and female climacteric states were the primary diseases. The pattern descriptors distribution within chapter XIV also varied by sex which may indicate an association between these CM diagnoses and TM patterns—males were more likely to be associated with kidney qi deficiency, while females were more likely to be associated with blood stasis and fluid disturbance. We report the characteristics of patients with similar CM and TM diagnoses in **Supplementary Tables S6–S8** and visually represent their differences in **Supplementary Figures S3–S5**.

Reference guide of ICD-11 articulates that ICD-11's chapter on Traditional Medicine disorders and patterns is designed to be integrated with coding of cases in conjunction with the CM concepts of ICD Chapters. The relationship between TM pattern descriptors and CM diagnosis has been an established research topic within the East Asian TM community (Lu and Chen, 2011; Lu et al., 2012). Studies have shown the relationship of specific TM pattern descriptors with a diverse array of CM presentations, including knee osteoarthritis (Tian et al., 2016) and open-angle glaucoma (Yang et al., 2018). However, before the advent of the standardized TM ICD-11 pattern descriptors as in this study, it has been difficult to conduct a comparative analysis between differing medical traditions with unique terminology and classifications, and such as between Kampo and traditional Chinese medicine. These types of

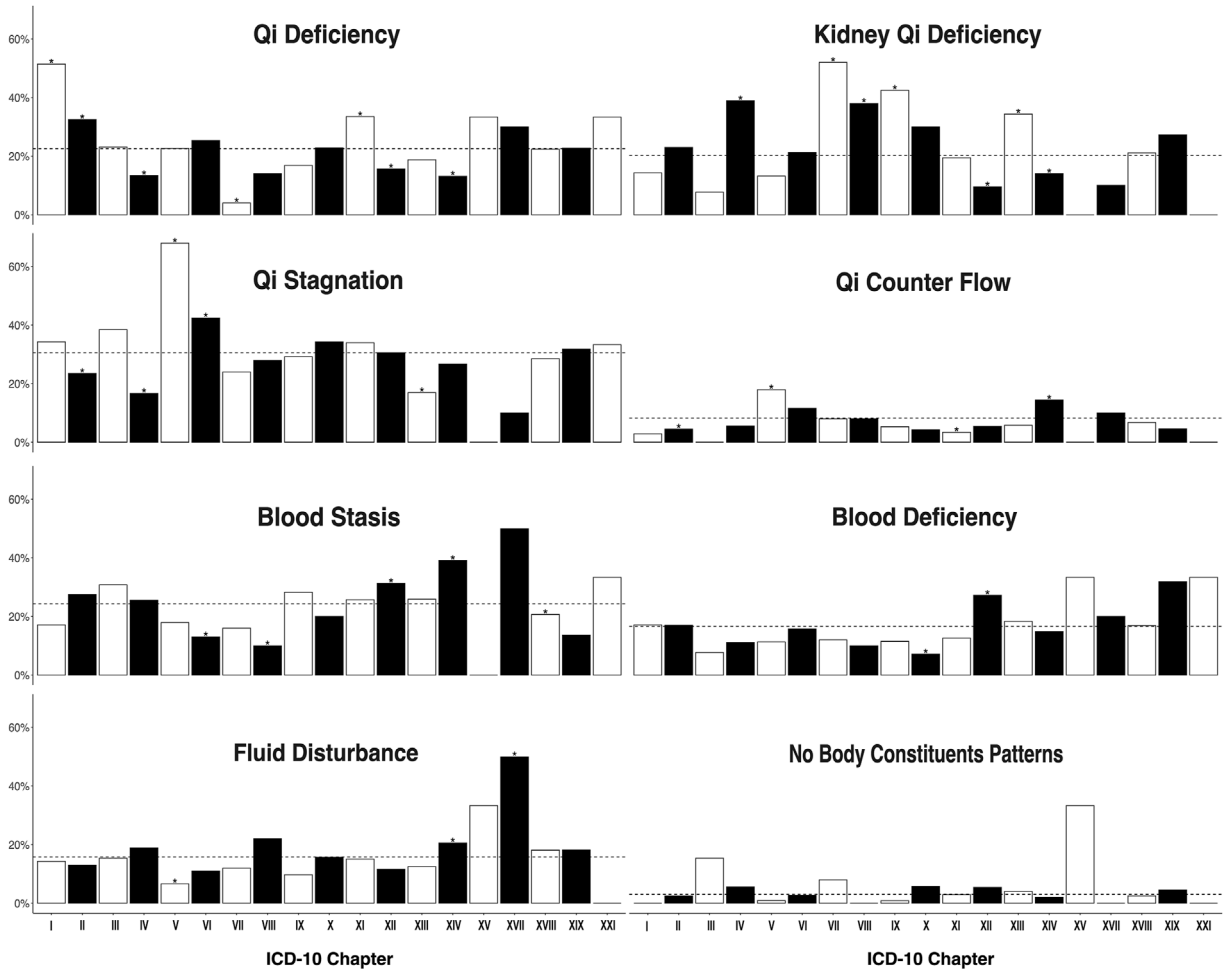

FIGURE 3 | Proportion of participants with body constituents pattern in each the 10th version of the International Classification of Diseases chapters. *$p < 0.05$ ICD = International Classification of Diseases. The facet of fluid deficiency is hidden due to its limited participant number ($n = 4$). I: Infection (35); II: Neoplasm (200); III: Hemotology/Immunology (13); IV: Endocrine/metabolism (90); V: Psychiatry (106); VI: Neurology (146); VII: Ophthalmology (25); VIII:Audiology (50); IX: Circulatology (113); X: Pulmonology (70); XI: Gastroenterology (206); XII: Dermatology (147); XIII: Musculoskeletal (224); XIV: Genitourology (243); XV: Obstetrics (3); XVII: Congenital (10); XVIII: Symptoms/signs (474); XIX: Injury/poisoning (22); XXI: Health state (3).

studies could be beneficial in-patient care. One study found that in patients with colorectal cancer, patients with deficiency descriptors had a higher survival rate than that of patients with excess descriptors—the authors hypothesized that patients diagnosed with excess could benefit from postoperative adjuvant chemotherapy (Li et al., 2020).

The standardized terminology in ICD-11 is a tool that allows for these cross-disciplinary comparisons to be made, both within TM in the different Asian modalities and between TM and CM. Regarding Kampo, while there exists much research into the efficacy of Kampo herbal formulas (Watanabe et al., 2011), much less is known about the significance between pattern diagnoses and their implications for CM diseases amongst TM patients. This will be a research field not only for Kampo practitioners, but for all TM practitioners in the future. The standardized terminology in the ICD-11 has already been utilized in other contexts by our group, such as in predictive modeling. Using the standardized TM pattern descriptors, we made a prediction model for cold-heat and deficiency-excess using data from a nation-wide sample of Kampo patients, and with the end goal to assist non-Kampo practitioners in making these Kampo diagnoses (Maeda-Minami et al., 2019; Maeda-Minami et al., 2020).

This study has some limitations. One limitation is its single-institutional design, reducing generalizability to the broader practice of Kampo in Japan and the world. It was also difficult to conduct stratified analyses for specific CM diagnoses due to our limited sample size. One future area of further research would be comparing TM with different CM disease severities–however there are no classifications for the disease severity in the ICD-10. In the ICD-11, severity of disease will be able to be classified using extension codes. Furthermore, once the ICD-11 is officially launched in 2022,

and future research could utilize larger datasets from multiple countries for similar studies.

Our study was cross-sectional, not allowing us to capture how TM patterns may change over time. Also, mild diseases with short duration, such as the common cold or acute gastroenteritis, are not commonly seen in our clinic due to its affiliation in a university hospital—in our clinic, it is more common to see complex, and chronic disease states. We did not include adolescent and infant patients, limiting generalizability to the population below age 20. Finally, in this study, we only focused on the TM pattern descriptor relationship with CM chapters in ICD-10. Considering the holistic nature of TM and Kampo, future studies may seek to investigate the interactive relationships between different combinations of the three modules of Kampo patterns and CM diseases (Hu et al., 2011).

CONCLUSION

We report Kampo pattern descriptors characteristics in TM ICD-11 pattern descriptors and compare these with CM chapters in ICD-10. Our findings show that certain Kampo pattern descriptors are related to specific CM ICD-10 chapters, especially after stratified by age, sex, and BMI. In the future, we hope that the results from this exploratory study motivate further investigation into the relationships between CM and TM diagnoses. This will be of importance to understand the relationship not only between Kampo and other TM modalities, but also the relationship between CM and TM, and how CM and TM can together advance patient care.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors on request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Keio University School of Medicine Institutional Review Board approved this study (Approval No. 20100144), and the protocol is available at the UMIN clinical trials registry (Unique ID: UMIN000020478). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization KW; Formal analysis XW, AM-M, and TY; Supervision TY, MM, and KW; Funding and resources acquisition KW, MM, TY, and YH; Data acquisition XW, AM-M, TY, and MM; Project design XW, AM-M, and TY; Project administration TY, MM; Original draft writing XW, TL; Review and editing TY, KW; All authors contributed significantly to the editing and revising of the final draft.

FUNDING

XW is funded by the Japanese government (Monbukagakusho: MEXT) scholarship (https://www.mext.go.jp/). MM received research grant supports from Tsumura and Co and applied it to English editing fee and article processing charge. The funding source was not involved in the interpretation of data, writing of the report, and the decision to submit the article for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.751403/full#supplementary-material

REFERENCES

Hu, J., Qiao, J., Kang, D., and Liu, B. (2011). Analysis on the Distinguishing Features of Traditional Chinese Therapeutics and Related Statistical Issues. *Front. Med.* 5 (2), 203–207. doi:10.1007/s11684-011-0138-6

Hyun, M. K., Yoon, H. Y., Yoshino, T., and Park, M. J. (2019). Japanese Government Research grants for Kampo Medicine: aAn Overview of 10 Years (1997-2017). *Integr. Med. Res.* 8 (4), 279–283. doi:10.1016/j.imr.2019.11.006

Li, D., Wang, W., Xiang, L., Ni, T., Tao, L., Lv, M., et al. (2020). The Type of Traditional Chinese Medicine Syndrome Predicts Prognosis and Chemotherapeutic Outcomes in Colorectal Cancer. *Eur. J. Integr. Med.* 33, 101026. doi:10.1016/j.eujim.2019.101026

Lu, A., Jiang, M., Zhang, C., and Chan, K. (2012). An Integrative Approach of Linking Traditional Chinese Medicine Pattern Classification and Biomedicine Diagnosis. *J. Ethnopharmacol* 141 (2), 549–556. doi:10.1016/j.jep.2011.08.045

Lu, A. P., and Chen, K. J. (2011). Chinese Medicine Pattern Diagnosis Could lead to Innovation in Medical Sciences. *Chin. J. Integr. Med.* 17 (11), 811–817. doi:10.1007/s11655-011-0891-z

Maeda-Minami, A., Yoshino, T., Horiba, Y., Nakamura, T., and Watanabe, K. (2021). Inter-Rater Reliability of Kampo Diagnosis for Chronic Diseases. *J. Altern. Complement. Med.* 27, 613–616. doi:10.1089/acm.2020.0298

Maeda-Minami, A., Yoshino, T., Katayama, K., Horiba, Y., Hikiami, H., Shimada, Y., et al. (2020). Discrimination of Prediction Models between Cold-Heat and Deficiency-Excess Patterns. *Complement. Ther. Med.* 49, 102353. doi:10.1016/j.ctim.2020.102353

Maeda-Minami, A., Yoshino, T., Katayama, K., Horiba, Y., Hikiami, H., Shimada, Y., et al. (2019). Prediction of Deficiency-Excess Pattern in Japanese Kampo Medicine: Multi-centre Data Collection. *Complement. Ther. Med.* 45, 228–233. doi:10.1016/j.ctim.2019.07.003

Ministry of Health Labor and Welfare (2020). Statistical Classification of Diseases, Injuries and Causes of Death. Available at: https://www.mhlw.go.jp/toukei/sippei/(Accessed Dec 14, 2020).

Moschik, E. C., Mercado, C., Yoshino, T., Matsuura, K., and Watanabe, K. (2012). Usage and Attitudes of Physicians in Japan Concerning Traditional Japanese Medicine (Kampo Medicine): A Descriptive Evaluation of a Representative Questionnaire-Based Survey. *Evid. Based Complement. Alternat Med.* 2012, 139818. doi:10.1155/2012/139818

Tian, X., Zhu, G., Wang, J., Wang, Q., Guan, L., Tan, Y., et al. (2016). Study on the Relation between Tissues Pathologies and Traditional Chinese Medicine Syndromes in Knee Osteoarthritis: Medical Image Diagnostics by Preoperative X-ray and Surgical Arthroscopy. *J. Xray Sci. Technol.* 24 (4), 509–519. doi:10.3233/XST-160567

Watanabe, K., Matsuura, K., Gao, P., Hottenbacher, L., Tokunaga, H., Nishimura, K., et al. (2011). Traditional Japanese Kampo Medicine: Clinical Research between Modernity and Traditional Medicine—The State of Research and Methodological Suggestions for the Future. *Evid-based Complement. Altern. Med. ECAM* 2011–513842. doi:10.1093/ecam/neq067

Watanabe, K., Plotnikoff, G. A., Sakiyama, T., and Reissenweber-Hewel, H. (2014). Collaboration of Japanese Kampo Medicine and Modern Biomedicine. *Evidence-Based Complement. Altern. Med.* 2014, e646947. doi:10.1155/2014/646947

WHO (2019). International Classification of Diseases, 11th Revision (ICD-11) WHO. Available at: http://www.who.int/classifications/icd/en/(Accessed Mar 21, 2019).

Yakubo, S., Ito, M., Ueda, Y., Okamoto, H., Kimura, Y., Amano, Y., et al. (2014). Pattern Classification in Kampo Medicine. *Evid Based. Complement. Altern. Med.* 2014, 535146. doi:10.1155/2014/535146

Yang, Y., Ma, Q., Yang, Y., He, Y., Ma, C., Li, Q., et al. (2018). Evidence-based Practice Guideline of Chinese Herbal Medicine for Primary Open-Angle Glaucoma (Qingfeng -neizhang). *Medicine (Baltimore)* 97 (13), e0126. doi:10.1097/MD.0000000000010126

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 reviewer (TN) declared past co-authorships with several of the authors (TY, MM, KW, and AM-M) to the handling editor.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 18 January 2022
doi: 10.3389/fphar.2021.695039

The Japanese Herbal Medicine Hangeshashinto Induces Oral Keratinocyte Migration by Mediating the Expression of CXCL12 Through the Activation of Extracellular Signal-Regulated Kinase

*Kanako Miyano[1,2], Seiya Hasegawa[3], Noriho Asai[2,3], Miaki Uzu[4], Wakako Yatsuoka[5], Takao Ueno[5], Miki Nonaka[2], Hideaki Fujii[3] and Yasuhito Uezono[2,6]**

[1]Division of Cancer Pathophysiology, National Cancer Research Institute, Tokyo, Japan, [2]Department of Pain Control Research, The Jikei University School of Medicine, Tokyo, Japan, [3]Laboratory of Medicinal Chemistry, School of Pharmacy, Kitasato University, Tokyo, Japan, [4]Vitrigel Project Research Team, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan, [5]Dental Division, National Cancer Center Hospital, 5-1-1, Tsukiji, Japan, [6]Supportive and Palliative Care Research Support Office, National Cancer Center Hospital East, Kashiwa, Japan

OPEN ACCESS

Edited by:
Yukihiro Shoyama, Nagasaki International University, Japan

Reviewed by:
Yu Chiang Hung, Kaohsiung Chang Gung Memorial Hospital, Taiwan
Won-Kyo Jung, Pukyong National University, South Korea

***Correspondence:**
Yasuhito Uezono
yuezono@jikei.ac.jp

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

Received: *14 April 2021*
Accepted: *09 December 2021*
Published: *18 January 2022*

Citation:
Miyano K, Hasegawa S, Asai N, Uzu M, Yatsuoka W, Ueno T, Nonaka M, Fujii H and Uezono Y (2022) The Japanese Herbal Medicine Hangeshashinto Induces Oral Keratinocyte Migration by Mediating the Expression of CXCL12 Through the Activation of Extracellular Signal-Regulated Kinase. Front. Pharmacol. 12:695039. doi: 10.3389/fphar.2021.695039

Several clinical studies have reported that Japanese herbal medicine Hangeshashinto (HST) has beneficial effects on chemotherapy-induced oral ulcerative mucositis (OUM). Our previous research demonstrated that HST improves chemotherapy-induced OUM through human oral keratinocyte (HOK) migration, which was suppressed by mitogen-activated protein kinase (MAPK) and C-X-C chemokine receptor 4 (CXCR4) inhibitors. However, the association between these molecules and HOK migration was unclear. Here, we examined the effects of HST on the expression of CXCR4/CXCR7 and C-X-C motif chemokine ligands 11 and 12 (CXCL11/CXCL12) in HOKs. Our results indicated that HST upregulated CXCL12, but not CXCR4, CXCR7, nor CXCL11 in HOKs. HST-induced expression of CXCL12 was significantly suppressed by an inhibitor of extracellular signal-regulated kinase (ERK), but not of p38 and c-Jun N-terminal kinase (JNK). In addition, HST induced phosphorylation of ERK in HOKs. These findings suggest that HST enhances HOK migration by upregulating CXCL12 via ERK.

Keywords: hangeshashinto, oral ulcerative mucositis, oral keratinocytes, CXCL12, extracellular signal-regulated kinase

1 INTRODUCTION

Cancer patients receiving chemotherapy, radiotherapy, hematopoietic stem cell transplant, or terminal care often experience severe oral ulcerative mucositis (OUM), which evokes painful inflammation and limits their basic day-to day activities, such as "eating, drinking, and talking" (McGuire et al., 1993; Sonis, 1998; Dodd et al., 2000; Dörr et al., 2002; Sonis, 2004; Duncan et al., 2005; Jones et al., 2006; Vera-Llonch et al., 2006; Barber et al., 2007; El-Housseiny et al., 2007; Vera-Llonch et al., 2007; Bensinger et al., 2008; Sonis, 2010a; Sonis, 2010b; Miyano et al., 2016; Miyano et al., 2020). Additionally, OUM increases the risk of systemic infection via opportunistic microorganisms, which may lead to extension of hospitalization (Elting et al., 2003; Sonis, 2004;

Elting et al., 2007; Yeoh et al., 2007; Lalla et al., 2014; Miyano et al., 2016). Further, OUM often forces patients with cancer to discontinue or modify their therapy regimen, which adversely affects their prognosis (Elting et al., 2003; Trotti et al., 2003; Sonis, 2010a; Miyano et al., 2016). Therefore, effective management of OUM is indispensable for improving both patient quality of life and prognosis (Miyano et al., 2016).

Although chemotherapy-induced OUM is associated with the use of various anti-cancer drugs, there are not many effective prevention methods or therapeutic modalities (Miyano et al., 2016). Hangeshashinto (HST), a traditional Japanese medicine (Kampo medicine) that contains extracts of seven botanical drugs, was approved by Japan's Ministry of Health, Labour and Welfare as a prescription treatment for OUM. From the 16th century to the present, HST has been used in Japan to treat inflammatory diarrhea, gastritis, and oral mucositis (Uezono et al., 2012; Miyano et al., 2016). A recent double-blind, placebo-controlled, randomized study reported that the repetitive use of HST-containing mouthwash effectively improved chemotherapy-induced OUM in patients with colorectal cancer or gastric cancer (Matsuda et al., 2015). Basic research indicated that HST enhanced OUM healing through multiple pharmacological actions, such as anti-oxidant, anti-inflammatory, anti-bacterial, and analgesic activities (Fukamachi et al., 2015; Matsumoto et al., 2015; Hiroshima et al., 2016; Hitomi et al., 2016; Hitomi et al., 2017). With regard to anti-inflammatory effects, we previously determined that various ingredients in HST decrease interleukin 1β-induced prostaglandin E2 (PGE2) production in human oral keratinocytes (HOKs) with multi-targeting effects, such as dual suppression of cyclooxygenase-2 expression and PGE2 metabolic activity (Kono et al., 2014). Moreover, our recent *in vitro* and *in vivo* studies revealed that HST directly affects OUM and enhances tissue repair through migration of HOKs, involving activation of mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), and C-X-C chemokine receptor 4 (CXCR4) (Miyano et al., 2020). In the present study, we investigated the effects of HST on the expression of endogenous CXCR4 agonists (C-X-C chemokine ligands CXCL11 and CXCL12) and the receptors CXCR4 and CXCR7 to clarify how MAPKs and CXCR4 induce HOK migration. We analyzed the effects of several MAPK inhibitors on the expression of CXCL12, and also examined the effects of HST treatment on MAPK phosphorylation in migrating HOKs.

2 MATERIALS AND METHODS

2.1 Chemicals and Reagents

The following reagents were used: fetal bovine serum (FBS) and Keratinocyte-Serum Free Medium (SFM) (1X) (Gibco, Carlsbad, CA, United States); trypsin and trypsin neutralizing solution (TNS; Lonza, Basel, Switzerland); penicillin/streptomycin, dimethyl sulphoxide (DMSO), and U0126 (Nacalai Tesque, Kyoto, Japan); poly-L-lysine (PLL), SB202190 (Sigma-Aldrich, St. Louis, MO, United States); Cellmatrix® I-P (Nitta Gelatin Inc., Osaka, Japan); phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Osaka, Japan); BDPA-Zn (Fujifilm Wako Pure Chemical, Osaka, Japan); and JNK inhibitor II (Calbiochem, San Diego, CA, United States).

HST extract powder (Lot No. 2180014010), the base powder without excipients, was obtained from Tsumura & Co. (Ibaraki, Japan), manufactured as an aqueous extract mixture of seven botanical drugs. All items prescribed in Hangeshashinto are listed as in the Japanese Pharmacopoeia: *Pinellia tuber* (5.0 g, tuber of *Pinellia ternate* (Thunb.) Makino (Araceae)), *Scutellaria root* (2.5 g, root of *Scutellaria baicalensis* Georgi (Lamiaceae)), *Processed ginger* (2.5 g, rhizome of *Zingiber officinale* Roscoe (Zingiberaceae)), *Glycyrrhizae Radix* (2.5 g, root of *Glycyrrhiza uralensis* Fisch. ex DC. (Fabaceae) or *Glycyrrhiza glabra* L. (Fabaceae)), *Ziziphi Fructus* (2.5 g, fruit of *Ziziphus jujuba* Mill. (Rhamnaceae)), *Ginseng Radix* (2.5 g, root of *Panax ginseng* C. A. Mey. (Araliaceae)), and *Coptis rhizome* (1.0 g, rhizome of *Coptis japonica* (Thunb.) Makino (Ranunculaceae), *Coptis chinensis* Franch. (Ranunculaceae), *Coptis deltoidei* C. Y. Cheng and P. K. Hsiao (Ranunculaceae) or *Coptis teeta* Wall. (Ranunculaceae)). Briefly, the mixture of the seven raw materials was extracted in boiling water for 1 h, and the extract was then separated from insoluble waste. The separated extract was concentrated under reduced pressure and then spray-dried to produce the extract powder of HST. The yield of the extract was about 24.3%. The three-dimensional high-performance liquid chromatograph (3D-HPLC) profile of HST was created by Tsumura & Co., showing at **Supplementary Figure S1**. For the analysis of components, the dried extract (1.0 g) of HST was extracted with methanol (20 ml) under ultrasonication for 30 min and was centrifuged at 3,000 rpm for 5 min. The supernatants were filtered with a membrane filter (0.45 μm) and then submitted for HPLC analysis (30 μL). HPLC apparatus consisted of a Shimadzu LC 10A (analysis system software: CLASS-M10A ver. 1.64, Tokyo, Japan) equipped with a multiple wavelength detector (UV 200–400 nm) (Shimadzu SPD-M10Avp, diode array detector), an auto injector (Shimadzu CTO-10AC). HPLC conditions were described as follows: column, ODS (TSK-GEL 80TS, 250 × 4.6 mm i.d., TOSOH, Tokyo, Japan); eluent, (A) 0.05M AcONH4 (pH 3.6) (B) 100% CH3CN. A linear gradient of 90% of A and 10% of B changing over 60 min to 0% A and 100% B was used. (And 100% B was continued for 20 min); temperature, 40°C; flow rate, 1.0 ml/min. The quality of HST was confirmed to fulfill the standard of the Japanese Pharmacopoeia. Specifically, the following marker compounds were included in the extract within the parenthesized rages: baicalin (70–210 mg), glycyrrhizic acid (22–66 mg), and berberine (7–21 mg). All voucher specimens of raw materials used were deposited in the herbarium of Tsumura & Co., with batch numbers (**Supplementary Table S1**).

HST extract powder was suspended in DMSO at 100 mg/ml, diluted 100 fold with culture medium, and filtered through a 0.45 μm membrane (ADVANTEC, Tokyo, Japan) to give a final concentration of 100 μg/ml.

TABLE 1 | Primer sequences of human CXCR4, CXCR7, CXCL12, and GAPDH.

	Forward primers (5′→3′)	Reverse primers (3′→5′)
CXCR4	CGTCTCAGTGCCCTTTTGTTC	CTGAAGTAGTGGGCTAAGGGC
CXCR7	CTATGACACGCACTGCTACATC	CTGTACGAGACTGACCACC
CXCL12	ACACTCCAAACTGTGCCCTT	CTGTAAGGGTTCCTCAGGCG
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC

2.2 Cell Culture

Primary HOKs (ScienCell Research Laboratories, Carlsbad, CA, United States) were cultured on poly-L-lysine-coated dishes in Keratinocyte-SFM (1X) supplemented with 10% FBS and penicillin (100 U/mL).

2.3 Scratch-Induced Migration Assay

HOK migration was evaluated using the IncuCyte ZOOM® system (ESSEN BioScience, Ann Arbor, MI, United States), which enables real-time and quantitative live-cell analysis, as previously described (Miyano et al., 2020). HOKs were seeded at a concentration of 3.0×10^4 cells/0.1 ml/well onto a 96-well ImageLock microplate (ESSEN BioScience), coated with 300 μg/ml Cellmatrix® I-P (Nitta Gelatin Inc., Osaka, Japan). The following day, the cells were scratched using a 96-well WoundMaker (ESSEN BioScience), and the culture medium was changed to assay medium (Keratinocyte-SFM (1X) containing 2% FBS). The cells were then treated with HST (100 μg/ml) and visually monitored every 2 h for 72 h. The area occupied by HOKs on the scratched area was quantified using IncuCyte™ scratch wound cell migration software (ESSEN BioScience).

2.4 Real-Time Quantitative PCR

HOKs were seeded at 6.5×10^5 cells/2 ml/well onto a 6-well microplate (Thermo Fisher Scientific, Inc., Waltham, MA, United States), coated with 300 μg/ml Cellmatrix® I-P. The following day, the cells were scratched with a 1 ml syringe (Terumo Corporation, Tokyo, Japan) and the culture medium was changed to assay medium. The cells were then treated with HST for 48 h. Total RNA was extracted using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Hilden, Germany). Sample RNA (1.5 μg) was reverse-transcribed using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) analysis was conducted using LightCycler FastStart DNA Master PLUS SYBR Green I (Roche, Basel, Switzerland) on a LightCycler 2.0 system (Roche). Thermal cycling was initiated at 95°C for 1 min, followed by 50 cycles of 20 s at 95°C, 10 s at 58°C, and 10 s at 72°C. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference gene to normalize expression levels in RT-qPCR analysis. The primer sequences of CXCR4, CXCR7, CXCL12, and GAPDH are listed in **Table 1**. The PCR products were analyzed on 1.5% agarose gel and had the sizes expected from the known cDNA sequences. CXCL11 primers were purchased from Sino Biological (Beijing, China). RNA quantities of target genes were calculated using the Ct method (Livak and Schmittgen, 2001).

2.5 Western Blotting

Sample proteins were extracted using the AllPrep DNA/RNA/Protein Mini Kit and diluted in sodium dodecyl sulfate (SDS) sample buffer (Nacalai Tesque). After heating for 5 min at 95°C, equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with Blocking One solution (Nacalai Tesque) for 1 h at room temperature, and incubated overnight at 4°C with primary rabbit IgG antibodies against ERK1/2 (1:1,000; Cell Signaling Technology Inc., Danvers, MA, United States) and primary rabbit IgG antibodies against phospho-ERK1/2 (1:1,000; Cell Signaling Technology, Inc.). After washing, the membranes were further incubated with horseradish peroxidase-linked anti-rabbit IgG antibody (1:2,000; Cell Signaling Technology Inc.) for 2 h at room temperature. Immunoreactivity was detected using the Western Lightning ECL Pro system (Perkin Elmer Co., Ltd., Waltham, MA, United States). Finally, the band densities of both pERK and ERK were measured using ImageJ software (National Institutes of Health, Bethesda, MD, United States). ERK expression was calculated using the ratio of the phospho-ERK-specific band density/ERK-specific band density.

2.6 Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM) for at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni's multiple comparisons test (**Figures 1**, **2**, **5**) or unpaired *t*-test (**Figures 2**, **4**), using GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA, United States). A probability value (p) < 0.05 was considered statistically significant.

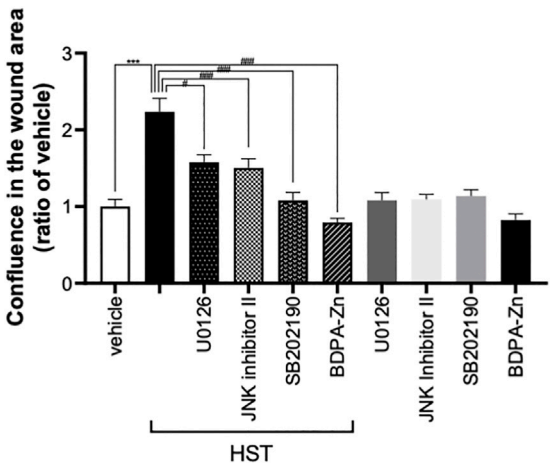

FIGURE 1 | Effect of MAPK inhibitors on Hangeshashinto (HST)-induced migration of human oral keratinocyte (HOKs). HOKs were scratched and co-treated with HST, CXCR4 inhibitor BDPA-Zn, ERK inhibitor U0126, JNK inhibitor II, or p38 inhibitor SB202190 for 72 h. Data are expressed as the mean ± SEM (bars, n = 12–50). *** indicates $p < 0.001$, compared with vehicle; #, ##, #### indicates $p < 0.05$, $p < 0.01$, $p < 0.001$ compared with HST alone. Bonferroni's comparison test following ANOVA.

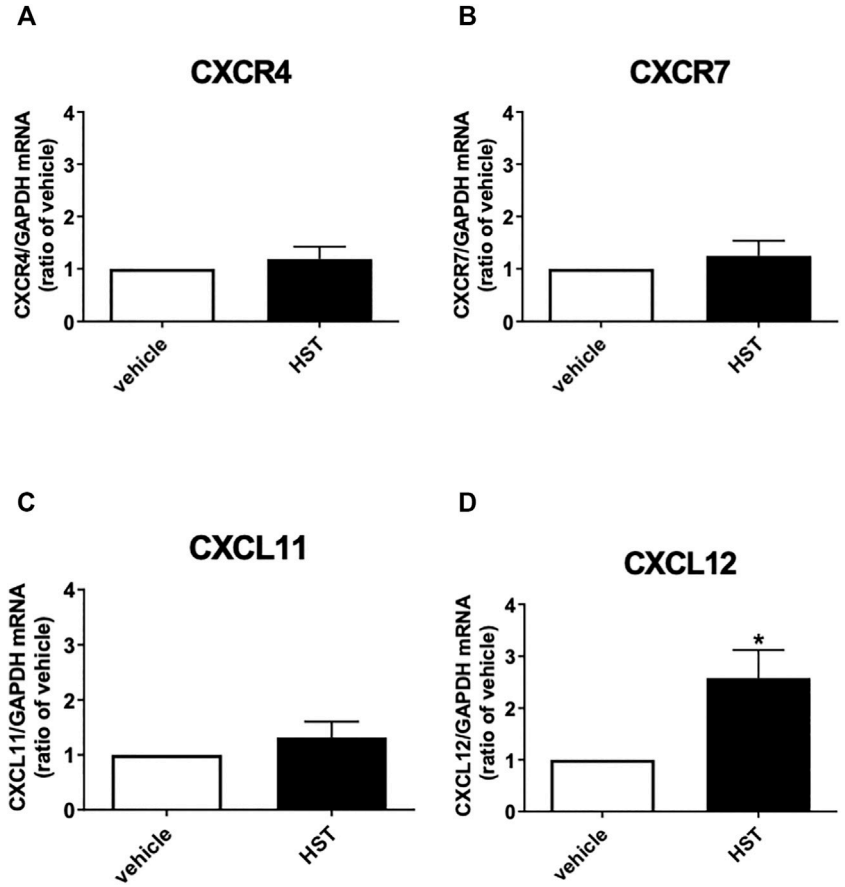

FIGURE 2 | Effect of Hangeshashinto (HST) on the mRNA expression of CXCR4, CXCR7, CXCL11, or CXCL12 in human oral keratinocytes (HOKs). HOKs were scratched and treated with vehicle or HST for 48 h mRNA expression was determined by RT-qPCR. **(A)** CXCR4 (n = 5), **(B)** CXCR7 (n = 6), **(C)** CXCL11 (n = 5), or **(D)** CXCL12 (n = 6). Data are presented as the mean ± SEM (bars). * indicates $p < 0.05$, compared with vehicle; unpaired t-test.

3 RESULTS

3.1 HST Enhanced Scratch-Induced HOK Migration via MAPKs and CXCR4

We previously reported that treatment with 1–100 μg/ml HST enhanced scratch-induced wound healing in dose- and time-dependent manners, which involved HOK migration (Miyano et al., 2020). As shown in **Figure 1**, treatment with HST for 72 h significantly induced HOK migration. Conversely, this effect was significantly suppressed by treatment with a CXCR4 inhibitor (BDPA-Zn, 3 μM), an ERK inhibitor (U0126, 10 μM), a JNK inhibitor (JNK inhibitor II, 1 μM), and a p38 inhibitor (SB202190, 10 μM). Compared with vehicle treatment alone, treatment with each inhibitor did not significantly affect HOK migration (**Figure 1**).

3.2 HST Upregulated CXCL12, But Not CXCR4, CXCR7, Nor CXCL11 in HOKs

To clarify the molecular mechanism responsible for HST-induced HOK migration, we first investigated the effects of HST on the expression of endogenous CXCR4 agonists (CXCL11 and CXCL12) and the receptors CXCR4 and CXCR7 in HOKs. As shown in **Figure 2**, treatment with HST for 48 h significantly increased mRNA expression of CXCL12, but not that of CXCR4, CXCR7, nor CXCL11, compared with vehicle treatment.

3.3 HST Upregulated CXCL12 via ERK Activation in HOKs

To elucidate the involvement of MAPKs in HST-induced upregulation of CXCL12, we examined the effects of MAPK inhibitors on HST-induced upregulation of CXCL12 in HOKs. The ERK inhibitor (U0126, 10 μM) completely suppressed HST-induced CXCL12 mRNA expression (**Figure 3A**), but this phenomenon was not observed when HOKs were treated with the JNK inhibitor (JNK inhibitor II, 1 μM, **Figure 3B**) nor with the p38 inhibitor (SB202190, 10 μM, **Figure 3C**). We then examined whether HST affected phosphorylation of ERK in HOKs using western blotting. The results indicated that HST treatment

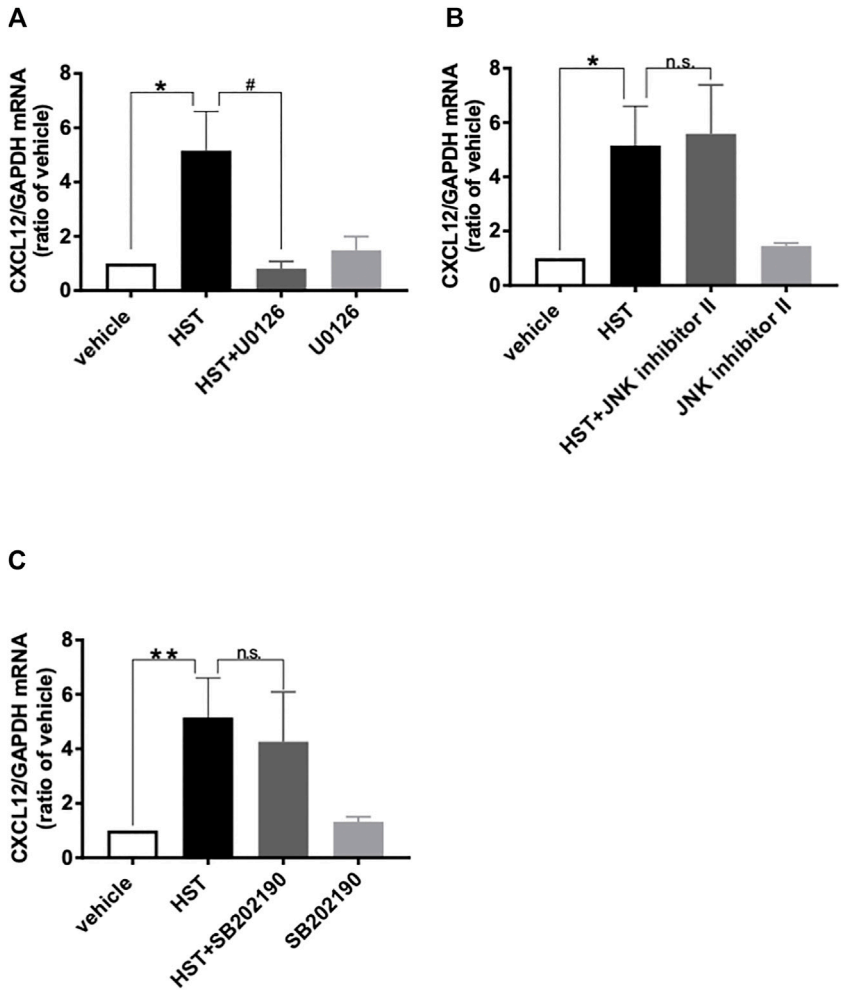

FIGURE 3 | Involvement of MAPKs in Hangeshashinto (HST)-induced expression of CXCL12 mRNA in human oral keratinocytes (HOKs). HOKs were scratched and co-treated with HST and inhibitors of **(A)** ERK (U0126; $n = 3–5$), **(B)** JNK (JNK inhibitor II; $n = 3–5$) or **(C)** p38 (SB202190; $n = 3–8$) for 48 h. mRNA expression was determined by RT-qPCR. Data are expressed as the mean ± SEM (bars). *, ** indicates $p < 0.05$, $p < 0.01$ compared with vehicle, respectively; # indicates $p < 0.05$ compared with HST alone. Bonferroni's comparison test following ANOVA **(A–C)**. n.s. indicates not significant **(B,C)**.

significantly increased ERK phosphorylation in HOKs compared with vehicle treatment (**Figure 4**).

3.4 10-gingerol Upregulated CXCL12 in HOKs

Our previous study revealed that 6-shogaol, 10-gingerol and glycyrrhetinic acid, which are the typical components of HST, enhanced the scratch-induced HOK migration (Miyano et al., 2020). We examined the effects of these components on the level of CXCL12 mRNA expression in HOKs. The doses of these compounds used in the present study were determined according to our previous study (Miyano et al., 2020), which have highest efficacy in the scratch-induced migration. As shown in **Figure 5**, 10-gingerol (10 μM) significantly induced CXCL12 expression, compared with vehicle. Although 6-shogaol (1 μM) and glycyrrhetinic acid (10 μM) slightly increase CXCL12 expression, these responses were not significant.

4 DISCUSSION

In this study, we revealed for the first time that treatment with 100 μg/ml HST activated ERK and upregulated CXCL12 in HOKs, which subsequently caused their migration. OUM treatment in clinical practice involves dissolving HST in hot water to a final concentration 50 mg/ml, followed by mouth washing (Matsuda et al., 2015). Our previous study determined that the doses of HST-derived compounds required for effective HST-induced HOK migration were higher than the concentrations of HST-derived compounds found in patient plasma (Miyano et al., 2020). However, the effective doses in the HST-induced HOK migration assay were lower than those measured in the HST

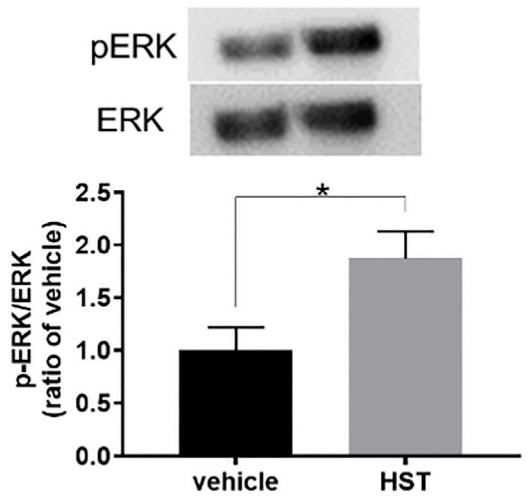

FIGURE 4 | Effect of Hangeshashinto (HST) on ERK phosphorylation in human oral keratinocytes (HOKs). HOKs were scratched and treated with vehicle or HST for 48 h. ERK expression was calculated using the ratio of the pERK-specific band density/ERK-specific band density determined by western blotting (n = 3). Data are expressed as the mean ± SEM. (bars). * indicates $p < 0.05$ compared with vehicle. Unpaired t-test.

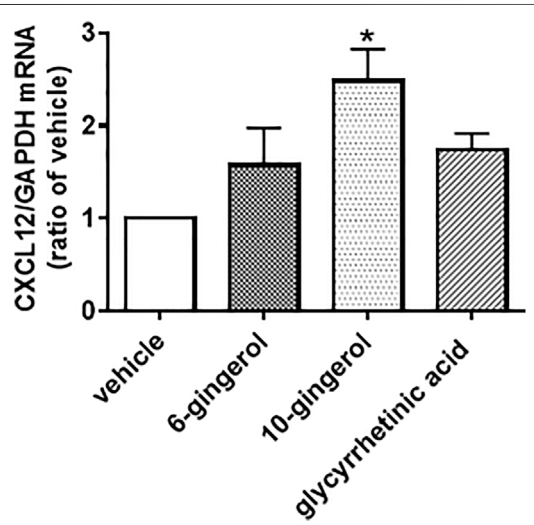

FIGURE 5 | Effect of components of Hangeshashinto (HST) on the expression of CXCL12 mRNA in human oral keratinocytes (HOKs). HOKs were scratched and treated with vehicle, 6-shogaol (1 μM), 10-gingerol (10 μM), or glycyrrhetinic acid (10 μM) for 48 h. mRNA expression was determined by RT-qPCR. Data are expressed as the mean ± SEM. (bars). * indicates $p < 0.05$ compared with vehicle. Bonferroni's comparison test following ANOVA.

solution used in clinical practice (Matsuda et al., 2015; Miyano et al., 2020). Taken together, these findings suggest that HST-induced CXCR12 expression via ERK activation is evoked by direct action of HST on OUM, not following absorption in the blood.

Many studies have reported that MAPK, CXCR, and CXCL play important roles in cell migration (Kukreja et al., 2005; Huang et al., 2009; Yuan et al., 2013; Cui et al., 2016). Concurring with the results of

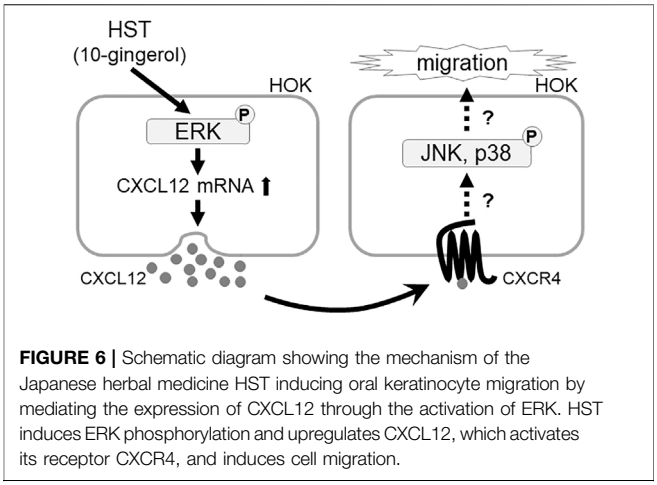

FIGURE 6 | Schematic diagram showing the mechanism of the Japanese herbal medicine HST inducing oral keratinocyte migration by mediating the expression of CXCL12 through the activation of ERK. HST induces ERK phosphorylation and upregulates CXCL12, which activates its receptor CXCR4, and induces cell migration.

previous studies, our results demonstrated that ERK, JNK, p38, and CXCR4 inhibitors significantly suppressed HST-induced HOK migration (**Figure 1**). Shi et al. determined that CXCL12 was upregulated via ERK activation (Shi et al., 2013). In addition, some reports have shown that cell migration induced by the CXCL12/CXCR4 axis is the result of ERK, JNK, and p38 activation (Sun et al., 2002; Kukreja et al., 2005; Huang et al., 2009; Yuan et al., 2013; Cui et al., 2016). We found that HST-induced CXCL12 expression was involved in the activation of ERK, but not that of JNK and p38 (**Figure 3**). HST increased the phosphorylation level of ERK in HOKs (**Figure 4**). Taken together, these data suggest that HST induces ERK phosphorylation and upregulates CXCL12, which activates its receptor CXCR4, and consequently induces cell migration through phosphorylation of JNK and p38 (**Figure 6**). However, further studies are needed to clarify the effects of CXCL12 on HOK migration via JNK and/or p38.

Our previous study revealed that Scutellaria root (baicalein), processed ginger (6-shogaol, 8-shogaol, 10-shogaol, 6-gingerol, 8-gingerol, and 10-gingerol), and Glycyrrhiza (glycyrrhetinic acid) were the active constituents among the seven botanical drugs comprising HST, suggesting that these ingredients could cooperatively enhance scratch-induced HOK migration (Miyano et al., 2020). Some studies have reported that baicalein activates JNK and/or p38 (Chao et al., 2007; Su et al., 2018), while 6-shogaol and 10-gingerol activate ERK, JNK, and p38 (Kim et al., 2009; Kim et al., 2015; Ryu and Chung, 2015). These studies suggest that 6-shogaol and 10-gingerol induce ERK phosphorylation, resulting in production of CXCL12 in HOKs. In fact, our present study revealed that 10-gingerol significantly induced mRNA expression of CXCL12 in HOKs (**Figure 5**). Taken together, these data suggest 10-gingerol induced CXCL12 expression via activation of ERK in HOKs. Further investigation is warranted to elucidate which ingredients including 10-gingerol activate ERK, JNK, and p38.

Our previous study indicated that HST enhances tissue repair using animal models of chemotherapy-induced OUM (Miyano et al., 2020). The migration of keratinocytes is the basis for re-epithelialization during wound healing (Castellano-Pellicena and Thornton, 2020). In our wound healing assay using HOKs, the scratched HOKs produced inflammatory mediators such as PGE2

(data not shown), which were elicited during chemotherapy and such inflammatory mediators induced OUM (Miyano et al., 2016). These data suggest that our cell culture model of HOK migration reflects one of mechanism of chemotherapy-induced OUM. However, further investigations are needed to reveal relationship between HOK migration induced by HST and this tissue repair using both *in vitro* and vivo assay.

In conclusion, the findings of the present study suggest that treatment with HST enhances tissue repair through oral keratinocyte migration likely induced by CXCR4 activation through upregulation of CXCL12 via activation of ERK. In addition, we identified 10-gingerol to induce CXCL12 expression in HOKs among components of HST. However, it is not clear whether 10-gingerol induces CXCL12 expression via ERK in HOKs. Further investigations using *in vivo* and *in vitro* assay are needed to reveal that 10-gingerol improves mucositis via the ERK-CXCL12-CXCR4 pathway. Nonetheless, this study provides scientific evidence supporting the use of HST in patients with cancer and comorbid OUM.

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

Conceptualization, KM and YU; Methodology, KM, SH, NA, and MU; Validation, MN and WY; Investigation, KM, SH, and NA; Data Curation, KM, SH, and NA; Writing-Original Draft Preparation, KM; Writing-Review and Editing, KM and YU; Supervision, TU and HF; Project Administration, YU; Funding Acquisition, TU and YU.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 18K07404 and 21K10059) from the Japan Society for the Promotion of Science and AMED (21ak01011160h0001). The authors declare that this study received funding from Tsumura & Co. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. The founder provided hangeshashinto and its information (e.g., list of raw materials with batch number).

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for their assistance with English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.695039/full#supplementary-material

REFERENCES

Barber, C., Powell, R., Ellis, A., and Hewett, J. (2007). Comparing Pain Control and Ability to Eat and Drink with Standard Therapy vs Gelclair: a Preliminary, Double centre, Randomised Controlled Trial on Patients with Radiotherapy-Induced Oral Mucositis. *Support Care Cancer* 15 (4), 427–440. doi:10.1007/s00520-006-0171-1

Bensinger, W., Schubert, M., Ang, K. K., Brizel, D., Brown, E., Eilers, J. G., et al. (2008). NCCN Task Force Report. Prevention and Management of Mucositis in Cancer Care. *J. Natl. Compr. Canc Netw.* 6 Suppl 1 (Suppl. S1), S1–S4. doi:10.6004/jnccn.2008.2001

Castellano-Pellicena, I., and Thornton, M. J. (2020). Isolation of Epidermal Keratinocytes from Human Skin: The Scratch-Wound Assay for Assessment of Epidermal Keratinocyte Migration. *Methods Mol. Biol.* 2154, 1–12. doi:10.1007/978-1-0716-0648-3_1

Chao, J. I., Su, W. C., and Liu, H. F. (2007). Baicalein Induces Cancer Cell Death and Proliferation Retardation by the Inhibition of CDC2 Kinase and Survivin Associated with Opposite Role of P38 Mitogen-Activated Protein Kinase and AKT. *Mol. Cancer Ther.* 6 (11), 3039–3048. doi:10.1158/1535-7163.MCT-07-0281

Cui, C., Wang, P., Cui, N., Song, S., Liang, H., and Ji, A. (2016). Sulfated Polysaccharide Isolated from the Sea Cucumber Stichopus Japonicas Promotes the SDF-1α/CXCR4 axis-induced NSC Migration via the PI3K/Akt/FOXO3a, ERK/MAPK, and NF-Kb Signaling Pathways. *Neurosci. Lett.* 616, 57–64. doi:10.1016/j.neulet.2016.01.041

Dodd, M. J., Dibble, S. L., Miaskowski, C., MacPhail, L., Greenspan, D., Paul, S. M., et al. (2000). Randomized Clinical Trial of the Effectiveness of 3 Commonly Used Mouthwashes to Treat Chemotherapy-Induced Mucositis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 90 (1), 39–47. doi:10.1067/moe.2000.105713

Dörr, W., Hamilton, C. S., Boyd, T., Reed, B., and Denham, J. W. (2002). Radiation-induced Changes in Cellularity and Proliferation in Human Oral Mucosa. *Int. J. Radiat. Oncol. Biol. Phys.* 52 (4), 911–917. doi:10.1016/s0360-3016(01)02721-3

Duncan, G. G., Epstein, J. B., Tu, D., El Sayed, S., Bezjak, A., Ottaway, J., et al. (2005). Quality of Life, Mucositis, and Xerostomia from Radiotherapy for Head and Neck Cancers: a Report from the NCIC CTG HN2 Randomized Trial of an Antimicrobial Lozenge to Prevent Mucositis. *Head Neck* 27 (5), 421–428. doi:10.1002/hed.20162

El-Housseiny, A. A., Saleh, S. M., El-Masry, A. A., and Allam, A. A. (2007). The Effectiveness of Vitamin "E" in the Treatment of Oral Mucositis in Children Receiving Chemotherapy. *J. Clin. Pediatr. Dent* 31 (3), 167–170.

Elting, L. S., Cooksley, C., Chambers, M., Cantor, S. B., Manzullo, E., and Rubenstein, E. B. (2003). The Burdens of Cancer Therapy. Clinical and Economic Outcomes of Chemotherapy-Induced Mucositis. *Cancer* 98 (7), 1531–1539. doi:10.1002/cncr.11671

Elting, L. S., Cooksley, C. D., Chambers, M. S., and Garden, A. S. (2007). Risk, Outcomes, and Costs of Radiation-Induced Oral Mucositis Among Patients with Head-And-Neck Malignancies. *Int. J. Radiat. Oncol. Biol. Phys.* 68 (4), 1110–1120. doi:10.1016/j.ijrobp.2007.01.053

Fukamachi, H., Matsumoto, C., Omiya, Y., Arimoto, T., Morisaki, H., Kataoka, H., et al. (2015). Effects of Hangeshashinto on Growth of Oral Microorganisms. *Evid. Based Complement. Alternat Med.* 2015, 512947. doi:10.1155/2015/512947

Hiroshima, Y., Bando, M., Inagaki, Y., Kido, R., Kataoka, M., Nagata, T., et al. (2016). Effect of Hangeshashinto on Calprotectin Expression in Human Oral Epithelial Cells. *Odontology* 104 (2), 152–162. doi:10.1007/s10266-015-0196-3

Hitomi, S., Ono, K., Terawaki, K., Matsumoto, C., Mizuno, K., Yamaguchi, K., et al. (2017). [6]-gingerol and [6]-shogaol, Active Ingredients of the Traditional Japanese Medicine Hangeshashinto, Relief Oral Ulcerative Mucositis-Induced Pain via Action on Na+ Channels. *Pharmacol. Res.* 117, 288–302. doi:10.1016/j.phrs.2016.12.026

Hitomi, S., Ono, K., Yamaguchi, K., Terawaki, K., Imai, R., Kubota, K., et al. (2016). The Traditional Japanese Medicine Hangeshashinto Alleviates Oral Ulcer-Induced Pain in a Rat Model. *Arch. Oral Biol.* 66, 30–37. doi:10.1016/j.archoralbio.2016.02.002

Huang, C. Y., Lee, C. Y., Chen, M. Y., Yang, W. H., Chen, Y. H., Chang, C. H., et al. (2009). Stromal Cell-Derived factor-1/CXCR4 Enhanced Motility of Human Osteosarcoma Cells Involves MEK1/2, ERK and NF-kappaB-dependent Pathways. *J. Cel Physiol* 221 (1), 204–212. doi:10.1002/jcp.21846

Jones, J. A., Avritscher, E. B., Cooksley, C. D., Michelet, M., Bekele, B. N., and Elting, L. S. (2006). Epidemiology of Treatment-Associated Mucosal Injury after Treatment with Newer Regimens for Lymphoma, Breast, Lung, or Colorectal Cancer. *Support Care Cancer* 14 (6), 505–515. doi:10.1007/s00520-006-0055-4

Kim, J. H., Chang, J. H., Yoon, J. H., Kwon, S. H., Bae, J. H., and Kim, K. S. (2009). [6]-Gingerol Suppresses Interleukin-1 Beta-Induced MUC5AC Gene Expression in Human Airway Epithelial Cells. *Am. J. Rhinol Allergy* 23 (4), 385–391. doi:10.2500/ajra.2009.23.3337

Kim, S. M., Kim, C., Bae, H., Lee, J. H., Baek, S. H., Nam, D., et al. (2015). 6-Shogaol Exerts Anti-proliferative and Pro-apoptotic Effects through the Modulation of STAT3 and MAPKs Signaling Pathways. *Mol. Carcinog* 54 (10), 1132–1146. doi:10.1002/mc.22184

Kono, T., Kaneko, A., Matsumoto, C., Miyagi, C., Ohbuchi, K., Mizuhara, Y., et al. (2014). Multitargeted Effects of Hangeshashinto for Treatment of Chemotherapy-Induced Oral Mucositis on Inducible Prostaglandin E2 Production in Human Oral Keratinocytes. *Integr. Cancer Ther.* 13 (5), 435–445. doi:10.1177/1534735413520035

Kukreja, P., Abdel-Mageed, A. B., Mondal, D., Liu, K., and Agrawal, K. C. (2005). Up-regulation of CXCR4 Expression in PC-3 Cells by Stromal-Derived Factor-1alpha (CXCL12) Increases Endothelial Adhesion and Transendothelial Migration: Role of MEK/ERK Signaling Pathway-dependent NF-kappaB Activation. *Cancer Res.* 65 (21), 9891–9898. doi:10.1158/0008-5472.CAN-05-1293

Lalla, R. V., Bowen, J., Barasch, A., Elting, L., Epstein, J., Keefe, D. M., et al. (2014). MASCC/ISOO Clinical Practice Guidelines for the Management of Mucositis Secondary to Cancer Therapy. *Cancer* 120 (10), 1453–1461. doi:10.1002/cncr.28592

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25 (4), 402–408. doi:10.1006/meth.2001.1262

Matsuda, C., Munemoto, Y., Mishima, H., Nagata, N., Oshiro, M., Kataoka, M., et al. (2015). Double-blind, Placebo-Controlled, Randomized Phase II Study of TJ-14 (Hangeshashinto) for Infusional Fluorinated-Pyrimidine-Based Colorectal Cancer Chemotherapy-Induced Oral Mucositis. *Cancer Chemother. Pharmacol.* 76 (1), 97–103. doi:10.1007/s00280-015-2767-y

Matsumoto, C., Sekine-Suzuki, E., Nyui, M., Ueno, M., Nakanishi, I., Omiya, Y., et al. (2015). Analysis of the Antioxidative Function of the Radioprotective Japanese Traditional (Kampo) Medicine, Hangeshashinto, in an Aqueous Phase. *J. Radiat. Res.* 56 (4), 669–677. doi:10.1093/jrr/rrv023

McGuire, D. B., Altomonte, V., Peterson, D. E., Wingard, J. R., Jones, R. J., and Grochow, L. B. (1993). Patterns of Mucositis and Pain in Patients Receiving Preparative Chemotherapy and Bone Marrow Transplantation. *Oncol. Nurs. Forum* 20 (10), 1493–1502.

Miyano, K., Eto, M., Hitomi, S., Matsumoto, T., Hasegawa, S., Hirano, A., et al. (2020). The Japanese Herbal Medicine Hangeshashinto Enhances Oral Keratinocyte Migration to Facilitate Healing of Chemotherapy-Induced Oral Ulcerative Mucositis. *Sci. Rep.* 10 (1), 625. doi:10.1038/s41598-019-57192-2

Miyano, K., Ueno, T., Yatsuoka, W., and Uezono, Y. (2016). Treatment for Cancer Patients with Oral Mucositis: Assessment Based on the Mucositis Study Group of the Multinational Association of Supportive Care in Cancer in International Society of Oral Oncology (MASCC/ISOO) in 2013 and Proposal of Possible Novel Treatment with a Japanese Herbal Medicine. *Curr. Pharm. Des.* 22 (15), 2270–2278. doi:10.2174/1381612822666160219120842

Ryu, M. J., and Chung, H. S. (2015). [10]-Gingerol Induces Mitochondrial Apoptosis through Activation of MAPK Pathway in HCT116 Human colon Cancer Cells. In Vitro *Cel Dev Biol Anim* 51 (1), 92–101. doi:10.1007/s11626-014-9806-6

Shi, C. H., Jiang, Y. N., Shan, L. J., Lu, Y., Zhang, Y., and Gao, Y. G. (2013). Advanced Oxidation Protein Products Promote Expression of Stromal-Cell Derived Factor-1alpha of ECV304 Cells through ERK Signal Pathway. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 29 (2), 142–146.

Sonis, S. T. (2010). Efficacy of Palifermin (Keratinocyte Growth Factor-1) in the Amelioration of Oral Mucositis. *Core Evid.* 4, 199–205. doi:10.2147/ce.s5995

Sonis, S. T. (1998). Mucositis as a Biological Process: a New Hypothesis for the Development of Chemotherapy-Induced Stomatotoxicity. *Oral Oncol.* 34 (1), 39–43. doi:10.1016/s1368-8375(97)00053-5

Sonis, S. T. (2010). New Thoughts on the Initiation of Mucositis. *Oral Dis.* 16 (7), 597–600. doi:10.1111/j.1601-0825.2010.01681.x

Sonis, S. T. (2004). Pathobiology of Mucositis. *Semin. Oncol. Nurs.* 20 (1), 11–15. doi:10.1053/j.soncn.2003.10.003

Su, M. Q., Zhou, Y. R., Rao, X., Yang, H., Zhuang, X. H., Ke, X. J., et al. (2018). Baicalein Induces the Apoptosis of HCT116 Human colon Cancer Cells via the Upregulation of DEPP/Gadd45a and Activation of MAPKs. *Int. J. Oncol.* 53 (2), 750–760. doi:10.3892/ijo.2018.4402

Sun, Y., Cheng, Z., Ma, L., and Pei, G. (2002). Beta-arrestin2 Is Critically Involved in CXCR4-Mediated Chemotaxis, and This Is Mediated by its Enhancement of P38 MAPK Activation. *J. Biol. Chem.* 277 (51), 49212–49219. doi:10.1074/jbc.M207294200

Trotti, A., Bellm, L. A., Epstein, J. B., Frame, D., Fuchs, H. J., Gwede, C. K., et al. (2003). Mucositis Incidence, Severity and Associated Outcomes in Patients with Head and Neck Cancer Receiving Radiotherapy with or without Chemotherapy: a Systematic Literature Review. *Radiother. Oncol.* 66 (3), 253–262. doi:10.1016/s0167-8140(02)00404-8

Uezono, Y., Miyano, K., Sudo, Y., Suzuki, M., Shiraishi, S., and Terawaki, K. (2012). A Review of Traditional Japanese Medicines and Their Potential Mechanism of Action. *Curr. Pharm. Des.* 18 (31), 4839–4853. doi:10.2174/138161212803216924

Vera-Llonch, M., Oster, G., Ford, C. M., Lu, J., and Sonis, S. (2007). Oral Mucositis and Outcomes of Autologous Hematopoietic Stem-Cell Transplantation Following High-Dose Melphalan Conditioning for Multiple Myeloma. *J. Support. Oncol.* 5 (5), 231–235.

Vera-Llonch, M., Oster, G., Hagiwara, M., and Sonis, S. (2006). Oral Mucositis in Patients Undergoing Radiation Treatment for Head and Neck Carcinoma. *Cancer* 106 (2), 329–336. doi:10.1002/cncr.21622

Yeoh, A. S., Gibson, R. J., Yeoh, E. E., Bowen, J. M., Stringer, A. M., Giam, K. A., et al. (2007). A Novel Animal Model to Investigate Fractionated Radiotherapy-Induced Alimentary Mucositis: the Role of Apoptosis, P53, Nuclear Factor-kappaB, COX-1, and COX-2. *Mol. Cancer Ther.* 6 (8), 2319–2327. doi:10.1158/1535-7163.MCT-07-0113

Yuan, L., Sakamoto, N., Song, G., and Sato, M. (2013). Low-level Shear Stress Induces Human Mesenchymal Stem Cell Migration through the SDF-1/CXCR4 axis via MAPK Signaling Pathways. *Stem Cell Dev* 22 (17), 2384–2393. doi:10.1089/scd.2012.0717

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

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

frontiers in Pharmacology

REVIEW
published: 01 March 2022
doi: 10.3389/fphar.2021.713228

Overview of Cannabis including Kampo Medicine and Therapy for Treatment of Dementia: A Review

*Tibor Wenger[1], Kazuhito Watanabe[2], Yui Sasaki[3], Keiko Kanazawa[3], Koichi Shimizu[3], Supaart Sirikantaramas[4], Yoshinari Shoyama[5], Futoshi Taura[6], Satoshi Morimoto[7] and Yukihiro Shoyama[8]**

[1]Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary, [2]Daiichi University of Pharmacy, Fukuoka, Japan, [3]Association for Health Economics Research and Social Insurance and Welfare, Tokyo, Japan, [4]Molecular Crop Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, [5]Bonac Corporation, BIO Factory, Fukuoka, Japan, [6]Faculty of Pharmacy and Pharmaceutical Science, Toyama, Japan, [7]Faculty of Pharmaceutical Science, Fukuoka, Japan, [8]Faculty of Pharmacy, Nagasaki International University, Nagasaki, Japan

Cannabis sativa L. is an annual herb oldest cultivated plants as a source of fiber since about 5000 B.C. On the other hand, the cannabis flower and seed are listed in Shennong's classic Materia Medica approximately 2000 years ago. The formulas prescribed with cannabis in Kampo medicine have been summarized. Cannabidiol (CBD) and tetrahydrocannabinol (THC) are the major neurological and psychiatric cannabinoids, and develop to drugs. It becomes evident that the therapeutic CBD and/or THC are the important candidate of anti-dementia drugs having different mechanism for Alzheimer's patients. Two receptors and endocannabinoids are also discussed for underlying mechanism of action. In order to promote the breeding of cannabis plant containing higher concentration of target cannabinoid the biosynthetic enzymes were isolated, cloning and the tertiary structure of THCA synthase determined by x-ray analysis resulting in the possibility of molecular breeding for cannabinoids.

Keywords: *Cannabis sativa*, kampo medicine, cannabinoid biosynthetic enzyme, CB1 and CB2 receptor, anti-dementia

OPEN ACCESS

Edited by:
Pulok Kumar Mukherjee, Institute of Bio-Resources and Sustainable Development (IBSD), India

Reviewed by:
Cláudia Pereira, University of Coimbra, Portugal
Michihito Deguchi, Penn State Harrisburg, United States

****Correspondence:***
Yukihiro Shoyama
shoyama@niu.ac.jp

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

Received: *22 May 2021*
Accepted: *21 December 2021*
Published: *01 March 2022*

Citation:
Wenger T, Watanabe K, Sasaki Y, Kanazawa K, Shimizu K, Sirikantaramas S, Shoyama Y, Taura F, Morimoto S and Shoyama Y (2022) Overview of Cannabis including Kampo Medicine and Therapy for Treatment of Dementia: A Review. Front. Pharmacol. 12:713228. doi: 10.3389/fphar.2021.713228

INTRODUCTION

Cannabis sativa L. (family Cannabaceae, formerly Moraceae) is an annual herb, commonly called marijuana or cannabis in addition to many regional names (**Table 1**). *C. sativa* L. is used by approximately 3% of the global population as a relaxant (United Nations Office on Drugs and Crime, 2017). Tetrahydrocannabinol (THC) and cannabidiol (CBD) are the major constituents of cannabis, known as cannabinoids, and both exhibit neurological and psychiatric activities (Cohen et al., 2019; Friedman et al., 2019).

Cannabis is among the oldest cultivated plants as has been used as a source of fiber since about 5000 B.C. At present, cannabis is cultivated worldwide, as the plant is adaptable to a wide range of temperatures. The cannabis seed is considered one of five grains together with bean, corn, millet, and Japanese barnyard millet, and was first recorded in China around 1000 B.C. Three herbs, including cannabis, safflower, and indigo, together with four trees, including the tea tree, mulberry plant, lacquer plant, and paper mulberry, have been cultivated in Japan for at least 1,000 years. Among these plants, cannabis is an important source of fiber, while safflower and indigo are used to obtain dyes for the production of clothing. The cannabis flower and seed are listed among 120 safe medicinals in

TABLE 1 | Name of marihuana and its products in the world.

Country	Name of marihuana and its products
Algeria	Kif
Brasil	Diamba, Djamba, Liamba, Riamba, Maconha, Meconha
Edypt	Kamonga
Greek	Mavron
India	Ganja, Bhang, Charas
Jamaica	Ganga
Lebanon	Hashish el Keif
Madagascar	Rongony
Mexico	Mariguana, Marihuana, Marijuana
Morocco	Kif
Mozambique	Bangue, Suruma
Northwest Africa	Chira, Chiras
South Africa	Dagga
Syria	Hashish el Keif
Tunisia	Takrouri
Turkey	Kobak
United States	Mariguana, Marihuana, Marijuana
West indies	Mariguana, Marihuana, Marijuana

Shennong's classic Materia Medica, composed during the Eastern Han Dynasty (25–220 C.E.), which lists 365 species that are divided into three categories: safe (120 items), medicinal use (120 items), and toxic substances (125 items). Therefore, cannabis can be classified as a useful plant as a source of fiber and medicine.

Cannabis species contain 110 cannabinoids and 440 non-cannabinoid compounds, including terpenoids, flavonoids, and sterols (Solymosi and Köfalvi, 2017). Therapeutic studies suggest that cannabis is clinically useful for the treatment of a wide range of pathological conditions, including neurological and psychiatric disorders (Cohen et al., 2019; Friedman et al., 2019). The major cannabinoids of cannabis include CBD, THC, cannabinol, and cannabichromene (**Figure 1**). Adams et al. (1940) were first to describe the structure of cannabinol, while Mechoulam and Shvo (1963) and Gaoni and Mechoulam (1964) determined the structures of THC and CBD, including the positions of the double bonds and stereochemistries. Turner et al. (1980) noted the existence of 60 cannabinoids, which was increased to 70 in 2005. Elsohly et al. (2017) confirmed the isolation of 120 cannabinoids and elucidated the structures following the rapid development of analytical technologies, such as high-sensitivity mass spectrometry. It is believed that cannabinoids are sensitive against autooxidation, high temperature, lite and so on. In fact cannabinolic acid (CBNA) (Shoyama et al., 1970) and cannabicyclolic acid (CBLA) (Shoyama et al., 1972) are transformed from tetrahydrocannabinolic acid (THCA) and cannabichromenic acid (CBCA), respectively by photooxidation. However, recently Pacifici et al. (2017) confirmed that major cannabinoids, THCA and CBDA which are easily decarboxylated to give THC and CBD, respectively by heating are still stable after 1 year storage as a plant form under dark condition. This phenomenon might be occurred from the evidence that THCA and CBDA are contained in the resin composed of mainly essential oil in glandular trichomes on leaves as a sealed form (Morimoto et al., 2007).

The cannabinoids THC and CBD are the only pharmacologically active constituents of cannabis (**Figure 1**) and are now widely used for the treatment of chemotherapy-induced nausea and vomiting, and weight loss and intractable seizure due to HIV/AIDS.

KAMPO MEDICINE PRESCRIBED WITH CANNABIS

The cannabis flower is classified as a safe substance in the Chinese medical text Shennong Ben Cao Jing for the treatment of trauma and to activate memory. In appropriate amounts, cannabis has psychological activities, but can cause mental illness excessive

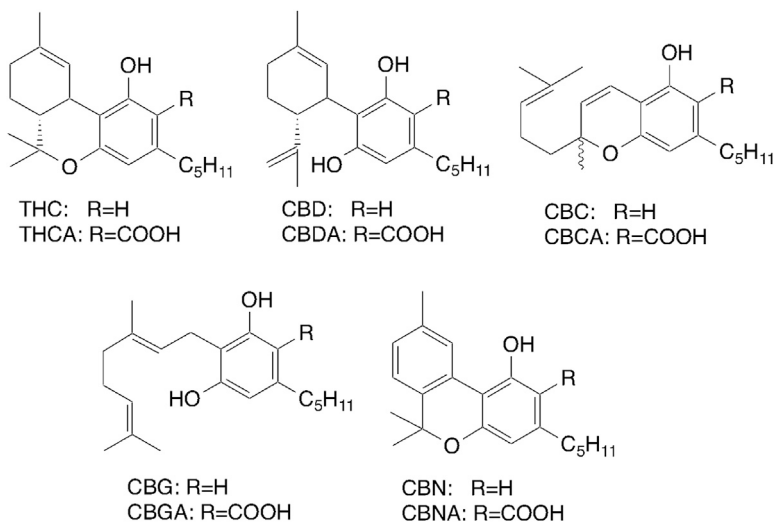

FIGURE 1 | Structures of major cannabinoids.

amounts. Long-term intake of the seed and flower of cannabis has been proposed to maintain health. The Compendium of Materia Medica documented that the cannabis flower, called mafen, is applied as a psychotropic medication to stabilize the spirit and body to reach an immortal stage with a clear head when used in suitable amounts which might be related to anti-dementia activity as discussing in the next section. Leaves can be used for fiver and malaria. Recently Brand and Zhao (2017) introduced the mental effects of cannabis and long-term use linked to hallucinations and psychotic behaviors reported by Li Shizhen sixteen century. He Chinese surgeon Hua Tuo (140~208 C.E.) was recorded as the first physician to use cannabis with alcohol and herbs, known as mafeisan, as an anesthetic prior to surgery. The historical details of the use of mafeisan as an anesthetic drug remain a mystery (Rafe De Crespigny, 2007). However, recently Brand and Zhao (2017) confirmed that the anesthetic prescription for decreasing paint in China was the combination of cannabis and *Datura* species flower documented in the text *Heart Text of Bian Que* (1127–1270 AD).

The cannabis seed, known as mazi in Kampo medicine, is also classified as a safe substance that can enhance visceral functions and mood. When continuously consumed for prolonged periods, body fat is reduced allowing the user to have a more youthful appearance. Kampo medicines prescribed with cannabis seeds are widely used for treatment of constipation.

Mashiningan is composed of the *Shojokito* formulation, which includes immature orange, magnolia bark, peony root, and rhubarb to promote excretion. The addition of cannabis seed and apricot kernel, which are oily seeds, promotes smooth and mild excretion. *Junchogan* resembles the *Mashiningan* formulation with the addition of Japanese angelica root, rehmannia root, scutellaria root, and grilled licorice, which is used to promote fluid retention and mucus secretion in the large intestine, especially in the elderly. Meanwhile, the *Junchogan* formulation is used to treat habitual constipation in the elderly with no side effects.

Junsoto is similar to the *Junchogan* formulation with the addition of safflower to promote the circulation of body fluids (oketsu) and has almost same pharmacological activity as the Junchogan formulation. In these formulations, anthraquinone glycosides in rhubarb promote peristalsis in the large intestine, similar to sennosides, which can transfer a sugar moiety to anthraquinone via intestinal bacteria, resulting in the production of anthracene under anaerobic conditions. Anthracene derivatives stimulate excretion by the large intestine. In this formulation, cannabis seeds promote smooth over stimulation of the intestine because of the relatively high oil content.

On the other hand, *Shakanzoto* contains cannabis seed (to moisten the intestine), ginseng, broiled licorice and cinnamon, rehmannia root, ophiopogon tuber, and donkey-hide gelatin without rhubarb, which is used for shortness of breath and palpitations with constipation-like symptoms, lack of nutrients, dry skin, and fatigue. In this formulation, cannabis seeds function to moisten the intestine and induce detoxification. Long-term use is believed to promote youthfulness and lucidness as with the *Shennong* formulation.

THERAPEUTIC MARIJUANA AND DEMENTIA

The increase in human life expectancy has led to a surge in the prevalence of neurodegenerative disorders, especially dementia, worldwide. The incidence of dementia is projected to reach 81.1 million by the year 2040 (Ferri et al., 2005). In Japan, the number of dementia patients is expected to reach 7.3 million in the year 2025 and 10.2 million in 2050 (**Figure 2**) (National Institute of Public Health, 2015). Due to the rapid increase in the number of dementia patients, innovaventative strategies are urgently needed.

Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, the major types of dementia are Alzheimer's disease (AD), vascular dementia, frontal lobe hyperdermia, dementia with Lewy bodies, Parkinson's disease, Huntington's disease, and mixed dementia. Among these disorders, AD is the most common, followed by dementia with Lewy bodies and vascular dementia. AD, which accounts for an estimated 30% of dementia cases, affects 33 million people worldwide. Typical causes of AD include neuroinflammation and oxidative stress in the brain resulting in the accumulation of amyloid-β (Aβ) plaques and tau hyperphosphorylation. It is clear that the risk of AD increases with age, but lifestyle and dietary habits are closely related to the development of dementia.

Many studies have identified various compounds with anti-dementia activities, as determined by analysis of acetylcholinesterase inhibitors (Ho et al., 2011; Natarajan et al., 2013). However, galantamine (from Galanthus caucasicus and G. woronowii) is the only natural acetylcholinesterase inhibitor currently available for clinical use. Recently, donepezil and rivistagmine have been approved for clinical use as synthetic acetylcholinesterase inhibitors, and memantine as a N-methyl-D-aspartate receptor antagonist for AD patients (Mangialasche et al., 2010). However, the side effects of acetylcholinesterase inhibitors include nausea, diarrhea, and weight loss (Kaduszkiewicz et al., 2005), while those of memantine include hallucinations, dizziness, and fatigue (Parsons et al., 1999).

THC has been approved for medicinal use by the Food and Drug Administration as a safe and effective treatment for nausea and vomiting induced by chemotherapy, and weight loss due to HIV/AIDS. THC is marketed under the names Dronabinol, Adversa, Syndros, Marinol, and Reduvo for the treatment of nausea, vomiting, weight loss, and sleep apnea. CBD under the name Epidiolex was approved in the United States and European Union in 2018 and 2019, respectively, for the treatment of intractable seizures, especially intractable epilepsy.

THC inhibits acetylcholinesterase activity and prevents aggregation of Aβ-plaques *in vitro* (Eubanks et al., 2006). On the other hand, CBD might have antioxidant activities that could affect the metabolism of anandamide, although the underlying mechanism and receptor remain unclear (Campillo and Paez, 2009; Iuvone et al., 2009; Iuvone et al., 2009). Aβ-induced neurotoxicity was protected by CBD *in vitro*. Furthermore, following challenge with Aβ proteins *in vitro*, CBD inhibited intracellular signaling pathways and suppressed tau protein

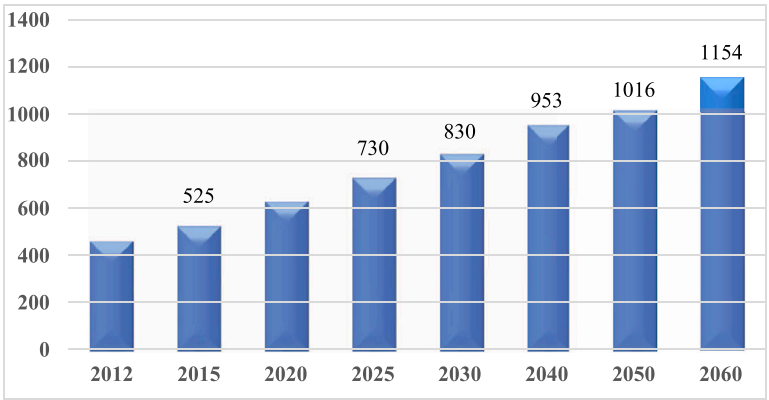

FIGURE 2 | Increases of dementia patients in Japan.

hyperphosphorylation (Esposito et al., 2006a) and nitric oxide production (Esposito et al., 2006b). When the mouse hippocampus was injected with Aβ proteins, CBD induced dose-dependent suppression of the proinflammatory factors interleukin-1β and nitric oxide (Esposito et al., 2007).

Therefore, CBD may act against oxidative stress and tau phosphorylation in AD without the risk of the psychological side effects of THC.

A recent study assessed CBD for the prevention and treatment of AD (Georgia et al., 2017). An *in vivo* study conducted by Watt and Karl (2017) reported that CBD has therapeutic potential for treatment of AD. CBD exhibited anti-oxidative, neuroprotective, anti-inflammatory activities in mice injected with human Aβ proteins, while transgenic APPxPS1 mice developed Aβ plaques in the hippocampus and cortex. CBD decreased Aβ plaque-induced reactive gliosis and decreased iNOS and interleukin-1β protein levels, resulting reduced inflammation of neuronal tissues and subsequent neurogenesis, while preventing cognitive deficits in an animal model of AD.

Moreover, administration of a combination of CBD and THC resulted in greater therapeutic activity than with CBD alone, likely due to the antagonistic response of CBD. Based on these findings, CBD is strong candidate as a prophylactic for AD because the pharmacological mechanism and efficacy are completely different than those of current drugs. Furthermore, CBD can be used clinically without the psychological side effects of THC and no concern about potential abuse.

Recently several systematic reviews have been reported. Kim et al. (2019) reviewed the publications related to the use of cannabinoid for dementia and evaluated that CBD was useful for the treatment and prevention for AD. For example CBD protects PC-12 cells against Aβ neurotoxicity and oxidation stress. Moreover, CBD inhibit acetylcholinesterase to promote memory, stimulate the neurogenesis of the hippocampus, strengthen cell survival by reducing ROS production and lipid peroxidation and so on. They concluded that the combination of CBD and THC was more effective in memory than CBD or THC alone although the psychotropic activity of THC appeared, Twelve studies were associated with inclusion criteria. Study designs were relatively scattered such as randomized controlled trials (50%) and the drug of cannabinoids, dronabinol (33%), nabilone (25%) or THC (42%). Among them dronabinol and THC were associated with significant improvements in a range of neuropsychiatric scores. The most common side effect reported was sedation. Studies under low doses of cannabinoids were evaluated to be not enough efficacy.

Although it was not proven in a randomized control trial, the observational studies showed promising results for patients having refractory symptoms. The safety evidence is good and mild. Authors suggested that dose increase and formulations having appropriated bioavailability will be needed in future (Hillen et al., 2019).

Ten female demented patients with severe behavior problems received an oral intact of higher dosages of THC and CBD likely increasing to 9.0 mg THC/18.0 mg CBD after 2 months compared to the other studies, and were well tolerated and improved behavior problems, rigidity, and daily care in severely demented patients (Broers et al., 2019).

A systematic review of randomized controlled trials reported that THC is effective against the cognitive symptoms of dementia, although evidence was less than convincing (Charernboon et al., 2021).

POTENTIAL BENEFITS

Cannabis, which can be classified as a fiber, grain, oil, and/or medicine, is used by only 3% of the global population. The cannabis flower and seed have been successfully used for the treatment of psychological disorders. As mentioned above, ligands for the two types of THC receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993) identified in the brain and macrophage, respectively, were identified as anandamide (Devane et al., 1992) and 2-arachidony glyceride (Sugiura et al., 1995). These findings have promoted the medical use of cannabinoids directly and further research of drug development from the cannabinoids THC and CBD.

Long-term use of marijuana can result in "cannabis psychosis," which is characterized by the development of psychotomimetic and psychiatric disorders and mental instability. Also, decreased short-term potentiation and a motivational syndrome have been reported in younger people who repeatedly use marijuana (George, 2017).

However, since decriminalization policy against marihuana have been spread worldwide, the potentiality of marihuana for therapeutic use are growing louder (World drug report, 2021). In fact Brunetti et al. suggested the appropriate dosages, the analytical way of cannabinoids and the concentration of cannabinoids in marihuana prepared from different sources of herbal cannabis for prescribing doctors (Brunetti et al., 2020).

CANNABINOID RECEPTORS AND ENDOCANNABINOID

Two receptors and endocannabinoids will be discussed for underlying mechanism of action of cannabinoids. The ligands of two types of THC receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993) in the brain and macrophage, respectively, were identified as arachidonoylethanolamide (anandamide) (Devane et al., 1992) and two- arachidony glyceride (Sugiura et al., 1995). It became evident that 2-arachidony glyceride is a potent ligand with high affinity for both the CB1 and CB2 receptors and the content is higher in rat brain than that of anandamide (Murielle et al., 1994). In 1998 Murielle et al. (1998) identified a selective antagonist for the CB2 receptor.

CB1 Receptor: Matsuda et al. (1990) was first to isolate, clone, and sequence a central cannabinoid receptor. Gerard et al. (1990) reported that cannabinoid receptors of the human and rat shared a 98% amino acid homology. The human cannabinoid receptor, which belongs to the seven *trans*-membrane spanning receptor family, assumes a three-dimensional conformation with 1) seven helices spanning from one side of the cell, 2) three extra-cellular and three intracellular loops, 3) a glycosylate extra-cellular N-terminal domain, and 4) an intracellular C-terminal domain involved in interactions with a G protein, which is responsible for the *trans*-membrane transduction of the receptor-mediated signal. Hua et al. (2016) elucidated the crystal structure of human CB1 and, Moldrich and Wenger (2000) used an immunohistochemical technique to identify a CB1 receptor in the rat brain and to elucidate the distribution in several other organs.

CB2 Receptor: A peripheral cannabinoid receptor, named CB2, was identified in the human spleen and was also identified as a G protein coupled 7-trans-membrane spanning receptor with 44% sequence identity with the CB1 receptor (Munro et al., 1993).

Recent advancements in endocannabinoid research have shown that most of pharmacological properties of anandamide are similar in the central nervous system (CNS) and peripheral systems with THC and other active cannabinoids, especially in regard to 1) the inhibitory effects on memory, motor activity and turning behavior (Lichtman et al., 1995; Romero et al., 1995; Terranova et al., 1995), 2) ocular blood pressure and heart rate (Varga et al., 1995; Weidenfeld et al., 1994, 3) regulation of hormones involved in the hypothalamus-pituitary-adrenal axis (Giannikou et al., 1995; Wenger et al., 1995), 4) neurotransmission mediated by dopamine, acetylcholine, noradrenalin, endorphin, glutamate, and gamma-aminobutyric acid (Wickens and Pertwee, 1993; Ishac et al., 1996, and 5) the immune response (Schuel et al., 1994; Schwarz et al., 1994). Since some of these functions are closely related to the CNS, therapeutic cannabinoids were discussed previously.

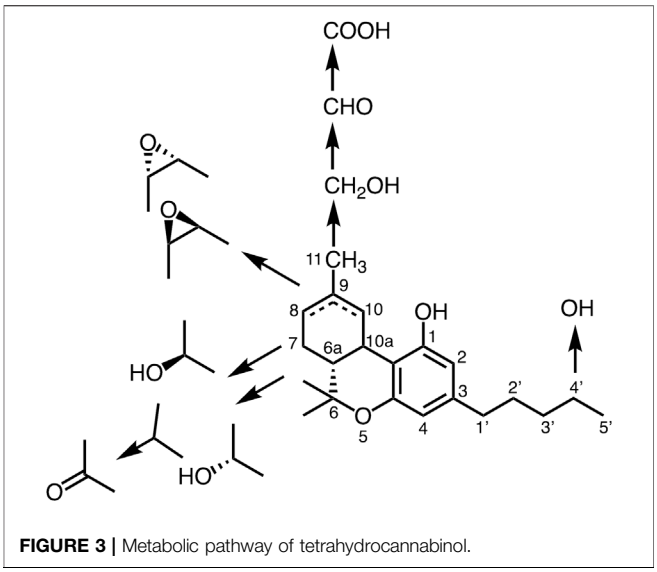

FIGURE 3 | Metabolic pathway of tetrahydrocannabinol.

CANNABINOID METABOLIC PATHWAYS

THC is transformed to the oxidative metabolites quickly as indicated in **Figure 3** and their activities are changeable (Watanabe et al., 2000). To detect various kind of metabolites against THC a monoclonal antibody (mAb) against THCA was prepared in our lab (Goto et al., 1994; Tanaka and Shoyama, 1999). Interestingly, the anti-THCA mAb was cross-reactive against all metabolites of THC (**Figure 3**), CBD, and cannabinol. Furthermore, *in vitro* and *in vivo* analyses revealed rapid oxidation of THC occurred at several sites and the molecule was metabolized into hydroxyl, epoxide, aldehyde and carboxylic acid derivatives in the body. Fortunately, the mAb recognized only limited cannabinoids, but not lipophilic compounds, such as cholesterol, testosterone, β-carotene, and androstene- 3,17-dione, or the endogenous cannabinoid anandamide (Watanabe et al., 2000). An eastern blotting system will allow for one-step analysis of cannabinoid metabolites, which would be useful for pharmacological and biological analyses of cannabinoids (Shan et al., 2001). Metabolites of THC in the body are important for the bioavailability and affinity of enzymes and/or receptors. The THC metabolic pathway is shown in **Figure 3** (Watanabe et al., 2005).

Elmes et al. (2015) reported a new finding related to cannabinoid metabolism that THC and CBD are carried by

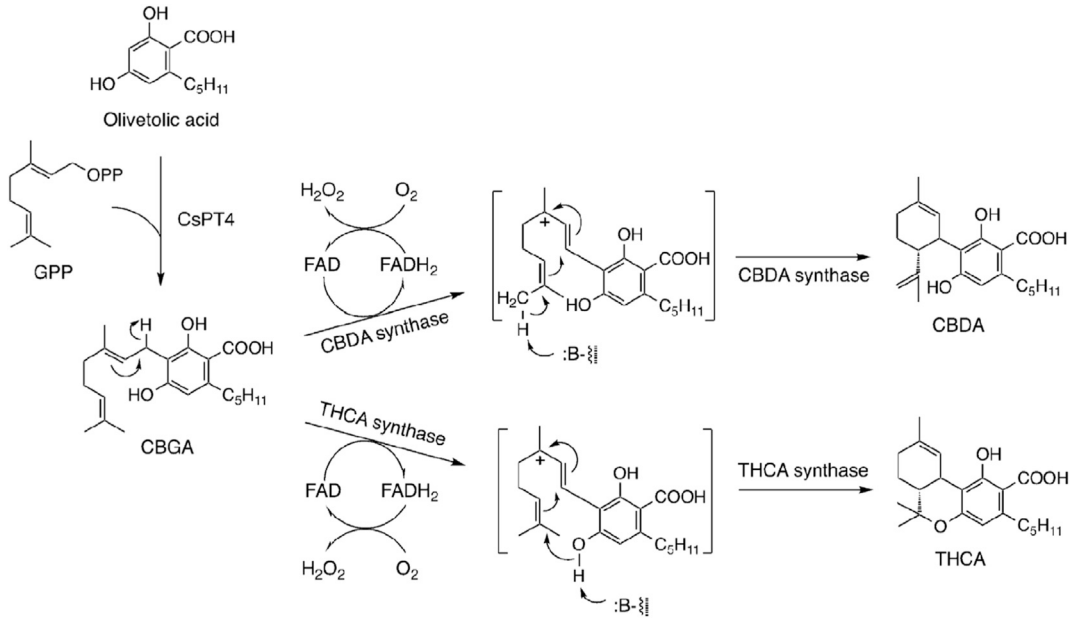

FIGURE 4 | Biosynthetic pathway of CBDA and THCA [Furthermore, recently cannabinoid prenyltransferase 4 was found in *C. sativa* (Luo et al., 2019)].

fatty acid-binding proteins (FABP1). Further, it becomes evident that FABP1 carries and preserves THC in ligand binding pocket temporarily, then transports to intracellular CYP450 enzymes for THC metabolism (Elmes et al., 2019) as described previously.

CANNABINOID BIOSYNTHESIS PATHWAY

Recently Luo et al. (2019) succeeded to fix the biosynthetic matrix for preparation of CBGA, CBDA, THCA, tetrahydrocannabivarinic acid (CBDVA) and cannabidivarinic acid (CBDVA) (Shoyama et al., 1977) in yeast. However, since the molecular breeding for cannabinoids is needed in future, the biosynthetic enzymes of cannabinoids including isolation, cloning and the tertiary structure of THCA synthase will be discussed for further investigation based on the newly developed methodology called as missile type molecular breeding (Putalun et al., 2003; Sakamoto et al., 2012; Putalun et al., 2015).

Studies on the biosynthesis of cannabinoids started in the 1960s with the use of isotope tracers. Cannabinoids are biosynthesized via the conjugation of acetyl-malonate and mevalonate (Shoyama et al., 1994). Stout et al. (2012) confirmed that the hexanoyl-CoA formed by the acyl-activating enzyme was a precursor of cannabinoids. The conjugation of olivetorate and monoterpene moiety was occurred by a transferase to yield CBGA, the key precursor of CBDA and THCA (Fellermeier and Zenk, 1988). Further it become evident that olivetol synthase, a polyketide synthase was an essential enzyme for the biosynthesis of cannabinoids (Taura et al., 2009).

Since the biosynthetic pathway was confirmed in the 1990s, three enzymes related to cannabinoid biosynthesis were isolated and purified from cannabis plants: THCA synthase (Taura et al., 1995), CBDA synthase (Taura et al., 1996), and cannabichromenic acid (CBCA) synthase (Morimoto et al., 1998). cDNA cloning of THCA synthase produces a transgenic protein (Sirikantaramas et al., 2004; Taura et al., 2007a), which is an important finding. THCA synthase is a flavoenzyme with high homology with the berberine bridge enzyme (Dittrich and Kutchan, 1991), which catalyzes oxidative cyclization of reticuline to scoulerine in the biosynthesis of benzylisoquinoline alkaloids (Zenk, 1985). These findings were confirmed by overexpression of recombinant enzymes using a baculovirus-insect cell system (Sirikantaramas et al., 2004; Sirikantaramas et al., 2005; Taura et al., 2009). CBDA synthase was also cloned and expressed as a

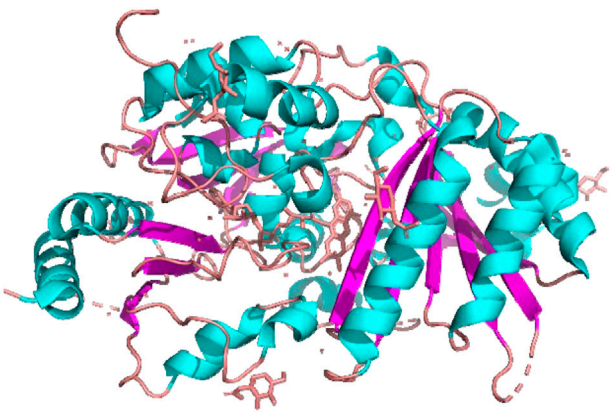

FIGURE 5 | Structure of THCA synthase by X-ray crystallography.

recombinant enzyme (Taura et al., 2007b; Taura et al., 2007c). The cannabinoid biosynthetic pathway involves direct biosynthesis of THCA and CBDA from CBGA by CBDA synthase and THCA synthase, respectively (**Figure 4**).

Information on biosynthesis of cannabinoids has resulted in the crystallization and structure of THCA synthase. The enzyme was overproduced in baculovirus, and crystalized (Shoyama et al., 2005; Taguchi et al., 2008). Then, the tertiary structure was determined by X-ray crystallography at 0.75 Å resolution (**Figure 5**) (Shoyama et al., 2012).

The most typical characteristic of the THCA synthase molecule is a residue that binds flavin adenine dinucleotide (FAD). Based on this analysis, CBGA was identified as a substrate and THCA as a product that competed to fit into the pocket of the active site (**Figure 3**). Mutation experiments at the active site resulted decreased enzymatic activity of THCA synthase, but not complete inhibition. These findings suggest that the active site may function by binding with CBGA as a substrate. Therefore, the tertiary structure explains the enzymatic reaction of THCA formation from CBGA (**Figure 2**).

Luo et al. (2019) succeeded to set up the biosynthetic system using yeast for CBGA, CBDA, THCA, tetrahydrocannabivarinic acid (CBDVA) (Shoyama et al., 1977) and cannabidivarinic acid (CBDVA) (Shoyama et al., 1977) production without the organic synthesis and/or *Cannabis* plant. This methodology may open a new platform for preparation of natural products. Further the authors reviewed biochemistry and biotechnology on cannabinoids including new advance (Taura et al., 2019).

Cannabis can be divided into CBDA- and THCA-type strains (Kushima et al., l980). Authors group previously successfully bred a CBDA strain by the repeated crossing that mainly produced CBDA as a precursor of CBD, which was simply transformed by hort-term heating, during 5 min (Shoyama, 1993). Moreover, a mutation to THCA synthase could completely inhibit THCA synthesis and increase the CBDA content (Sirikantaramas et al., 2004). The "missile-type molecular breeding" method is a unique breeding technology in which a single chain fragment variable (scFv) gene is transformed into the host plant (CBDA strain) resulting in a three-fold increase in antigen molecules (Putalun et al., 2003; Sakamoto et al., 2012; Putalun et al., 2015). In this case, the scFv gene targeted by an anti-CBDA mAb was induced into the CBDA strain, resulting in an increased the concentration of CBDA by up to three-fold as compared to that of the original plant. These technologies strongly support the development of CBD as a drug to prevent AD as documented previously.

CONCLUSION

C. sativa are listed as safe substances in the Chinese medical text Shennong Ben Cao Jing to maintain health and brain function at suitable dosages. The Kampo medicine such as Daiokanzoto prescribed with rhubarb and licorice can be used for the treatment of constipation in healthy persons as this formulation can cause diarrhea in the frail and elderly. Mashiningan formulation promotes milder defecation, although the high concentration of oil might be affected by the CNS to maintain mental stability without any adverse side effects.

CBD is an effective drug for the treatment of intractable seizures, especially intractable pilepsy. A recent neuronal investigation (Georgia et al., 2017) found that CBD blocked Aβ-induced neurotoxicity, tau protein hyperphosphorylation, and the activities of iNOS and interleukin-1β, highlighting a unique pharmacological mechanism as compared to currently approved anti-dementia drugs, resulting in reduced inflammation in neuronal tissues and neurogenesis for the treatment of dementia.

Two receptors, CB1 and CB2, and endocannabinoids, anandamide and 2-arachidony glyceride are discussed for underlying mechnism of action of cannabinoids in the brain. It became evident that 2-arachidony glyceride is a potent ligand with high affinity for both the CB1 and CB2 receptors. Endocannabinoid research have shown that most of pharmacological activities of anandamide are similar with THC and CBD in the CNS and peripheral systems. Metabolic pathway of THC is discussed because the metabolic speed is faster and the activity of THC decrease rapidly.

In order to make evident the biosynthetic enzyme system which has possibility for the production of higher concentration of cannabinoids, their enzymes were isolated and cloned. THCA synthase is a flavoenzyme that efficiently catalyzes oxidative cyclization of CBGA. The tertiary structure THCA synthase was determined by X-ray crystallography (Shoyama et al., 2012). This finding suggests the potential of molecular breeding of cannabis plants. For example, the cleavage of FAD from THCA synthase in the CBDA strain enhances biosynthesis of CBDA. This technology can increase the CBDA content in cannabis plants and further the development of new CBD-based drugs without the risks of psychological disorders and abuse for the prevention of dementia, especially AD.

AUTHOR CONTRIBUTIONS

WT prepared the section on endo-cannabinoid and its receptor. KW wrote the section on cannabinoid metabolism. YSa, KK, and KS collected and discussed the comprehensive data on marijuana. SS worked on the cloning of THCA synthase and the expression system. YoS investigated the crystallization and X-ray analysis of THCA synthase. FT worked on the cloning and gene construction. SM purified the biosynthetic enzymes related to cannabinoids. YkS prepared the manuscript and managed the study.

ACKNOWLEDGMENTS

The authors sincerely thank the Faculty of Pharmacy of Nagasaki International University for providing the facilities to support this project and the Association for Health Economics Research and Social Insurance and Welfare for financial support.

REFERENCES

Adams, R., Baker, B. R., and Wearn, R. B. (1940). Structure of Cannabinol. III. Synthesis of Cannabinol, 1-Hydroxy-3-N-Amyl-6,6,9-Trimethyl-6-Dibenzopyran1. *J. Am. Chem. Soc.* 62, 2204–2207. doi:10.1021/ja01865a083

Brand, E. J., and Zhao, Z. (2017). Cannabis in Chinese Medicine: Are Some Traditional Indications Referenced in Ancient Literature Related to Cannabinoids? *Front. Pharmacol.* 8, 108. doi:10.3389/fphar.2017.00108

Broers, B., Patà, Z., Mina, A., Wampfler, J., de Saussure, C., and Pautex, S. (2019). Prescription of a THC/CBD-Based Medication to Patients with Dementia: A Pilot Study in Geneva. *Med. Cannabis Cannabinoids.* 2 (1), 56–59. doi:10.1159/000498924

Brunetti, P., Pichini, S., Pacifici, R., Busardò, F. P., and Del Rio, A. (2020). Herbal Preparations of Medical Cannabis: A Vademecum for Prescribing Doctors. *Medicina (Kaunas).* 56, 237. doi:10.3390/medicina56050237

Campillo, N. E., and Páez, J. A. (2009). Cannabinoid System in Neurodegeneration: New Perspectives in Alzheimer's Disease. *Mini Rev. Med. Chem.* 9, 539–559. doi:10.2174/138955709788167628

Charernboon, T., Lerthattasilp, T., and Supasitthumrong, T. (2021). Effectiveness of Cannabinoids for Treatment of Dementia: A Systematic Review of Randomized Controlled Trials. *Clin. Gerontol.* 44, 16–24. doi:10.1080/07317115.2020.1742832

Cohen, K., Weizman, A., and Weinstein, A. (2019). Positive and Negative Effects of Cannabis and Cannabinoids on Health. *Clin. Pharmacol. Ther.* 105, 1139–1147. doi:10.1002/cpt.1381

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and Structure of a Brain Constituent that Binds to the Cannabinoid Receptor. *Science.* 258, 1946–1949. doi:10.1126/science.1470919

Dittrich, H., and Kutchan, T. M. (1991). Molecular Cloning, Expression, and Induction of Berberine Bridge Enzyme, an Enzyme Essential to the Formation of Benzophenanthridine Alkaloids in the Response of Plants to Pathogenic Attack. *Proc. Natl. Acad. Sci. U S A.* 88, 9969–9973. doi:10.1073/pnas.88.22.9969

Elmes, M. W., Kaczocha, M., Berger, W. T., Leung, K., Ralph, B. P., Wang, L., et al. (2015). Fatty Acid-Binding Proteins (FABPs) Are Intracellular Carriers for Δ9-tetrahydrocannabinol (THC) and Cannabidiol (CBD). *J. Biol. Chem.* 290 (14), 8711–8721. doi:10.1074/jbc.M114.618447

Elmes, M. W., Prentis, L. E., McGoldrick, L. L., Giuliano, C. J., Sweeney, J. M., Joseph, O. M., et al. (2019). FABP1 Controls Hepatic Transport and Biotransformation of Δ9-THC. *Sci. Rep.* 9, 7588. doi:10.1038/s41598-019-44108-3

ElSohly, M. A., Radwan, M. M., Gul, W., Chandra, S., and Galal, A. (2017). "Phytochemistry of Cannabis Sativa L," in *Phytocannabinoids, Progress in the Chemistry of Organic Natural Products*. Editors A. D. H. KinghornFalk, S. Gibbons, and J. Kobayashi, 103, 1–36. doi:10.1007/978-3-319-45541-9_1

Esposito, G., De Filippis, D., Carnuccio, R., Izzo, A. A., and Iuvone, T. (2006a). The Marijuana Component Cannabidiol Inhibits Beta-Amyloid-Induced Tau Protein Hyperphosphorylation through Wnt/beta-Catenin Pathway Rescue in PC12 Cells. *J. Mol. Med. (Berl).* 84, 253–258. doi:10.1007/s00109-005-0025-1

Esposito, G., De Filippis, D., Maiuri, M. C., De Stefano, D., Carnuccio, R., and Iuvone, T. (2006b). Cannabidiol Inhibits Inducible Nitric Oxide Synthase Protein Expression and Nitric Oxide Production in Beta-Amyloid Stimulated PC12 Neurons through P38 MAP Kinase and NF-kappaB Involvement. *Neurosci. Lett.* 399, 91–95. doi:10.1016/j.neulet.2006.01.047

Esposito, G., Scuderi, C., Savani, C., Steardo, L., Jr., De Filippis, D., Cottone, P., et al. (2007). Cannabidiol *In Vivo* Blunts Beta-Amyloid Induced Neuroinflammation by Suppressing IL-1beta and iNOS Expression. *Br. J. Pharmacol.* 151, 1272–1279. doi:10.1038/sj.bjp.0707337

Eubanks, L. M., Rogers, C. J., Beuscher, A. E., Koob, G. F., Olson, A. J., Dickerson, T. J., et al. (2006). A Molecular Link Between the Active Component of Marijuana and Alzheimer's Disease Pathology. *Mol. Pharm.* 3, 773–777. doi:10.1021/mp060066m

Fellermeier, M., and Zenk, M. H. (1988). Prenylation of Olivetolate by a Hemp Transferase Yields Cannabigerolic Acid, the Precursor of Tetrahydrocannabinol. *FEBS Lett.* 427 (2), 283–285. doi:10.1016/s0014-5793(98)00450-5

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., et al. (2005). Global Prevalence of Dementia: a Delphi Consensus Study. *Lancet.* 366, 2112–2117. doi:10.1016/S0140-6736(05)67889-0

Friedman, D., French, J. A., and Maccarrone, M. (2019). Safety, Efficacy, and Mechanisms of Action of Cannabinoids in Neurological Disorders. *Lancet Neurol.* 18, 504–512. doi:10.1016/S1474-4422(19)30032-8

Gaoni, Y., and Mechoulam, R. (1964). Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *J. Am. Chem. Soc.* 86, 1646–1647. doi:10.1021/ja01062a046

Georgia, W., and Karl, T. (2017). *In vivo* Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease. *Front. Pharmacol.* 8, 20. doi:10.3389/fphar.2017.00020

Gérard, C., Mollereau, C., Vassart, G., and Parmentier, M. (1990). Nucleotide Sequence of a Human Cannabinoid Receptor cDNA. *Nucleic Acids Res.* 18, 7142. doi:10.1093/nar/18.23.7142

Giannikou, P., Yiannakakis, N., Frangkakis, G., Probonas, K., and Wenger, T. (1995). Anandamide (Endogen Cannabinoid) Decreases Serum Prolactin in Pregnant Rat. *Neuro Endocrinol. Lett.* 17, 281–287.

Goto, Y., Shima, Y., Morimoto, S., Shoyama, Y., Murakami, H., Kusai, A., et al. (1994). Determination of Tetrahydrocannabinolic Acid-Carrier Protein Conjugate by Matrix-Assisted Laser Desorption/ionization Mass Spectrometry and Antibody Formation. *Org. Mass. Spectrom.* 29, 668–671. doi:10.1002/oms.1210291115

Hillen, J. B., Soulsby, N., Alderman, C., and Caughey, G. E. (2019). Safety and Effectiveness of Cannabinoids for the Treatment of Neuropsychiatric Symptoms in Dementia: a Systematic Review. *Ther. Adv. Drug Saf.* 10, 2042098619846993. doi:10.1177/2042098619846993

Ho, Y. S., So, K. F., and Chang, R. C. (2011). Drug Discovery from Chinese Medicine against Neurodegeneration in Alzheimer's and Vascular Dementia. *Chin. Med.* 6, 15. doi:10.1186/1749-8546-6-15

Hua, T., Vemuri, K., Pu, M., Qu, L., Han, G. W., Wu, Y., et al. (2016). Crystal Structure of the Human Cannabinoid Receptor CB1. *Cell.* 167, 750–e14. doi:10.1016/j.cell.2016.10.004

Ishac, E. J., Jiang, L., Lake, K. D., Varga, K., Abood, M. E., and Kunos, G. (1996). Inhibition of Exocytotic Noradrenaline Release by Presynaptic Cannabinoid CB1 Receptors on Peripheral Sympathetic Nerves. *Br. J. Pharmacol.* 118, 2023–2028. doi:10.1111/j.1476-5381.1996.tb15639.x

Iuvone, T., Esposito, G., De Filippis, D., Scuderi, C., and Steardo, L. (2009). Cannabidiol: a Promising Drug for Neurodegenerative Disorders? *CNS Neurosci. Ther.* 15, 65–75. doi:10.1111/j.1755-5949.2008.00065.x

Kaduszkiewicz, H., Zimmermann, T., Beck-Bornholdt, H. P., and van den Bussche, H. (2005). Cholinesterase Inhibitors for Patients with Alzheimer's Disease: Systematic Review of Randomised Clinical Trials. *BMJ.* 331, 321–327. doi:10.1136/bmj.331.7512.321

Kim, S. H., Yang, J. W., Kim, K. H., Kim, J. U., and Yook, T. H. (2019). A Review on Studies of Marijuana for Alzheimer's Disease—Focusing on CBD, THC. *J. Pharmacopuncture.* 22, 225–230. doi:10.3831/KPI.2019.22.030

Kushima, H., Shoyama, Y., and Nishioka, I. (1980). Cannabis. XII. Variations of Cannabinoid Contents in Several Strains of Cannabis Sativa L. With Leaf-Age, Season and Sex. *Chem. Pharm. Bull.* 28, 594–598. doi:10.1248/cpb.28.594

Lichtman, A. H., Dimen, K. R., and Martin, B. R. (1995). Systemic or Intrahippocampal Cannabinoid Administration Impairs Spatial Memory in Rats. *Psychopharmacology (Berl).* 119, 282–290. doi:10.1007/BF02246292

Luo, X., Reiter, M. A., d'Espaux, L., Wong, J., Denby, C. M., Lechner, A., et al. (2019). Complete Biosynthesis of Cannabinoids and Their Unnatural Analogues in Yeast. *Nature.* 567, 123–126. doi:10.1038/s41586-019-0978-9

Mangialasche, F., Solomon, A., Winblad, B., Mecocci, P., and Kivipelto, M. (2010). Alzheimer's Disease: Clinical Trials and Drug Development. *Lancet Neurol.* 9, 702–716. doi:10.1016/S1474-4422(10)70119-8

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990). Structure of a Cannabinoid Receptor and Functional Expression of the Cloned cDNA. *Nature.* 346, 561–564. doi:10.1038/346561a0

Mechoulam, R., and Shvo, Y. (1963). Hashish. I. The Structure of Cannabidiol. *Tetrahedron.* 19, 2073–2078. doi:10.1016/0040-4020(63)85022-x

Moldrich, G., and Wenger, T. (2000). Localization of the CB1 Cannabinoid Receptor in the Rat Brain. An Immunohistochemical Study. *Peptides.* 21, 1735–1742. doi:10.1016/s0196-9781(00)00324-7

Morimoto, S., Komatsu, K., Taura, F., and Shoyama, Y. (1998). Purification and Characterization of Cannabichromenic Acid Synthase from Cannabis Sativa. *Phytochemistry.* 49, 1525–1529. doi:10.1016/s0031-9422(98)00278-7

Morimoto, S., Tanaka, Y., Sasaki, K., Tanaka, H., Fukamizu, T., Shoyama, Y., et al. (2007). Identification and Characterization of Cannabinoids that Induce Cell Death through Mitochondrial Permeability Transition in Cannabis Leaf Cells. *J. Biol. Chem.* 282, 20739–20751. doi:10.1074/jbc.M700133200

Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993). Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature.* 365, 61–65. doi:10.1038/365061a0

Natarajan, S., Shunmugiah, K. P., and Kasi, P. (2013). Plants Traditionally Used in Age-Related Brain Disorders (Dementia): an Ethanopharmacological Survey. *Pharm. Biol.* 51, 492–523. doi:10.3109/13880209.2012.738423

National rInstitute of Public Health (2015). MHLW GRANTS SYSTEM [in Japanese]. Available at: https://mhlw-grants.niph.go.jp/node/55558 (Accessed April 26, 2021).

Pacifici, R., Marchei, E., Salvatore, F., Guandalini, L., Busardò, F. P., and Pichini, S. (2017). Evaluation of Long-Term Stability of Cannabinoids in Standardized Preparations of Cannabis Flowering Tops and Cannabis Oil by Ultra-high-performance Liquid Chromatography Tandem Mass Spectrometry. *Clin. Chem. Lab. Med.* 56, 94–96. doi:10.1515/cclm-2017-0758

Parsons, C. G., Danysz, W., and Quack, G. (1999). Memantine Is a Clinically Well Tolerated N-Methyl-D-Aspartate (NMDA) Receptor Antagonist-Aa Review of Preclinical Data. *Neuropharmacology.* 38 (6), 735–767. doi:10.1016/s0028-3908(99)00019-2

Putalun, W., Taura, F., Qing, W., Matsushita, H., Tanaka, H., and Shoyama, Y. (2003). Anti-solasodine Glycoside Single-Chain Fv Antibody Stimulates Biosynthesis of Solasodine Glycoside in Plants. *Plant Cell Rep* 22, 344–349. doi:10.1007/s00299-003-0689-3

Putalun, W., Tanaka, H., Wakana, A., Uto, T., and Shoyama, Y. (2015). Missile-type Molecular Breeding of Medicinal Plant Using Compact Monoclonal Antibody Gene. *EC Agr.* 2, 358–365.

Rafe De Crespigny (2007). *A Biographical Dictionary of Later Han to the Three Kingdoms (23–220 AD)*. Leiden: Brill Academic Pub.

Romero, J., García, L., Cebeira, M., Zadrozny, D., Fernández-Ruiz, J. J., and Ramos, J. A. (1995). The Endogenous Cannabinoid Receptor Ligand, Anandamide, Inhibits the Motor Behavior: Role of Nigrostriatal Dopaminergic Neurons. *Life Sci.* 56, 2033–2040. doi:10.1016/0024-3205(95)00186-a

Sakamoto, S., Putalun, W., Pongkitwitoon, B., Juengwatanatrakul, T., Shoyama, Y., Tanaka, H., et al. (2012). Modulation of Plumbagin Production in Plumbago Zeylanica Using a Single-Chain Variable Fragment Antibody against Plumbagin. *Plant Cell Rep.* 31, 103–110. doi:10.1007/s00299-011-1143-6

Schuel, H., Goldstein, E., Mechoulam, R., Zimmerman, A. M., and Zimmerman, S. (1994). Anandamide (Arachidonylethanolamide), a Brain Cannabinoid Receptor Agonist, Reduces Sperm Fertilizing Capacity in Sea Urchins by Inhibiting the Acrosome Reaction. *Proc. Natl. Acad. Sci. U S A.* 91, 7678–7682. doi:10.1073/pnas.91.16.7678

Schwarz, H., Blanco, F. J., and Lotz, M. (1994). Anadamide, an Endogenous Cannabinoid Receptor Agonist Inhibits Lymphocyte Proliferation and Induces Apoptosis. *J. Neuroimmunol.* 55, 107–115. doi:10.1016/0165-5728(94)90152-x

Shan, S., Tanaka, H., and Shoyama, Y. (2001). Enzyme-linked Immunosorbent Assay for Glycyrrhizin Using Anti-glycyrrhizin Monoclonal Antibody and an Eastern Blotting Technique for Glucuronides of Glycyrrhetic Acid. *Anal. Chem.* 73, 5784–5790. doi:10.1021/ac0106997

Shoyama, Y., Takeuchi, A., Taura, F., Tamada, T., Adachi, M., Kuroki, R., et al. (2005). Crystallization of Delta1-tetrahydrocannabinolic Acid (THCA) Synthase from Cannabis Sativa. *Acta Crystallogr. Sect F Struct. Biol. Cryst. Commun.* 61, 799–801. doi:10.1107/S1744309105023365

Shoyama, Y., Tamada, T., Kurihara, K., Takeuchi, A., Taura, F., Arai, S., et al. (2012). Structure and Function of Δ1-tetrahydrocannabinolic Acid (THCA) Synthase, the Enzyme Controlling the Psychoactivity of Cannabis Sativa. *J. Mol. Biol.* 423, 96–105. doi:10.1016/j.jmb.2012.06.030

Shoyama, Y. (1993). "Biological Aspects on Marihuana, Cannabis Sativa L, and Biotechnological Investigation on Medicinal Plants," in *Winged Been and Some Other Vegetables and Medicinal Plants in the Tropics and Subtropics*. Editors W. Herath and S. Uemoto (Tokyo: Tokushu Nousanbutsu Kyokai), 141–200.

Shoyama, Y., Hirano, H., Makino, H., Umekita, N., and Nishioka, I. (1977). Cannabis. X. The Isolation and Structures of Four New Propyl Cannabinoid Acids, Tetrahydrocannabivarinic Acid, Cannabidivarinic Acid, Cannabichromevarinic Acid and Cannabigerovarinic Acid, from Thai Cannabis, 'Meao Variant'. *Chem. Pharm. Bull.* 25, 2306–2311. doi:10.1248/cpb.25.2306

Shoyama, Y., Hirano, H., and Nishioka, I. (1994). Biosynthesis of Propyl Cannabinoid Acid and its Biosynthetic Relationship with Pentyl and Methyl Cannabinoid Acids. *Phytochemistry.* 23, l909–1912. doi:10.1016/S0031-9422(00)84939-0

Shoyama, Y., Oku, R., Yamauchi, T., and Nishioka, I. (1972). Cannabis. VI. Cannabicyclolic Acid. *Chem. Pharm. Bull.* 20, 1927–1930. doi:10.1248/cpb.20.1927

Shoyama, Y., Yamauchi, T., and Nishioka, I. (1970). Cannabis. V. Cannabigerolic Acid Monomethyl Ether and Cannabinolic Acid. *Chem. Pharm. Bull.* 18, 1327–1332. doi:10.1248/cpb.18.1327

Sirikantaramas, S., Morimoto, S., Shoyama, Y., Ishikawa, Y., Wada, Y., Shoyama, Y., et al. (2004). The Gene Controlling Marijuana Psychoactivity: Molecular Cloning and Heterologous Expression of Delta1-tetrahydrocannabinolic Acid Synthase from Cannabis Sativa L. *J. Biol. Chem.* 279, 39767–39774. doi:10.1074/jbc.M403693200

Sirikantaramas, S., Taura, F., Tanaka, Y., Ishikawa, Y., Morimoto, S., Shoyama, Y., et al. (2005). Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, Is Secreted into the Storage Cavity of the Glandular Trichomes. *Plant Cell Physiol.* 46, 1578–1582. doi:10.1093/pcp/pci166

Solymosi, K., and Köfalvi, A. (2017). Cannabis: A Treasure Trove or Pandora's Box? *Mini Rev. Med. Chem.* 17, 1223–1291. doi:10.2174/1389557516666161004162133

Stout, J. M., Boubakir, Z., Ambrose, S. J., Purves, R. W., and Page, J. E. (2012). The Hexanoyl-CoA Precursor for Cannabinoid Biosynthesis Is Formed by an Acyl-Activating Enzyme in Cannabis Sativa Trichomes. *Plant J.* 71 (3), 353–365. doi:10.1111/j.1365-313X.2012.04949.x

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., et al. (1995). 2-Arachidonoylglycerol: a Possible Endogenous Cannabinoid Receptor Ligand in Brain. *Biochem. Biophys. Res. Commun.* 215, 89–97. doi:10.1006/bbrc.1995.2437

Taguchi, C., Taura, F., Tamada, T., Shoyama, Y., Shoyama, Y., Tanaka, H., et al. (2008). Crystallization and Preliminary X-ray Diffraction Studies of Polyketide Synthase-1 (PKS-1) from Cannabis Sativa. *Acta Crystallogr. Sect F Struct. Biol. Cryst. Commun.* 64, 217–220. doi:10.1107/S1744309108003795

Tanaka, H., and Shoyama, Y. (1999). Monoclonal Antibody against Tetrahydrocannabinolic Acid Distinguishes Cannabis Sativa Samples from Different Plant Species. *Forensic Sci. Int.* 106, 135–146. doi:10.1016/s0379-0738(99)00193-0

Taura, F., Morimoto, S., and Shoyama, Y. (1996). Purification and Characterization of Cannabidiolic-Acid Synthase from Cannabis Sativa L. *J. Biol. Chem.* 271, 17411–17416. doi:10.1074/jbc.271.29.17411

Taura, F., Dono, E., Sirikantaramas, S., Yoshimura, K., Shoyama, Y., and Morimoto, S. (2007a). Production of Delta(1)-tetrahydrocannabinolic Acid by the Biosynthetic Enzyme Secreted from Transgenic Pichia pastoris. *Biochem. Biophys. Res. Commun.* 361, 675–680. doi:10.1016/j.bbrc.2007.07.079

Taura, F., Sirikantaramas, S., Shoyama, Y., Shoyama, Y., and Morimoto, S. (2007b). Phytocannabinoids in Cannabis Sativa: Recent Studies on Biosynthetic Enzymes. *Chem. Biodivers.* 4, 1649–1663. doi:10.1002/cbdv.200790145

Taura, F., Sirikantaramas, S., Shoyama, Y., Yoshikai, K., Shoyama, Y., and Morimoto, S. (2007c). Cannabidiolic-acid Synthase, the Chemotype-Determining Enzyme in the Fiber-type Cannabis Sativa. *FEBS Lett.* 581, 2929–2934. doi:10.1016/j.febslet.2007.05.043

Taura, F., Tanaka, S., Taguchi, C., Fukamizu, T., Tanaka, H., Shoyama, Y., et al. (2009). Characterization of Olivetol Synthase, a Polyketide Synthase Putatively Involved in Cannabinoid Biosynthetic Pathway. *FEBS Lett.* 583, 2061–2066. doi:10.1016/j.febslet.2009.05.024

Taura, F., Morimoto, S., Shoyama, Y., and Mechoulam, R. (1995). First Direct Evidence for the Mechanism of .DELTA.1-tetrahydrocannabinolic Acid Biosynthesis. *J. Am. Chem. Soc.* 117, 9766–9767. doi:10.1021/ja00143a024

Taura, F., Tanaya, R., and Sirikantaramas, S. (2019). Recent Advances in Cannabinoid Biochemistry and Biotechnology. *ScienceAsia.* 45 (5), 399–407. doi:10.2306/scienceasia1513-1874.2019.45.399

Terranova, J. P., Michaud, J. C., Le Fur, G., and Soubrié, P. (1995). Inhibition of Long-Term Potentiation in Rat Hippocampal Slices by Anandamide and

WIN55212-2: Reversal by SR141716 A, a Selective Antagonist of CB1 Cannabinoid Receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* 352, 576–579. doi:10.1007/BF00169393

Turner, C. E., Elsohly, M. A., and Boeren, E. G. (1980). Constituents of Cannabis Sativa L. XVII. A Review of the Natural Constituents. *J. Nat. Prod.* 43, 169–234. doi:10.1021/np50008a001

United Nations Office on Drugs and Crime (2017). *World Drug Report 2017*. (ISBN: 978-92-1-148291-1, eISBN: 978-92-1-060623-3. Vienna: United Nations publication. Sales No. E.17.XI.6).

Varga, K., Lake, K., Martin, B. R., and Kunos, G. (1995). Novel Antagonist Implicates the CB1 Cannabinoid Receptor in the Hypotensive Action of Anandamide. *Eur. J. Pharmacol.* 278, 279–283. doi:10.1016/0014-2999(95)00181-j

Watanabe, K., Matsunaga, T., Kimura, T., Funahashi, T., Yamaori, S., Shoyama, Y., et al. (2005). Stereospecific and Regioselective Hydrolysis of Cannabinoid Esters by ES46.5K, an Esterase from Mouse Hepatic Microsomes, and its Differences from Carboxylesterases of Rabbit and Porcine Liver. *Biol. Pharm. Bull.* 28, 1743–1747. doi:10.1248/bpb.28.1743

Watanabe, K., Matsuda, M., Tateoka, Y., Kimura, T., Matsunaga, T., Tanaka, H., et al. (2000). Cross-Reactivity of Various Tetrahydrocannabinol Metabolites with a Monoclonal Antibody against Tetrahydrocannabinolic Acid. *J. Health Sci.* 46, 310–313. doi:10.1248/jhs.46.310

Watt, G., and Karl, T. (2017). *In Vivo* Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease. *Front. Pharmacol.* 8, 20. doi:10.3389/fphar.2017.00020

Weidenfeld, J., Feldman, S., and Mechoulam, R. (1994). Effect of the Brain Constituent Anandamide, a Cannabinoid Receptor Agonist, on the Hypothalamo-Pituitary-Adrenal axis in the Rat. *Neuroendocrinology.* 59, 110–112. doi:10.1159/000126646

Wenger, T., Tóth, B. E., and Martin, B. R. (1995). Effects of Anandamide (Endogen Cannabinoid) on Anterior Pituitary Hormone Secretion in Adult Ovariectomized Rats. *Life Sci.* 56, 2057–2063. doi:10.1016/0024-3205(95)00189-d

Wickens, A. P., and Pertwee, R. G. (1993). delta 9-Tetrahydrocannabinol and Anandamide Enhance the Ability of Muscimol to Induce Catalepsy in the Globus Pallidus of Rats. *Eur. J. Pharmacol.* 250, 205–208. doi:10.1016/0014-2999(93)90646-y

World drug report (2021). *Booklet 3-Drug Market Trends: Opioids, Cannabis*. Vienna: United Nations, Office on Drugs and Crime.

Conflict of Interest: YoS was employed by the company Bonac Corporation.

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

frontiers in Pharmacology

REVIEW
published: 04 March 2022
doi: 10.3389/fphar.2021.779821

Preventing Dementia Using Saffron, The Kampo Medicine, Kamiuntanto, and Their Combination, Kamiuntantokabankoka

*Kenny Kuchta[1], Kosuke Aritake[2], Yoshihiro Urade[2], Nguyen Huu Tung[3], Chun-Su Yuan[4], Yui Sasaki[5], Koichi Shimizu[5] and Yukihiro Shoyama[6]**

[1]*Forschungsstelle für Fernöstliche Medizin, Department of Vegetation Analysis and Phytodiversity, Albrecht von Haller Institute of Plant Sciences, Georg August University, Göttingen, Germany,* [2]*Daiichi University of Pharmacy, Fukuoka, Japan,* [3]*Faculty of Pharmacy, Phenikaa University, Hanoi, Vietnam,* [4]*Department of Anesthesia and Critical Care, The University of Chicago, Chicago, IL, United States,* [5]*Association for Health Economics Research and Social Insurance and Welfare, Tokyo, Japan,* [6]*Faculty of Pharmacy, Nagasaki International University, Sasebo, Japan*

The objective of this review is to evaluate the anti-dementia activities of saffron and its combination with Kampo medicine. The Kampo formula Kamiuntanto composed of 13 crude drugs is well known for its anti-dementia activity. A significant increase in choline acetyltransferase activity and mRNA levels were observed. *Polygala* radix was identified as the most essential component drug in Kamiuntanto, probably due to the saponins, tenuifolin, and sinapinic acid. Ginseng was also identified as an essential Kamiuntanto component in terms of its synergistic functions with *Polygala* radix. Saffron, which was recommended in the Bencao Gangmu for memory and dementia, and is used as an anti-spasmodic, anti-catarrhal, and sedative herbal drug. Saffron and its major constituent, crocin were shown to enhance learning-memory, non-rapid eye movement (rem) sleep, and inhibit depression and neuronal cell death due to strong anti-oxidant and anti-inflammation activities. In addition based on the epidemiological studies such as the treatment of sleeping disorders and the clinical trials of saffron for Alzheimer patients, we demonstrated the indirect and direct anti-dementia activities of crocin and saffron.

Keywords: kamiuntanto, saffron, kamiuntantokabankoka, kampo (traditional Japanese herbal medicine), dementia prevention and control

OPEN ACCESS

Edited by:
Pinarosa Avato,
University of Bari Aldo Moro, Italy

Reviewed by:
Stefania Schiavone,
University of Foggia, Italy
Jong Hoon Ryu,
Kyung Hee University, South Korea

****Correspondence:***
Yukihiro Shoyama
shoyama@niu.ac.jp

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

Received: *19 September 2021*
Accepted: *29 December 2021*
Published: *04 March 2022*

Citation:
Kuchta K, Aritake K, Urade Y, Tung NH, Yuan C-S Sasaki Y, Shimizu K and Shoyama Y (2022) Preventing Dementia Using Saffron, The Kampo Medicine, Kamiuntanto, and Their Combination, Kamiuntantokabankoka. Front. Pharmacol. 12:779821. doi: 10.3389/fphar.2021.779821

INTRODUCTION

Globally, the incidence of neurodegenerative disorders such as dementia has increased with increased life expectancy. Previously, it was estimated there could be 81.1 million dementia patients by 2040 (Ferri et al., 2005). In Japan, 7.3 million cases are speculated by 2025, and by 2050, 10.2 million cases are expected (National Institute of Public Health, 2015). Consequently, speedy and rapid dementia innovations and prevention methods are required.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FB1, Fumonisin B1; c-GCS, c-Glutamylcysteinyl synthase; GPx, Glutathione peroxidase; GR, Glutathione reductase; GSH, Glutathione; JNK, c-Jun kinase; LTP, Long-term potentiation; NGF, Nerve growth factor; NMDA, N-methyl-D-aspartate; PS, Phosphatidylserine; SAPK, Stress-activated protein kinase; SM, Sphingomyelin.

The Diagnostic and Statistical Manual of Mental Disorders (Battle, 2013) indicates that major dementia cases are classified as Alzheimer's disease, vascular dementia, frontal lobe hyperthermia, dementia with Lewy bodies, Parkinson's disease with dementia, Huntington's disease with dementia, or a combination of the above. Of these conditions, Alzheimer's disease is the most common, with aging the most important single risk factor. However, epidemiological studies have identified that lifestyle and eating habits influence the condition, suggesting that wine (Orgogozo et al., 1997) or fish (Kalmijn et al., 1997) reduce the probability of developing Alzheimer's disease in old age. Similarly, Kampo medicine or other herbal remedies may also prevent dementia. Several studies have shown, via acetylcholine esterase inhibitor assays, that numerous pure compounds have anti-dementia activities (Ho et al., 2011; Natarajan et al., 2013), but no compounds have been clinically approved. In this review, phytochemicals such as Kamiuntanto and saffron which have anti-dementia activities are discussed.

Anti-Dementia Active Compounds From Medicinal Plants

The alkaloid galantamine (brand name: Razadyne and GalantaMind™) was originally isolated from Galanthus nivalis L., but is now produced by chemical synthesis, and is a global anti-dementia drug for mild and moderate Alzheimer's disease. Since 2016, Polygala tenuifolia Willd. root extract is also marketed as an over the counter (OTC) drug for memory improvement in Japan. Another similar product, Ginkgo biloba L. extract fraction (EGb761), which contains 24% flavonoid glycosides and 6% diterpene lactones, is an OTC drug for vascular dementia prevention in Europe (Clostre, 1999). Interestingly, Valeriana officinalis L. root, an OTC medicine for sleep disorders traditionally used in Europe, is reported to exert anti-dementia activity (Chen, 2016).

Of the many plants that exhibit pharmacological activities (e.g., acetylcholine esterase and monoamine oxidase inhibition) related to anti-dementia effects, anti-inflammatory activities, and learning and memory effects in animal models (Natarajan et al., 2013; Ho et al., 2011), the following are also commonly found in Kampo prescriptions: *Acorus gramineus* Aiton, *Angelica dahurica* (Hoffm.) Benth. & Hook.f.ex Franch. & Sav., *Aralia cordata* Thunb., *Codonopsis pilosula* (Franch.) Nannf., *Crocus sativus* L., *Curcuma longa* L., *Epimedium brevicornu* Maxim., *Gardenia jasminoides* J. Ellis, *Glycyrrhiza glabra* L., *Lycium chinense* Mill., *Magnolia officinalis* Rehder & E.H.Wilson, *Panax ginseng* C.A.Mey., *Perilla frutescens* (L.) Britton, *Polygala tenuifolia* Willd., *Zingiber offinale* Roscoe, *Rhodiola rosea* L., *Salvia miltiorrhiza* Bunge and *Uncaria rhynchophylla* (Miq.) Miq. (Li and Zhang, 2009).

Kampo Formulas for Dementia

Kampo theory was originally based on Ancient Chinese Medicine (ACM) from the fifth and sixth centuries in China and Korea. ACM was primarily based on ancient empirical knowledge of diseases and treatments, that was collected in classics such as the Shānghán Lùn (傷寒論, Jap. Shokanron) and the Shénnóng Běn Cǎo Jīng (神農本草経, Jap. Shinnohonzokyo). ACM is the base of all later forms of East Asian Traditional Medicine such as Korean Medicine, contemporary Traditional Chinese Medicine (TCM), and Japanese Kampo Medicine. Currently, 148 Kampo formulas as finished pharmaceutical products (FPP) are covered by the Japanese National Health Insurance and widely used for many diseases. In traditional Kampo philosophy, dementia is caused by "Oketsu" (瘀血) or "blood stagnation," which may be interpreted as circulatory disorders of the brain. Thus, therapies that enhance blood circulation are important for dementia therapy and prevention.

As indicated (**Table 1**), Kamiuntanto (Yabe et al., 1996; Yabe and Yamada, 1997a; Yabe and Yamada, 1997b; Yabe et al., 1997), Tokishakuyakusan (Kim and Cho, 2020), Yokukansanchinpihange (Okamoto, 2017), Hachimijiogan (Iwasaki et al., 2004), Chotosan (Terasawa et al., 1997), and Orengedokuto (Fujiwara et al., 2018) have been used for dementia therapy and/or prevention in Japan. Of these medicines, Kamiuntanto has been the most studied.

Kamiuntanto

Kamiuntanto is traditionally used for neurosis, insomnia, gastroptosis, gastroparesis, and weakness after a major illness. Kamiuntanto formula (**Table 1**) (Yabe et al., 1996; Yabe and Yamada, 1997a; Yabe and Yamada, 1997b; Yabe et al., 1997) enhances nerve growth factor (NGF) secretion and choline acetyltransferase (ChAT) activity. In a cell culture model of rat embryo basal forebrain cells cultured in medium containing Kamiuntanto extract, a significant increase in ChAT activity and mRNA levels was recorded (Yamada et al., 1997; Yabe and Yamada, 1997a). Furthermore, Kamiuntanto ameliorated cholinergic shortages in aging rats (Yabe et al., 1996) and subsequent investigations reported that cAMP and c-fos mRNA were closely related to NGF biosynthesis (Yabe and Yamada, 1997b). To make clear the mechanism for the memory improving activity of Kamiuntanto, Hong et al. confirmed the expressions of protein kinase B, cAMP response element-binding protein, brain-derived neurotrophic factor and doublecortin in the hippocampal CA1 and dentate gyrus regions in mice by immunohistochemical and blotting techniques (Hong et al., 2011). Interestingly, while a variant extract of Kamiuntanto without *Polygala* radix displayed no increased ChAT activity, an oral administration of *Polygala* radix extract alone induced ChAT activity. Subsequently, onjisaponin and sinapinic acid were independently tested and generated the same results as Kamiuntanto formulas (Yabe et al., 1997). In studies, the 13 Kamiuntanto components were individually removed so the effects of the remaining 12 could be assayed. This methodology is similar to knockout extract strategies which remove a target compound (antigen) from crude extracts using one step immunoaffinity separation techniques (Shoyama, 2011; Uto et al., 2012; Hsu et al., 2020).

The *P. tenuifolia* Willd. constituent, tenuifolin also inhibited β-amyloid secretion both *in vivo* and *in vitro* (Lv et al., 2009). Furthermore, tenogenic reduced antibody production by inhibiting β-secretase activity (Jia et al., 2004). Recently, multiple neuroprotective effects induced by *P. tenuifolia* Willd.

TABLE 1 | Clinically used anti-dementia Kampo formulae in Japan.

Type of dementia	Kampo prescription	Raw drugs in kampo formulas (scientific names are incorporated from Japanese pharmacopoeia)	References
Alzheimer's disease	Kamiuntanto (加味温胆湯)	*Pinellia ternata* Breitenbach (Araceae), *Polygala tenuifolia* Willd. (Polygalaceae), *Zizyphus jujuba* Miller var. *spinosa* (Bunge) Hu ex H. F. Chou (Rhamnaceae), Processed *Rhemania glutinosa* Liboschitz (Scrophulariaceae), *Panax ginseng* C.A. Meyer (Alariaceae), *Z. jujuba* var. *inermis* Rehder (Rhamnaceae), *Bambusa tuldoides* Munro (Gramineae), *Poria cocos* Wolf (Polyporaceae), Immature *Citrus aurantium* Linn. var. *daidai* Makio or *C. natsudaidai* Hayata (Rutaceae), *C. unshiu* Markovich or *C. reticulata* Blanco (Rutaceae), *Glycyrrhiza glabra* Linn. or *G. uralensis* Fisher (Leguminosae), *Zingiber offinale* Roscoe (Zingiberaceae), *Scrophularia ningpoensis* Hemsl. (Scrophulariaceae)	Yabe et al. (1997) Yabe and Yamada (1997b) Yabe and Yamada (1997a) Yabe et al. (1996)
Alzheimer's disease	Tokishakuyakusan (当帰芍薬散)	*Angelica acutiloba* (Siebold and Zucc.) Kitag. (Umbelliferae), *Cnidium offcinale* Makino (Umbelliferae), *Paeonia lactiflora* Pallas (Paeoniaceae), *Atractylodes lancea* De Candolle or *A. chinensis* Koidzumi (Compositae), *Alisma orientale* Juzepczuk (Alismataceae)	Kim and Cho (2020)
Vascular dementia	Chotosan (釣藤散)	*Uncaria rhynchophyla* Miquel. or *U. macophylla* Wallich (Rubiacerae), *P. ginseng* C.A. Meyer (Alariaceae), *P. cocos* Wolf (Polyporaceae), *P. ternata* Breitenbach (Araceae), *Ophiopogon japonicus* Ker-Gawler (Liliaceae), *C. unshiu* Markovich (Rutaceae), *Saposhnikovia divaricata* Schischkin (Umlelliferae), *G. glabra* Linn. or *G. uralensis* Fisher (Leguminosae), *Gypsum*, *Z. offinale* Roscoe (Zingiberaceae), *Chrysanthemum morifolium* Ramatulle or *C. indicum* Linn. (Compositae)	Terasawa et al. (1997)
Vascular dementia	Yokukansanchinpihange (抑肝散加陳皮半夏)	*A. lancea* De Candolle (Compositae), *P. cocos* Wolf (Polyporaceae), *A. acutiloba* (Siebold and Zucc.) Kitag. (Umbelliferae), *C. offcinale* Makino (Umbelliferae), *U. rhynchophyla* Miquel. or *U. macophylla* Wallich (Rubiacerae), *Bupleurum falcatum* Linn. (Umbelliferae), *G. glabra* Linn. or *G. uralensis* Fisher (Leguminosae), *P. ternata* Breitenbach (Araceae), *C. unshiu* Markovich or *C. reticulata* Blanco (Rutaceae)	Okamoto (2017)
Vascular dementia	Orengedokuto (黄連解毒湯)	*Coptis japonica* Makino or *C. chinensis* Franchet (Ranunculaceae), *Phellodendron amurense* Ruprecht or *P. chinense* Schneider (Rutaceae), *Gardenia jasminoides* J.Ellis, *Scutellaria baicalensis* Georgi (Labiatae)	Fujiwara et al. (2018)
Mix of the above	Hachimijiogan (八味地黄丸)	*R. glutinosa* Liboschitz (Scrophulariaceae), *Cornus officinalis* Siebold et Zuccarini (Cornaceae), *Dioscorea japonica* Thunberg or *D. batatas* Decaisne (Dioscoreaceae), *P. cocos* Wolf (Polyporaceae), *A. orientale* Juzepczuk (Alismataceae), *P. lactiflora* Pallas (Paeoniaceae), *Cinnamon cassia* Blume (Lauraceae), *Aconitum japonicum* Thunberg or *A. carmichaeli* Debeaux (Ranunculaceae)	Iwasaki et al. (2004)

were reported as a potential preventative therapy for Alzheimer's disease (Deng et al., 2020). Thus, since 2016, *P. tenuifolia* Willd. root extract has been marketed as an OTC drug for memory preservation in Japan.

Approximately 2000 years ago, *P. ginseng* C.A.Meyer, *P. tenuifolia* Willd., *A. gramineus* Aiton, and *P. cocos* Wolf were listed in the Shennong Ben Cao Jing (神農本草経) as having memory enhancement and related psychological effects. Among the characteristic constituents of ginseng, ginsenoside was shown to exhibit a wide pharmacological activity spectrum, including analgesic, cholesterol biosynthesis and neural lipid synthesis, and adrenal cortex hormone enhancing activities. Furthermore, it improves memory and learning, central nervous system excitation, and promotes DNA and RNA synthesis (Leung and Wong, 2010). Ginseng also promotes neuronal cell growth and survival, and was shown to rescue neuronal cell death both *in vivo* and *in vitro* (Lee et al., 2001). Ginseng increases *in vivo* choline acetyltransferase levels suggesting that ginsenosides strengthen central cholinergic functions, and could be used to treat dementia (Rudakewich et al., 2001). Furthermore, Yamaguchi et al. reported that ginsenosides enhanced learning and memory performances in both brain damaged and/or aging mice (Yamaguchi et al., 1996). Itoh et al. reported that ginsenosides activated norepinephirine and dopamine in the cerebral cortex, to facilitate increased attention, processing cognition, and motor function activation (Itoh et al., 1989). Ginsenoside Rg1 increased neuronal precursors and was mechanically important in terms of its anti-aging activity effects, such as learning and memory (Shen and Zhang, 2003). Zhang et al. demonstrated that the anti-apoptosis activity of ginsenoside-Rg2 counteracted vascular dementia in an *in vivo* animal model (Zhang et al., 2008). Huang et al. showed that ginsenoside Rc activated SIRT1, which protected neurons from mitochondrial damage (Huang et al., 2021). Hence, not only *P. tenuifolia*, but also ginseng and its constituents displayed anti-dementia activities.

Ginseng, ginsenoside, and ginsenoside Rg3 also increased the postoperative life span of patients with non-small cell lung cancer (Lu et al., 2008). Within this therapeutic context, Yang et al. reviewed 257 dammarane-type ginsenosides from numerous *Panax* species, many of which showed promising pharmacological activities (Yang et al., 2014). For example, since ginsenoside Rc has anti-dementia activity (see above) but its concentration as determined by eastern blotting fingerprint for

various *Panax* spp. (Sasaki et al., 2021) is quite low, it might be interesting to develop a method to induce the transformation of other minor ginsenosides into ginsenoside Rg3 in order to develop antidementia drugs.

We discussed the anti-dementia activities of two major crude drugs in the Kamiuntanto prescription, *P. tenuifolia* Willd. and *P. ginseng* C.A. Meyer previously. It became evident that the other crude drugs prescribed in Kamiuntanto showed anti-dementia activities as follows. *G. glabra* Linn. (Licorice root) is known to promote light movements, extend life span, and improve both physical and mental health as described in the Shennong Bao Jing. The pharmacological activities of licorice root can also improve mental function in patients with dementia, including AD. In fact aqueous extract of licorice root enhanced learning and memory in different type of models. Among its components, glycyrrhizin is used as a therapeutic drug for the treatment of liver disease and allergies in Japan. Regarding dementia Soo et al. investigated that glycyrrhizin significantly attenuated mitochondria-mediated cell death and decrease of glutathione due to neurotoxin, 1-methyl-4-phenylpyridinium resulting in the protective effect of glycyrrhizin on mitochondrial damage and cell death in PC-12 cells associated with dementia (Yim et al., 2007). The constituents of licorice root, especially flavones and isoflavones such as liquiritin, isoliquiritin, and coumestrol, are naturally occurring bioactive compounds although the ability of flavonoid glycosides to pass through the blood brain barrier (BBB) and reach the central nervous system is unclear. Therefore, several flavonoid glycosides and aglycones in *Glycyrrhiza* species root might be developed as functional compounds for the treatment of dementia. Liquiritigenin shows a selective estrogen receptor-β which are distributed in the brain centers of learning and memory, agonist (Mersereau et al., 2008). Further, liquiritigenin has neuroprotective activity against β-amyloid peptide (Aβ) in rat hippocampal neurons indicating that the pretreated neurons with liquiritigenin in the presence of Aβ increased cell viability and the treatment decreases Aβ-induced intracellular Ca^{2+} concentration and ROS level resulting in the decrease of apoptotic rate (Liu et al., 2009). Regarding *P. cocos* Smriga et al. showed that a single oral administration of *P. cocos* significantly intensified the formation of long-term potentiation (LTP) which is deeply involved to memory in the dentall gyrus (Smriga et al., 1995). Ban et al. reported that the rat cortical neurons pretreated by young *Phyllostachys nigra* (Bambu tree) methanol extracts were protected from Aβ-induced increase of cytosolic calcium concentration resulting that *P. nigra* prevents Aβ-induced neuronal cell damage *in vitro* (Ban et al., 2005). Pretreatment with *C. unshu* immature peel and its component, nobiletin inhibited individually cell death due to hydrogen peroxide induced the expression of phospho-Jun N-terminal kinases and p-p38 proteins in HT22 cells although the peel and nobiletin suppressed p-JNK and p-p38 without changing JNK or p38. These evidences confirm that the peel and nobiletin can protect against hydrogen peroxide-induced cell death in HT22 neurons *via* mitogen-activated protein kinases and apoptotic pathways (Cho et al., 2015). When rats were injected with $Aβ_{1-40}$ into the hippocampus, the ability of spatial learning and memory decreased. However, the treatment with harpagoside, a constituent of *Scrophularia ningpoensis* improved $Aβ_{1-40}$-induced behavioral damage (Li et al., 2015). The human monocytic cell line resemble human microglial cells (THP-1 cells) was incubated with ginger extract or with LPS, TNF-α, IL-1β or Aβ-protein resulted that the addition of ginger extract prohibited the expression of TNF-α, IL-1β, COX-2 and MCP-1. From this result the ginger extract could be used for delaying the onset and the progression of neurodegenerative disorders (Wang et al., 2015). When the methanol extract of dry ginger was tested for DPPH assay and FRAP assay, respectively to show the antioxidant activity and the Ellman's assay for the extract indicated the cholinesterase inhibition. Furthermore, the extract ameliorated the cell survival for Aβ-induced toxicity in primary rat hippocampal cell culture. This result together with the above evaluation suggested that the extract of dry ginger is effective for Alzheimer's disease (Mathew and Subramanian, 2014). The extract of steamed *R. glutinosa* Liboschitz root was investigated daily dose for rats injected scopolamine before 1 h for 14 days. The results were evaluated by a passive avoidance test and the Morris water maze test, and the activities of choline acetyltransferase and acetylcholinesterase in the hippocampus. The extract improved memory dysfunction behaviorally and cholinergicaly resulted that the extract could be used to improve cognitive function by activation of cholinergic enzyme (Lee et al., 2011).

The effect of spinosin isolated from the seeds of *Z. jujuba* Miller var. *spinosa* (Bunge) Hu ex H. F. was investigated on cholinergic induced memory dysfunction and behavioral task using the passive avoidance, Y-maze, and Morris water maze tasks. Spinosin significantly improved scopolamine-induced cognitive performance on behavioral tasks. In order to confirm the mechanism for improving activity of spinosyn, the survey of receptor antagonism and Western blotting were examined resulted that the improving effect of spinosin on scopolamine-induced memory impairment was significantly antagonized by 8-hydroxy-2-(di-N-propylamino) tetralin, a 5-HT1A receptor agonist and spinosin significantly increased the expression levels of phosphorylated extracellular signal-regulated kinases and cAMP response element-binding proteins in the hippocampus. From these results it became clear that the memory-improving activity of spinosin might be depend on the serotonergic neurotransmitter system. Therefore, spinosin and/or *Z. jujuba* Miller var. *spinosa* (Bunge) Hu ex H. F. could be applied for cognitive dysfunction like Alzheimer's disease (Jung et al., 2014). The crude drugs except *Pinellia ternate* and *Citrus aurantium* prescribed in Kamiuntanto formula have been surveyed their pharmacological activities regarding cognitive performance. They all indicated the activities for solving cognitive problem. So that their synergistic effects might be important for the documented effects of the Kamiuntanto formula.

Saffron Pharmacological Activities

Crocus sativus L. (Iridaceae; a perennial herb) was documented as cultivated on Crete approximately 3,500 years ago, but today, it is widely cultivated in Iran, Greece, Spain, Morocco, and domestically in Japan, for its red stigmata and saffron (**Figure 1**).

FIGURE 1 | *Crocus sativus* L. **(A)** and saffron **(B)**. Indoor crocus cultivation in Oita-ken, Japan **(C)**.

As far back as 1578 AD, saffron was identified in the Bencao Gangmu (本草綱目) for its neurological functions on memory and dementia. Saffron is known for its anti-convulsion, sedative, and heart-blood disorder qualities (Rigobello et al., 2002; Lee et al., 2005; Aung et al., 2007). The major constituent, crocin was shown to exert anti-cancer (Aung et al., 2007; Chryssanthi et al., 2007), anti-hypolipidemic (Lee et al., 2005; Sheng et al., 2006), anti-atherosclerotic (Xu et al., 2005; Xu et al., 2006; Xu et al., 2009), and anti-inflammatory effects (Ochiai et al., 2007; Naghizadeh et al., 2008). Crocin's neuro-protective activities related to dementia were investigated using cerebral ischemia, (Papandreou et al., 2006), memory impairment (Sugiura et al., 1994; Sugiura et al., 1995a), and N-methyl-D-aspartate receptor (NMDA) malfunction models (Abe et al., 1998).

The medical used of imported saffron in Japan has been documented since the start of the Edo-Bakufu in the early 1600s. In 1886, the first Japanese Pharmacopoeia accepted saffron as a non-prescription drug, a status that has remained unchanged to the present. Between 1830 and 1844, domestic saffron cultivation commenced in Oita-ken in western Japan (**Figure 1**).

The blooming period of the crocus is once a year and the stigma harvest time is very short; therefore, saffron prices are very high when compared with other herbal medicines (Morimoto et al., 1994). Moreover, saffron quality depends on weather conditions, however to alleviate this, indoor cultivation systems were developed in Japan in the early 20th century (1910) (**Figure 1**). Under these conditions, approximately 90,000–100,000 flowers generate 5.0 kg fresh saffron, in turn generating 1.0 kg dried drug. Indoor cultivation systems facilitate the easy collection of saffron adjusting most suitable full blooming season, therefore indoor cultivation is less labor intensive and advantageous for quality control measures (Morimoto et al., 1994).

The dominant components of saffron are carotenoids, picrocrocin, and safranal (**Figure 2**). Recently, the novel crocetin glycoside trans-crocetin-1-al 1-O-β-gentiobiosyl ester was isolated in our laboratory (Tung and Shoyama, 2013). Drying saffron should be completed within 30–45 min; β-glucosidase remains active as long as moisture is contained in the plant material and may destroy the typical ester glycoside conjugation of carotenoid pigments, e.g., crocetin-diglucoside, -2, -3, -4 and crocetin di-(β-D-digentiobiosyl)-ester. The dried saffron is then chilled and preserved free from moisture, as β-glucosidase remains active under moisture conditions thereby causing hydrolysis (Morimoto et al., 1994). High performance liquid chromatography (Morimoto et al., 1994) and monoclonal antibody (MAb) (Xuan et al., 1999) technologies are commonly

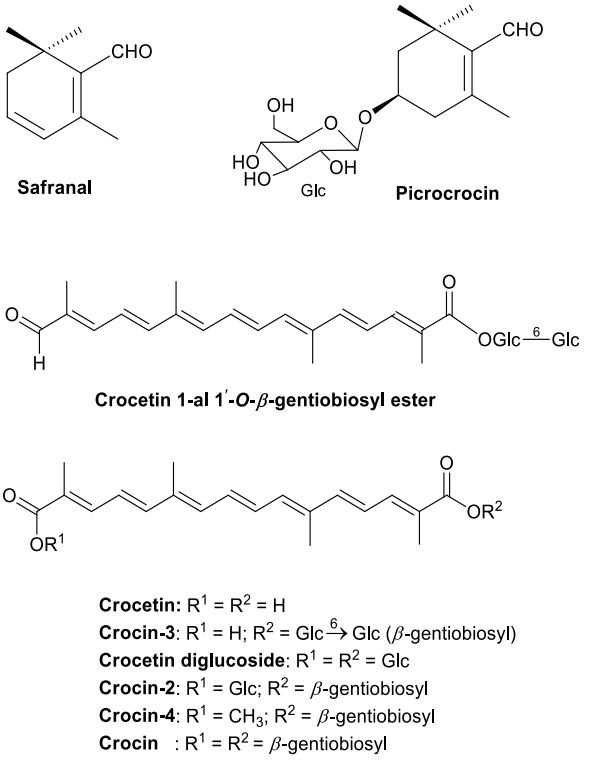

FIGURE 2 | The major saffron phytochemical constituents.

used for saffron quality control. Crocin levels in official saffron extracts are approximately 30% higher than other components. Crocin is a major contributor to the pharmacological activity of saffron as identified by extract fractionation bioactivity assays (Morimoto et al., 1994).

Saffron may be used as an anti-spasmodic and anti-catarrhal therapy in neuronal and heart-blood disorders (Rigobello et al., 2002; Lee et al., 2005; Aung et al., 2007). Crocin exerts anti-oxidant (Rigobello et al., 2002; Ochiai et al., 2004; Lee et al., 2005), anti-cancer (Aung et al., 2007; Chryssanthi et al., 2007), hypolipidemic (Lee et al., 2005; Sheng et al., 2006), anti-atherosclerotic (Xu et al., 2005; Xu et al., 2006; Xu et al., 2009), and anti-inflammatory effects (Ochiai et al., 2007; Naghizadeh et al., 2008). Brain dysfunction models performed the protection of neuronal function such as cerebral ischemia (Papandreou et al., 2006), Alzheimer's disease (Lechtenberg et al., 2008), NMDA receptor (Abe et al., 1998), and memory impairment (Sugiura et al., 1994; Sugiura et al., 1995a), which are closely related to dementia. It is accepted that learning and memory processes via long-term potentiation (LTP) occur in the hippocampus (Abe et al., 1991).

Previously, several studies investigated saffron extracts and crocin in mice with learning behaviors (Zhang et al., 1994; Sugiura et al., 1995b; Dashtira et al., 2009; Akbari et al., 2019; Roustazade et al., 2021), and LTP in the CA1 region of the rat hippocampus (Zhang et al., 1994; Sugiura et al., 1995b).

Crowe et al. reported that programmed cell death (apoptosis) in neurons occurred in the brains stripped of oxygen by stroke (Crowe et al., 1997), trauma (Hill et al., 1995), and patients with Alzheimer's disease (Pettmann and Henderson, 1998). Although the value of this approach is not yet proven beyond reasonable doubt, the prevention of neuronal apoptosis could become a therapeutic strategy for neurodegenerative disease.

Learning and Memory Activities Induced by Saffron and Crocin

We observed that mice with saffron extract and the control group on memory and learning behavior were nothing of differences supporting no effect of saffron for healthy control mice but improving potentiality time induced by ethanol (Sugiura et al., 1995b). Saffron extracts prevented impairments in memory induced by ethanol in passive avoidance studies (Zhang et al., 1994). Saffron also dose-dependently and significantly ameliorated increased memory errors induced by ethanol (Sugiura et al., 1995b). As an active constituent in saffron extracts it is easily suggested to be crocin because higher concentration. In fact, approximately 15% of crocin is contained in fresh dried saffron and results in 30% crocin levels in ethanol extracts (Morimoto et al., 1994). The activity of saffron extract appeared from 125 mg/kg which contained nearly 40 mg of crocin and dose-dependently increased (Sugiura et al., 1995b). Further studies have indicated that the oral administration of at least 50 mg/kg crocin improved impaired memory induced by ethanol (Sugiura et al., 1995c). Thus, crocin is a major active component of saffron and exerts similar pharmacological effects as saffron extract, although its activity is slightly different (Sugiura et al., 1995c).

Saffron and Crocin Activities Against LTP

In LTP studies, the intracerebroventricular injection of saffron extract dose-dependently decreased the negative effects of ethanol on LTP (Sugiura et al., 1995a; Sugiura et al., 1994). A crocin injection (50 mg/kg) at 5 min before ethanol treatment exhibited 84% LTP when compared with controls. As previously discussed, several crocetin glucose esters were isolated, and included crocin with 2-gentiobiose ester, crocetin gentiobiose glucose ester, and crocetin di-glucose ester (**Figure 2**) in molecules. When compared with the LTP blocking effects of ethanol the others rather than crocin were lower resulting that the improvement effect against blocking by ethanol was relatively reflected to the sugar number. This tendency was reported that the saponin's haemolytic activity of di- and triglycoside saponins were higher than that of monoglycoside (Voutquenne et al., 2002). Also, the hemolytic activity of saikosaponins was dependent on sugar numbers (Abe et al., 1978). From these observations, crocin appears to be the major active component in saffron in terms of its impact on learning and memory.

Crocin Activity on Pheochromocytoma Cell Death Induced by Serum/Glucose Deprivation in PC-12 Cells

To confirm crocin incorporation on PC-12 cells, a MAb against crocin was generated for immunostaining (Xuan et al., 1999). The

method confirmed crocin incorporation into PC-12 cells when compared with control cells (Ochiai et al., 2004).

When cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing glucose and serum (DMEM+), a normal morphology was observed after 24 h culture. However, cells cultured in serum- and glucose-free medium (DMEM-) for 24 h were rounded, causing a necrotic or apoptotic morphology and 60% cell death. However, this was recovered to 85% cell survival by crocin (10 μM) addition to DMEM-, and was reportedly *via* TNF-α inhibition, in a dose-dependent manner (Soeda et al., 2001). However, it is accepted that serum (Batistatou and Green, 1991; Oppenheim, 1991; Rukenstein et al., 1991) or NGF (Mesner et al., 1992; Pittman et al., 1993) removal induces apoptosis in PC-12 cells. It was reported that serum elimination from culture medium increased intracellular ceramide levels in undifferentiated HN9.10e cells and induced apoptosis (Colombaioni et al., 2002). In fact, when PC-12 cells were cultured for 3 h in DMEM-media, ceramide levels increased 3.5-fold when compared with DMEM+ conditions. Although fumonisin B1 (FB1) inhibits the *de novo* synthesis of ceramide at 10–30 μM (Wang et al., 1991; Merrill et al., 1993), FB1 exerted no decrease in ceramide levels. This phenomenon may have occurred via the combinatorial function of sphingomyelin (SM) and SAPK/JNK signaling pathways in the stress-induced apoptosis of U937 and BAE cells (Verheij et al., 1996). However, this hypothesis requires further investigation.

The Anti-apoptotic Activities of Crocin

Previously, PC-12 cells in DMEM-media displayed morphological changes and membrane peroxidation leading to decreased superoxide dismutase (SOD) activity (Ochiai et al., 2004). Annexin V is typically used to stain phosphatidylserine (PS) lipids in peroxidized membrane lipids. While PS lipids are usually fixed to inner membranes, they become morphologically altered and bind to outer membranes under oxidative stress. PS externalized membranes are detected by annexin V as ring-like staining reflective of apoptotic activity. PC-12 cells cultured in DMEM-for 6 h exhibited 1.8-fold increased peroxidized membrane lipid levels, whereas SOD activity had decreased to 14% when compared with control cells in DMEM+.

To confirm the anti-oxidant activity of crocin, peroxidized membrane lipids and restored SOD activity in PC-12 cells in DMEM-plus crocin were analyzed and compared to α-tocopherol as a positive control. Crocin significantly weakened peroxidized membrane lipid formation and preserved SOD activity when compared with α-tocopherol treated cells (Soeda et al., 2001).

The Effects of Crocin on Neural Sphingomyelinase in PC-12 Cells

PC-12 cell homogenate supernatants after substituting the reaction medium for 50 mM sodium acetate buffer (pH 5.6) were investigated to search the activity of magnesium-dependent neural sphingomyelinase for the determination of origin of accumulated ceramide. It became evident that the activity in DMEM-cells reached a maximum 1 h culturing and 3 h late backed to the revel of control cells without the time dependent change. This phenomenon indicated no effects of serum and glucose withholding for 3 h. In contrast, crocin supplementation to the medium dose-dependently inhibited enzyme activities at 1 and 2 h of culturing. When 1 or 10 μM crocin was added to DMEM-medium and cultured for 2 h, no inhibition of neural sphingomyelinase activity occurred in this medium. Previously, we had indicated that GSH functioned as a physiological inhibitor of magnesium-dependent neural sphingomyelinase in plasma membranes (Ochiai et al., 2004), 1 and 10 mM GSH added in the medium inhibited the enzyme activity dose-dependently.

Increased Intracellular GSH Levels in Serum and Glucose Deprived PC-12 Cells

We investigated intracellular GSH levels in serum and glucose deprived PC-12 cells and observed that GSH levels 3 h decreased by 50% when compared with control cells, and recovering to the constant revel. In contrast, 10 μM crocin significantly boosted intracellular GSH levels and maintained high levels to inactivate neural sphingomyelinase. To make sure the mechanism of increasing GSH level by crocin, the addition of 10 μM crocin in PC-12 cells cultured in DMEM-induced the higher GC activity time-dependently although the GR activity in PC-12 cells in EMEM-decreasing time-dependently. Nakajima et al. (2002) suggested that NGF increased c-GCS activity at the transcription level and extended the half-life of c-GCS mRNA. Urata et al. (1996) indicated that GSH synthesis was regulated by c-GCS, whose activity was related to increased TNF-α or interleukin (IL)-1β in mouse endothelial cells, and further related to mRNA expression. IL-6 also stimulated c-GCS mRNA expression and increased enzyme activity resulting in increased GSH levels in PC-12 cells. These data showed that crocin had no significant effects on GPx activity in cells. Ten μM crocin increased the c-GCS mRNA expression twice in PC-12 cells cultured in DMEM-inducing the higher enzyme activity in the cells although the mRNA levels in the control PC-12 cells did not increase. From this evidences, crocin increased GSH levels in PC-12 cells in DMEM-media, resulting in survival against the PC-12 cell death.

Non-Rapid Eye Movement Sleeping Effects of Crocin

Saffron and Kampo medicines promoted sleep during mental disorder therapy. The combination of saffron and Saikokaryukotsuboreito or Sansoninto was previously used as a sleep promoter in Japan (Matsuhashi, 1993). Therefore, we investigated the sleeping efficacy of crocin in mice after intraperitoneal administration with crocin at 20:00 in the evening. Wakefulness, non-REM sleep, and REM sleep after prescribed crocin (100 mg/kg) or vehicle were compared (**Figure 3**). Non-REM sleep occurred due to 100 mg/kg crocin administration and the intensity increased immediately after injection and the efficacy was statistically significant during 4 h after the injection. The extension of non-REM sleeping times was induced by the reduction of wake continuing 4 h after injection.

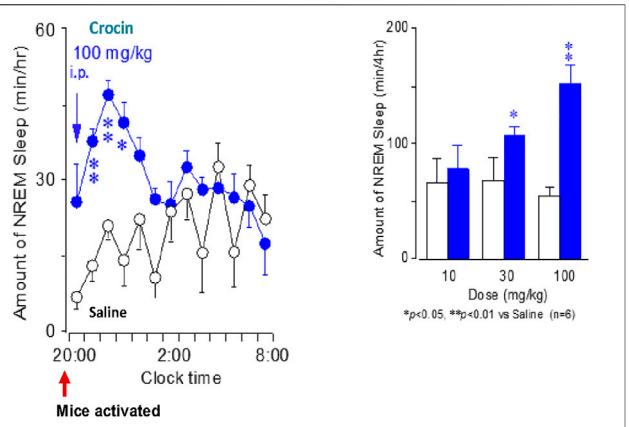

FIGURE 3 | The effect of crocin on sleep architecture in a mouse model.

However, crocin did not affect REM sleep. In contrast, mice treated with the vehicle were awake for longer between 20:00 and 01:00. From these data, crocin induced non-REM sleep with no typical side effects, such as rebound insomnia. When a 30 mg/kg crocin injection was administered, the same evidence occurred with shorter non-REM sleeping term for 1–2 h after the injection. The total time of non-REM and REM sleep and wakefulness in the 4 h period after crocin injection was calculated; crocin at 10 mg/kg had no effect on the cumulative amount of non-REM and REM sleep and wakefulness at 4 h after injection. However, 30 and 100 mg/kg crocin injections significantly increased total non-REM sleep to 160 and 270%, respectively, and significantly decreased total wakefulness to 20 and 50%, respectively, without changing REM sleep levels during 4 h as compared with the control (Masaki et al., 2012).

Clinical Trials on the Effects of Saffron on Sleep Architecture

To confirm increased sleep quality during saffron therapy, a double-blinded clinical trial using 21 healthy adults randomly assigned to either a saffron extract group (0.6 mg/day) or a placebo group was conducted. The trial demonstrated a significant reduction in Pittsburgh Sleep Quality Index scores in the saffron group. Furthermore, a significant positive effect of the saffron extract on daytime dysfunction appeared in the extract group when compared with the placebo group (Nishida et al., 2018).

The Relationship Between Sleep and Dementia

Recent evidence has suggested that sleep disturbance may lead to increased inflammatory processes, which in turn may lead to Alzheimer's disease (Irwin and Vitiello, 2019). Several meta-analyses and systematic reviews indicated that sleep disturbance may be an important risk factor, and thus an important target for Alzheimer's disease prevention (Bubu et al., 2017; Shi et al., 2018; Livingston et al., 2020). Furthermore, the Hisayama epidemiological study in Fukuoka, Japan provided clear evidence that sleep disorders and the concomitant use of hypnotic drugs resulted in an increased risk of dementia in elderly patients. When patients with daily sleep durations shorter than 5.0 h were compared with those reporting more than 10 h, the risk of dementia, such as vascular dementia and Alzheimer's disease for the short duration cohort, was increased 2-fold when compared with the 10 h cohort. To explain this, poor sleep quality may induce brain aging and lead to β-amyloid accumulation and Alzheimer's disease. Alternatively, sleep disturbance may promote inflammation and induce dementia and depression (Ohara et al., 2018). As saffron and crocin improve sleep quality (Masaki et al., 2012), this sleep duration extension may critically decrease the risk of dementia. A recent survey reported a connection between sleep disorders and dementia (Livingston et al., 2020). In our saffron research, we observed a 3-fold stronger anti-oxidant activity for crocin when compared with α-tocopherol (Ochiai et al., 2004). We further showed that previously proven crocin activity against colorectal cancer in mice was based on its strong anti-inflammatory activities (Kawabata et al., 2012). These observations suggested that the anti-dementia activities of saffron may be based on the same anti-inflammatory activities in mice. Lastly, crocin also exerted direct effects on hippocampal neurons via the NMDA receptor (Abe et al., 1998) and may therefore exert direct anti-dementia activities.

In Japan, combinations of classical Kampo formulas with saffron have been used in several clinical disciplines such as obstetrics and gynecology, psychiatry, and cardiology. For example, saffron (100 mg–1 g/day) was clinically used for patients with sleep disorders, together with Kampo formulas such as Saikokaryukotsuboreito, Hangekobokuto, Sansoninto, Daijokito, and Chotosan (Matsuhashi, 1993) or combinations thereof. Therefore, these Kampo-saffron combinations offer new and interesting avenues to develop future anti-dementia therapies.

Saffron and Crocin Activities for Anti-Depression Therapy

The neuroprotective activities of crocin were investigated using several brain disorder models, such as cerebral ischemia (Ochiai et al., 2007), Alzheimer's disease (Akhondzadeh et al., 2010a), depression (Hausenblas et al., 2013), and memory impairment (Sugiura et al., 1994; Sugiura et al., 1995c) which are all closely related to dementia. Depression—depending on the specific variant and physiological mechanism—may be closely related to early stage dementia. Depression was identified as a risk factor for dementia after 2–17 years of meta-analysis (Prince et al., 2014). In the Whitehall Study in the United Kingdom, in a follow-up of 10,189 patients, depression increased dementia risk later in life (Almeida et al., 2017). A 14-years study, including 4,922 initially cognitive healthy men of 71–89 years old, reported that depression induced a significant incidence of dementia (Kelly et al., 2017).

Since sleeping disorders and depression are closely associated with dementia, the indirect relationship between saffron and crocin and dementia via sleeping disorders or depression was correctly determined.

Saffron in Anti-dementia Therapy

A double-blinded, phase II study on 55 year old or older Alzheimer's patients (54 patients) was performed over 22 weeks. Patients randomly received a 30 mg saffron capsule/day or 10 mg donepezil/day as a positive control. Saffron showed almost similar efficacy as donepezil in mild to moderate Alzheimer's patients, and vomiting side effects were much lower than the donepezil group (Akhondzadeh et al., 2010). Furthermore, these authors also investigated the effects of saffron on mild to moderate Alzheimer's patients in a placebo-controlled trial to confirm the effects of saffron when compared to placebo. The double-blinded randomized clinical trial compared saffron with memantine and showed almost the same effects and no side effects (Farokhnia et al., 2014).

Furthermore, combined saffron and Kamiuntanto—or Kamiuntantokabankoka in Kampo terminology—should be used to limit dementia.

CONCLUSIONS AND PERSPECTIVES

Kamiuntanto activity against dementia was determined by *in vitro* and *in vivo* model systems. Based on these findings, the major Kamiuntanto constituents were assayed for their contribution to these activities. For the *Polygala* radix drug, onjisaponin (**Figure 1**) and sinapinic acid were identified as major chemical contributors to Kampo activity. Furthermore, numerous anti-dementia related activities of the *P. ginseng* root drug in Kamiuntanto were also supported by experimental evidence. Thus, both drugs may play important synergistic roles in Kamiuntanto formulas.

For 3,500 years, saffron has not only been used as a medical drug but also as a food spice, and is "Generally Recognized as Safe" by the American Food And Drug Administration (FDA) (Department Of Health And Human Services; Subchapter B—Food For Human Consumption (Continued); Part 182—Substances Generally Recognized as Safe/https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=182.20).

Crocin increased intracellular glutathione levels and prevented cell death in PC-12 cells cultured in DMEM-in a brain ischemia model. In these cells, reactive oxygen species generation activated neural sphingomyelinase resulting in ceramide production, which induced cell death as ceramide-release activates the caspase system. However, glutathione directly inhibited neural sphingomyelinase activation. We hypothesize that crocin may prevent neural sphingomyelinase activation in PC-12 cells cultured in DMEM- *via* a GSH-dependent inhibition mechanism.

As indicated, crocin is a major active constituent of saffron which improves learning and memory and prevents LTP blockade by ethanol in an *in vivo* mouse model. However, the oral administrations of saffron and crocin had no effect on memory acquisition in control mice. Naghibi et al. investigated the effects of saffron extracts on morphine-induced memory impairment and concluded saffron extracts attenuated this impairment (Naghibi et al., 2012). We also demonstrated, for the first time, that crocin selectively antagonized the inhibitory effects of ethanol on NMDA-receptor-mediated responses in hippocampal neurons (Abe et al., 1998). We observed that the efficacy of individual crocetin glycosides toward the inhibition of LTP blocking activity by ethanol was directly proportional to the number of sugar moieties in the respective molecules with crocin—containing four glucose moieties—exhibiting the strongest overall effect (Abe et al., 1998). Interestingly, increased bioactivity levels proportional to the number of sugar moieties in a series of related glycosides were previously reported for several other natural product classes, such as cardiac steroids (Shimada et al., 1986), streptozotocin (Gunnarsson et al., 1974), ginsenosides (Takemoto et al., 1984), saikosaponins (Abe et al., 1978), and hemolytic saponins (Voutquenne et al., 2002).

Currently in Japan, 25% of the population has reported occasional sleeping problems for which saffron could be prescribed in combination with Kampo. Saffron has been tested as a sleep promoter and increased total time for non-REM sleep. Thus, the accumulated evidence suggests the clinical benefit of saffron. The indirect relationship between saffron and/or crocin and dementia, *via* sleeping or depression disorders, was characterized. Furthermore, the evidence suggests that saffron improves Alzheimer's disease symptoms in clinical trials where market medicines were used as controls. Importantly, in 2006, crocin was approved by the Chinese State FDA for clinical trials and became an officially registered drug for angina.

AUTHOR CONTRIBUTIONS

KA, YU, NT, and C-SY carried out experimental laboratory investigations and drafted parts of the manuscript. YiS and KS collected and discussed the data on dementia. KK arranged and corrected the manuscript. YkS prepared the manuscript and oversaw project management.

FUNDING

Further support was granted by the Association for Health Economics Research and Social Insurance and Welfare. The work in Göttingen, Germany was supported by the "Förderkreis der Forschungsstelle für Fernöstliche Medizin".

ACKNOWLEDGMENTS

The authors sincerely thank Toshiharu Ninomiya, Medical School of Kyushu University for kind suggestions concerning the epidemiological study. We also sincerely thank Emeritus at Tokyo University, Hiroshi Saito, and Emeriti at Fukuoka University, Hiroshi Shimeno and Shinji Soeda who carried out experimental laboratory investigations and provided discussion topics. The authors greatly appreciate the Faculty of Pharmacy, Nagasaki International University for providing laboratory facilities and their support of this project.

REFERENCES

Abe, H., Sakaguchi, M., Konishi, H., Tani, T., and Arichi, S. (1978). The Effects of Saikosaponins on Biological Membranes. 1. The Relationship between the Structures of Saikosaponins and Haemolytic Activity. *Planta Med.* 34, 160–166. doi:10.1055/s-0028-1097428

Abe, K., Sugiura, M., Shoyama, Y., and Saito, H. (1998). Crocin Antagonizes Ethanol Inhibition of NMDA Receptor-Mediated Responses in Rat Hippocampal Neurons. *Brain Res.* 787, 132–138. doi:10.1016/s0006-8993(97)01505-9

Abe, K., Xie, F. J., and Saito, H. (1991). Epidermal Growth Factor Enhances Short-Term Potentiation and Facilitates Induction of Long-Term Potentiation in Rat Hippocampal Slices. *Brain Res.* 547, 171–174. doi:10.1016/0006-8993(91)90589-N

Akbari, F., Moghadasi, M., Farsi, S., and Edalatmanesh, M. A. (2019). The Effect of Eight Weeks Moderate-Intensity Endurance Training with Saffron Intake on Memory and Learning in Rats with Trimethytin Model of Alzheimer's Disease. *J. Appl. Exer Physiol.* 30, 115–128. doi:10.22080/JAEP.2019.15252.1831

Akhondzadeh, S., Sabet, M. S., Harirchian, M. H., Togha, M., Cheraghmakani, H., Razeghi, S., et al. (2010a). Saffron in the Treatment of Patients with Mild to Moderate Alzheimer's Disease: a 16-week, Randomized and Placebo-Controlled Trial. *J. Clin. Pharm. Ther.* 35, 581–588. doi:10.1111/j.1365-2710.2009.01133.x

Akhondzadeh, S., Shafiee Sabet, M., Harirchian, M. H., Togha, M., Cheraghmakani, H., Razeghi, S., et al. (2010b). A 22-week, Multicenter, Randomized, Double-Blind Controlled Trial of Crocus Sativus in the Treatment of Mild-To-Moderate Alzheimer's Disease. *Psychopharmacology (Berl)* 207 (4), 637–643. doi:10.1007/s00213-009-1706-1

Almeida, O. P., Hankey, G. J., Yeap, B. B., Golledge, J., and Flicker, L. (2017). Depression as a Modifiable Factor to Decrease the Risk of Dementia. *Transl Psychiatry* 7, e1117. doi:10.1038/tp.2017.90

Aung, H. H., Wang, C. Z., Ni, M., Fishbein, A., Mehendale, S. R., Xie, J. T., et al. (2007). Crocin from Crocus Sativus Possesses Significant Anti-proliferation Effects on Human Colorectal Cancer Cells. *Exp. Oncol.* 29, 175–180.

Ban, J. Y., Cho, S. O., Kwon, S. H., Kim, J. B., Seong, N. S., Bae, K. W., et al. (2005). Protection of Amyloid β Protein (25-35)-induced Neuronal Cell Damage by Methanol Extract of New Stem of Phyllostachys Nigra Munro Var. Henonis Stapf in Cultured Rat Cortical Neuron. *Korean J. Med. Crop Sci.* 13, 95–102.

Batistatou, A., and Greene, L. A. (1991). Aurintricarboxylic Acid Rescues PC12 Cells and Sympathetic Neurons from Cell Death Caused by Nerve Growth Factor Deprivation: Correlation with Suppression of Endonuclease Activity. *J. Cel Biol* 115, 461–471. doi:10.1083/jcb.115.2.461

Battle, D. E. (2013). Diagnostic and Statistical Manual of Mental Disorders (DSM). *Codas* 25 (2), 191–192. doi:10.1590/s2317-17822013000200017

Bubu, O. M., Brannick, M., Mortimer, J., Umasabor-Bubu, O., Sebastião, Y. V., Wen, Y., et al. (2017). Sleep, Cognitive Impairment, and Alzheimer's Disease: A Systematic Review and Meta-Analysis. *Sleep* 40, 32. doi:10.1093/sleep/zsw032

Chakravarthi, K. K., and Avadhani, R. (2013). Beneficial Effect of Aqueous Root Extract of Glycyrrhiza Glabra on Learning and Memory Using Different Behavioral Models: An Experimental Study. *J. Nat. Sci. Biol. Med.* 4, 420–425. doi:10.4103/0976-9668.117025

Cho, H. W., Jung, S. Y., Lee, G. H., Cho, J. H., and Choi, I. Y. (2015). Neuroprotective Effect of Citrus Unshiu Immature Peel and Nobiletin Inhibiting Hydrogen Peroxide-Induced Oxidative Stress in HT22 Murine Hippocampal Neuronal Cells. *Pharmacogn Mag.* 11, S284–S289. doi:10.4103/0973-1296.166047

Chryssanthi, D. G., Lamari, F. N., Iatrou, G., Pylara, A., Karamanos, N. K., and Cordopatis, P. (2007). Inhibition of Breast Cancer Cell Proliferation by Style Constituents of Different Crocus Species. *Anticancer Res.* 27, 357–362.

Chu, G. X., and Chen, X. (1990). Anti-lipid Peroxidation and protection of Ginsenosides against Cerebral Ischemia-Reperfusion Injuries in Rats. *Zhongguo Yao Li Xue Bao* 11, 119–123.

Clostre, F. (1999). Ginkgo Biloba Extract (EGb 761). State of Knowledge in the Dawn of the Year 2000. *Ann. Pharm. Fr* 57 (Suppl. 1), 1S8–88.

Colombaioni, L., Frago, L. M., Varela-Nieto, I., Pesi, R., and Garcia-Gil, M. (2002). Serum deprivation increases ceramide levels and induces apoptosis in undifferentiated HN9.10e cells. *Neurochem. Int.* 40, 327–336. doi:10.1016/s0197-0186(01)00090-0

Crowe, M. J., Bresnahan, J. C., Shuman, S. L., Masters, J. N., and Beattie, M. S. (1997). Apoptosis and Delayed Degeneration after Spinal Cord Injury in Rats and Monkeys. *Nat. Med.* 3, 73–76. doi:10.1038/nm0197-73

Deng, X., Zhao, S., Liu, X., Han, L., Wang, R., Hao, H., et al. (2020). *Polygala Tenuifolia*: a Source for Anti-alzheimer's Disease Drugs. *Pharm. Biol.* 58, 410–416. doi:10.1080/13880209.2020.1758732

Farokhnia, M., Shafiee Sabet, M., Iranpour, N., Gougol, A., Yekehtaz, H., Alimardani, R., et al. (2014). Comparing the Efficacy and Safety of *Crocus Sativus* L. With Memantine in Patients with Moderate to Severe Alzheimer's Disease: a Double-Blind Randomized Clinical Trial. *Hum. Psychopharmacol.* 29 (4), 351–359. doi:10.1002/hup.2412

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., et al. (2005). Global Prevalence of Dementia: a Delphi Consensus Study. *Lancet* 366, 2112–2117. doi:10.1016/S0140-6736(05)67889-0

Fujiwara, H., Yoshida, J., Dibwe, D. F., Awale, S., Hoshino, H., Kohama, H., et al. (2018). Orengedokuto and San'oshashinto Improve Memory Deficits by Inhibiting Aging-dependent Activation of Glycogen Synthase Kinase-3β. *J. Tradit Complement. Med.* 9, 328–335. doi:10.1016/j.jtcme.2018.12.001

Grzanna, R., Phan, P., Polotsky, A., Lindmark, L., and Frondoza, C. G. (2004). Ginger Extract Inhibits Beta-Amyloid Peptide-Induced Cytokine and Chemokine Expression in Cultured THP-1 Monocytes. *J. Altern. Complement. Med.* 10, 1009–1013. doi:10.1089/acm.2004.10.1009

Gunnarsson, R., Berne, C., and Hellerström, C. (1974). Cytotoxic Effects of Streptozotocin and N-Nitrosomethylurea on the Pancreatic B Cells with Special Regard to the Role of Nicotinamide-Adenine Dinucleotide. *Biochem. J.* 140, 487–494. doi:10.1042/bj1400487

Hausenblas, H. A., Saha, D., Dubyak, P. J., and Anton, S. D. (2013). Saffron (Crocus Sativus L.) and Major Depressive Disorder: a Meta-Analysis of Randomized Clinical Trials. *J. Integr. Med.* 11, 377–383. doi:10.3736/jintegrmed2013056

Hill, I. E., MacManus, J. P., Rasquinha, I., and Tuor, U. I. (1995). DNA Fragmentation Indicative of Apoptosis Following Unilateral Cerebral Hypoxia-Ischemia in the Neonatal Rat. *Brain Res.* 676, 398–403. doi:10.1016/0006-8993(95)00145-g

Ho, Y. S., So, K. F., and Chang, R. C. (2011). Drug Discovery from Chinese Medicine against Neurodegeneration in Alzheimer's and Vascular Dementia. *Chin. Med.* 6, 15. doi:10.1186/1749-8546-6-15

Hsu, Y. C., Chang, P. J., Tung, C. W., Shih, Y. H., Ni, W. C., Li, Y. C., et al. (2020). De-Glycyrrhizinated Licorice Extract Attenuates High Glucose-Stimulated Renal Tubular Epithelial-Mesenchymal Transition via Suppressing the Notch2 Signaling Pathway. *Cells* 9, 125. doi:10.3390/cells9010125

Huang, Q., Su, H., Qi, B., Wang, Y., Yan, K., Wang, X., et al. (2021). A SIRT1 Activator, Ginsenoside Rc, Promotes Energy Metabolism in Cardiomyocytes and Neurons. *J. Am. Chem. Soc.* 143, 1416–1427. doi:10.1021/jacs.0c10836

Irwin, M. R., and Vitiello, M. V. (2019). Implications of Sleep Disturbance and Inflammation for Alzheimer's Disease Dementia. *Lancet Neurol.* 18, 296–306. doi:10.1016/S1474-4422(18)30450-2

Itoh, T., Zang, Y. F., Murai, S., and Saito, H. (1989). Effects of Panax Ginseng Root on the Vertical and Horizontal Motor Activities and on Brain Monoamine-Related Substances in Mice. *Planta Med.* 55, 429–433. doi:10.1055/s-2006-962058

Iwasaki, K., Kobayashi, S., Chimura, Y., Taguchi, M., Inoue, K., Cho, S., et al. (2004). A randomized, double-blind, placebo-controlled clinical trial of the Chinese herbal medicine "ba wei di huang wan" in the treatment of dementia. *J. Am. Geriatr. Soc.* 52, 1518–1521. doi:10.1111/j.1532-5415.2004.52415.x

Jia, H., Jiang, Y., Ruan, Y., Zhang, Y., Ma, X., Zhang, J., et al. (2004). Tenuigenin Treatment Decreases Secretion of the Alzheimer's Disease Amyloid Beta-Protein in Cultured Cells. *Neurosci. Lett.* 367, 123–128. doi:10.1016/j.neulet.2004.05.093

Jung, I. H., Lee, H. E., Park, S. J., Ahn, Y. J., Kwon, G., Woo, H., et al. (2014). Ameliorating Effect of Spinosin, a C-Glycoside Flavonoid, on Scopolamine-Induced Memory Impairment in Mice. *Pharmacol. Biochem. Behav.* 120, 88–94. doi:10.1016/j.pbb.2014.02.015

Kalmijn, S., Launer, L. J., Ott, A., Witteman, J. C., Hofman, A., and Breteler, M. M. (1997). Dietary Fat Intake and the Risk of Incident Dementia in the Rotterdam Study. *Ann. Neurol.* 42, 776–782. doi:10.1002/ana.410420514

Kawabata, K., Tung, N. H., Shoyama, Y., Sugie, S., Mori, T., and Tanaka, T. (20122012). Dietary Crocin Inhibits Colitis and Colitis-Associated Colorectal Carcinogenesis in Male ICR Mice. *Evid. Based Complement. Alternat Med.* 2012, 820415. doi:10.1155/2012/820415

Kell, G., Rao, A., Beccaria, G., Clayton, P., Inarejos-García, A. M., and Prodanov, M. (2017). affron® a Novel Saffron Extract (Crocus Sativus L.) Improves Mood in Healthy Adults over 4 Weeks in a Double-Blind, Parallel, Randomized, Placebo-Controlled Clinical Trial. *Complement. Ther. Med.* 33, 58–64. doi:10.1016/j.ctim.2017.06.001

Kelly, M. E., Duff, H., Kelly, S., McHugh Power, J. E., Brennan, S., Lawlor, B. A., et al. (2017). The Impact of Social Activities, Social Networks, Social Support and Social Relationships on the Cognitive Functioning of Healthy Older Adults: a Systematic Review. *Syst. Rev.* 6, 259. doi:10.1186/s13643-017-0632-2

Kim, D., Nguyen, M. D., Dobbin, M. M., Fischer, A., Sananbenesi, F., Rodgers, J. T., et al. (2007). SIRT1 Deacetylase Protects against Neurodegeneration in Models for Alzheimer's Disease and Amyotrophic Lateral Sclerosis. *EMBO J.* 26, 3169–3179. doi:10.1038/sj.emboj.7601758

Kim, Y., and Cho, S. H. (2020). Danggui-Shaoyao-San for Dementia: a PRISMA-Compliant Systematic Review and Meta-Analysis. *Medicine (Baltimore)* 99, e18507. doi:10.1097/MD.0000000000018507

Lee, B., Shim, I., Lee, H., and Hahm, D. H. (2011). Rehmannia Glutinosa Ameliorates Scopolamine-Induced Learning and Memory Impairment in Rats. *J. Microbiol. Biotechnol.* 21, 874–883. doi:10.4014/jmb.1104.04012

Lee, I. A., Lee, J. H., Baek, N. I., and Kim, D. H. (2005). Antihyperlipidemic Effect of Crocin Isolated from the Fructus of Gardenia Jasminoides and its Metabolite Crocetin. *Biol. Pharm. Bull.* 28, 2106–2110. doi:10.1248/bpb.28.2106

Lee, T. F., Shiao, Y. J., Chen, C. F., and Wang, L. C. (2001). Effect of Ginseng Saponins on Beta-Amyloid-Suppressed Acetylcholine Release from Rat Hippocampal Slices. *Planta Med.* 67, 634–637. doi:10.1055/s-2001-17366

Leung, K. W., and Wong, A. S. (2010). Pharmacology of Ginsenosides: a Literature Review. *Chin. Med.* 5, 20. doi:10.1186/1749-8546-5-20

Li, J., Ding, X., Zhang, R., Jiang, W., Sun, X., Xia, Z., et al. (2015a). Harpagoside Ameliorates the Amyloid-β-Induced Cognitive Impairment in Rats via Up-Regulating BDNF Expression and MAPK/PI3K Pathways. *Neuroscience* 303, 103–114. doi:10.1016/j.neuroscience.2015.06.042

Li, X. J., and Zhang, H. Y. (2009). Potential Anti-dementia Agents in Traditional Chinese Medicine. *Nat. Prod. Commun.* 4 (6), 877–886. doi:10.1177/1934578X0900400629

Li, Z., Li, H., Zhao, C., Lv, C., Zhong, C., Xin, W., et al. (2015b). Protective Effect of Notoginsenoside R1 on an APP/PS1 Mouse Model of Alzheimer's Disease by Up-Regulating Insulin Degrading Enzyme and Inhibiting Aβ Accumulation. *CNS Neurol. Disord. Drug Targets* 14, 360–369. doi:10.2174/1871527314666150225141521

Liu, R.-t., Zou, L.-b., and Lü, Q.-j. (2009). Liquiritigenin Inhibits Aβ25-35-Induced Neurotoxicity and Secretion of Aβ1-40 in Rat Hippocampal Neurons. *Acta Pharmacol. Sin* 30 (7), 899–906. doi:10.1038/aps.2009.74

Livingston, G., Huntley, J., Sommerlad, A., Ames, D., Ballard, C., Banerjee, S., et al. (2020). Dementia Prevention, Intervention, and Care: 2020 Report of the Lancet Commission. *Lancet* 396, 413–446. doi:10.1016/S0140-6736(20)30367-6

Lu, P., Su, W., Miao, Z. H., Niu, H. R., Liu, J., and Hua, Q. L. (2008). Effect and Mechanism of Ginsenoside Rg3 on Postoperative Life Span of Patients with Non-small Cell Lung Cancer. *Chin. J. Integr. Med.* 14, 33–36. doi:10.1007/s11655-007-9002-6

Lv, J., Jia, H., Jiang, Y., Ruan, Y., Liu, Z., Yue, W., et al. (2009). Tenuifolin, an Extract Derived from Tenuigenin, Inhibits Amyloid-Beta Secretion *In Vitro*. *Acta Physiol. (Oxf)* 196, 419–425. doi:10.1111/j.1748-1716.2009.01961.x

Masaki, M., Aritake, K., Tanaka, H., Shoyama, Y., Huang, Z. L., and Urade, Y. (2012). Crocin Promotes Non-rapid Eye Movement Sleep in Mice. *Mol. Nutr. Food Res.* 56, 304–308. doi:10.1002/mnfr.201100181

Mathew, M., and Subramanian, S. (2014). *In Vitro* evaluation of Anti-alzheimer Effects of Dry Ginger (Zingiber Officinale Roscoe) Extract. *Indian J. Exp. Biol.* 52, 606–612.

Matsuhashi, T. (1993). The Effect of Saffron for Sleep Induction. *J. New Rem Clin.* 42, 595–597.

Merrill, A. H., Jr., van Echten, G., Wang, E., and Sandhoff, K. (1993). Fumonisin B1 Inhibits Sphingosine (Sphinganine) N-Acyltransferase and De Novo Sphingolipid Biosynthesis in Cultured Neurons *In Situ*. *J. Biol. Chem.* 268, 27299–27306. doi:10.1016/S0021-9258(19)74249-5

Mersereau, J. E., Levy, N., Staub, R. E., Baggett, S., Zogovic, T., Zogric, T., et al. (2008). Liquiritigenin Is a Plant-Derived Highly Selective Estrogen Receptor Beta Agonist. *Mol. Cel Endocrinol* 283 (1–2), 49–57. doi:10.1016/j.mce.2007.11.020

Mesner, P. W., Winters, T. R., and Green, S. H. (1992). Nerve Growth Factor Withdrawal-Induced Cell Death in Neuronal PC12 Cells Resembles that in Sympathetic Neurons. *J. Cel Biol* 119, 1669–1680. doi:10.1083/jcb.119.6.1669

Morimoto, S., Umezaki, Y., Shoyama, Y., Saito, H., Nishi, K., and Irino, N. (1994). Post-harvest Degradation of Carotenoid Glucose Esters in Saffron. *Planta Med.* 60, 438–440. doi:10.1055/s-2006-959527

Naghibi, S. M., Hosseini, M., Khani, F., Rahimi, M., Vafaee, F., Rakhshandeh, H., et al. (20122012). Effect of Aqueous Extract of Crocus Sativus L. On Morphine-Induced Memory Impairment. *Adv. Pharmacol. Sci.* 2012, 494367. doi:10.1155/2012/494367

Naghizadeh, B., Boroushaki, M. T., Vahdati Mashhadian, N., and Mansouri, M. T. (2008). Protective Effects of Crocin against Cisplatin-Induced Acute Renal Failure and Oxidative Stress in Rats. *Iran Biomed. J.* 12, 93–100.

Nakajima, A., Yamada, K., Zou, L. B., Yan, Y., Mizuno, M., and Nabeshima, T. (2002). Interleukin-6 Protects PC12 Cells from 4-Hydroxynonenal-Induced Cytotoxicity by Increasing Intracellular Glutathione Levels. *Free Radic. Biol. Med.* 32, 1324–1332. doi:10.1016/s0891-5849(02)00845-6

Natarajan, S., Shunmugiah, K. P., and Kasi, P. D. (2013). Plants Traditionally Used in Age-Related Brain Disorders (Dementia): an Ethanopharmacological Survey. *Pharm. Biol.* 51, 492–523. doi:10.3109/13880209.2012.738423

National Institute of Public Health (2015). MHLW grants SYST Em [in Japanese]. NIDD00.Do?resrchNum=201405037A. Available at: https://mhlw-grants.niph.go.jp/niph/search (Accessed June 10, 2020).

Nishide, A., Fujita, T., Nagaregawa, Y., Shoyama, Y., Ohnuki, K., Shimizu, K., et al. (2018). Sleep Enhancement by Saffron Extract Affron® in Randomized Control Trial. *J. Pharmacol. Ther.* 46, 1407–1415.

Nishiyama, N., Zhou, Y., and Saito, H. (1994b). Ameliorative Effects of Chronic Treatment Using DX-9386, a Traditional Chinese Prescription, on Learning Performance and Lipid Peroxide Content in Senescence Accelerated Mouse. *Biol. Pharm. Bull.* 17, 1481–1484. doi:10.1248/bpb.17.1481

Nishiyama, N., Zhou, Y., and Saito, H. (1994a). Beneficial Effects of DX-9386, a Traditional Chinese Prescription, on Memory Disorder Produced by Lesioning the Amygdala in Mice. *Biol. Pharm. Bull.* 17, 1679–1681. doi:10.1248/bpb.17.1679

Ochiai, T., Ohno, S., Soeda, S., Tanaka, H., Shoyama, Y., and Shimeno, H. (2004). Crocin Prevents the Death of Rat Pheochromyctoma (PC-12) Cells by its Antioxidant Effects Stronger Than Those of Alpha-Tocopherol. *Neurosci. Lett.* 362, 61–64. doi:10.1016/j.neulet.2004.02.067

Ochiai, T., Shimeno, H., Mishima, K., Iwasaki, K., Fujiwara, M., Tanaka, H., et al. (2007). Protective Effects of Carotenoids from Saffron on Neuronal Injury *In Vitro* and *In Vivo*. *Biochim. Biophys. Acta* 1770, 578–584. doi:10.1016/j.bbagen.2006.11.012

Ochiai, T., Soeda, S., Ohno, S., Tanaka, H., Shoyama, Y., and Shimeno, H. (2004). Crocin Prevents the Death of PC-12 Cells through Sphingomyelinase-Ceramide Signaling by Increasing Glutathione Synthesis. *Neurochem. Int.* 44, 321–330. doi:10.1016/s0197-0186(03)00174-8

Ohara, T., Honda, T., Hata, J., Yoshida, D., Mukai, N., Hirakawa, Y., et al. (2018). Association between Daily Sleep Duration and Risk of Dementia and Mortality in a Japanese Community. *J. Am. Geriatr. Soc.* 66, 1911–1918. doi:10.1111/jgs.15446

Okamoto, H. (2017). Treatment of Dementia-Related Symptoms with Japanese Traditional Medicine (Kampo): A Review of Clinical Studies. *J. Alzheimers Dis. Parkinsonism* 7, 326. doi:10.4172/2161-0460.1000326

Oppenheim, R. W. (1991). Cell Death During Development of the Nervous System. *Ann. Rev. Neurosci.* 14, 453–501. doi:10.1146/annurev.ne.14.030191.002321

Orgogozo, J. M., Dartigues, J. F., Lafont, S., Letenneur, L., Commenges, D., Salamon, R., et al. (1997). Wine Consumption and Dementia in the Elderly: a Prospective Community Study in the Bordeaux Area. *Rev. Neurol. (Paris)* 153, 185–192.

Papandreou, M. A., Kanakis, C. D., Polissiou, M. G., Efthimiopoulos, S., Cordopatis, P., Margarity, M., et al. (2006). Inhibitory Activity on Amyloid-Beta Aggregation and Antioxidant Properties of Crocus Sativus Stigmas Extract and its Crocin Constituents. *J. Agric. Food Chem.* 54, 8762–8768. doi:10.1021/jf061932a

Pettmann, B., and Henderson, C. E. (1998). Neuronal Cell Death. *Neuron* 20, 633–647. doi:10.1016/s0896-6273(00)81004-1

Pittman, R. N., Wang, S., DiBenedetto, A. J., and Mills, J. C. (1993). A System for Characterizing Cellular and Molecular Events in Programmed Neuronal Cell Death. *J. Neurosci.* 13, 3669–3680. doi:10.1523/JNEUROSCI.13-09-03669.1993

Prince, M., Albanese, E., Guerchet, M., and Prina, M. (2014). *World Alzheimer Report 2014 - Dementia and Risk Reduction: An Analysis of Protective and Modifiable Risk Factors*. London, UK: Alzheimer's Disease International.

Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., et al. (2006). Neuronal SIRT1 Activation as a Novel Mechanism Underlying the Prevention of Alzheimer Disease Amyloid Neuropathology by Calorie Restriction. *J. Biol. Chem.* 281, 21745–21754. doi:10.1074/jbc.M602909200

Rigobello, M. P., Scutari, G., Boscolo, R., and Bindoli, A. (2002). Inhibition of Lipid Peroxidation by S-Nitrosoglutathione and Copper. *Free Radic. Res.* 36, 1071–1077. doi:10.1080/1071576021000006680

Roustazade, R., Radahmadi, M., and Yazdani, Y. (2021). Therapeutic Effects of Saffron Extract on Different Memory Types, Anxiety, and Hippocampal BDNF and TNF-α Gene Expressions in Sub-chronically Stressed Rats. *Nutr. Neurosci.*, 1–15. doi:10.1080/1028415X.2021.1943138

Rudakewich, M., Ba, F., and Benishin, C. G. (2001). Neurotrophic and Neuroprotective Actions of Ginsenosides Rb(1) and Rg(1). *Planta Med.* 67, 533–537. doi:10.1055/s-2001-16488

Rukenstein, A., Rydel, R. E., and Greene, L. A. (1991). Multiple Agents rescue PC12 Cells from Serum-free Cell Death by Translation- and Transcription-independent Mechanisms. *J. Neurosci.* 11, 2552–2563. doi:10.1523/JNEUROSCI.11-08-02552.1991

Sasaki, Y., Shimizu, K., Watanabe, H., and Shoyama, Y. (2021). Application of Monoclonal Antibody against Ginsenoside in Ginseng Research: a Review. *Tradit Med. Res.* 6, 33. doi:10.12032/TMR20210118215 10.53388/tmr20210118215

Shen, L., and Zhang, J. (2003). Ginsenoside Rg1 Increases Ischemia-Induced Cell Proliferation and Survival in the Dentate Gyrus of Adult Gerbils. *Neurosci. Lett.* 344, 1–4. doi:10.1016/s0304-3940(03)00318-5

Sheng, L., Qian, Z., Zheng, S., and Xi, L. (2006). Mechanism of Hypolipidemic Effect of Crocin in Rats: Crocin Inhibits Pancreatic Lipase. *Eur. J. Pharmacol.* 543, 116–122. doi:10.1016/j.ejphar.2006.05.038

Shi, L., Chen, S. J., Ma, M. Y., Bao, Y. P., Han, Y., Wang, Y. M., et al. (2018). Sleep Disturbances Increase the Risk of Dementia: A Systematic Review and Meta-Analysis. *Sleep Med. Rev.* 40, 4–16. doi:10.1016/j.smrv.2017.06.010

Shimada, K., Ishii, N., Ohishi, K., Ro, J. S., and Nambara, T. (1986). Structure-activity Relationship of Cardiac Steroids Having a Doubly Linked Sugar and Related Compounds for the Inhibition of Na+,K+-adenosine Triphosphatase. *J. Pharmacobiodyn* 9, 755–759. doi:10.1248/bpb1978.9.755

Shinjyo, N., Waddell, G., and Green, J. (2020). Valerian Root in Treating Sleep Problems and Associated Disorders-A Systematic Review and Meta-Analysis. *J. Evid. Based Integr. Med.* 25, 2515690X20967323. doi:10.1177/2515690X20967323

Shoyama, Y. (2011). Monoclonal Antibodies against Small Molecule Natural Products and Their Applications, Eastern Blotting and Knockout Extract. *Pharmaceuticals* 4, 950–963. doi:10.3390/ph4070950

Smriga, M., Saito, H., and Nishiyama, N. (1995). Hoelen (Poria Cocos Wolf) and Ginseng (Panax Ginseng C. A. Meyer), the Ingredients of a Chinese Prescription DX-9386, Individually Promote Hippocampal Long-Term Potentiation *In Vivo*. *Biol. Pharm. Bull.* 18, 518–522. doi:10.1248/bpb.18.518

Soeda, S., Ochiai, T., Paopong, L., Tanaka, H., Shoyama, Y., and Shimeno, H. (2001). Crocin Suppresses Tumor Necrosis Factor-Alpha-Induced Cell Death of Neuronally Differentiated PC-12 Cells. *Life Sci.* 69, 2887–2898. doi:10.1016/s0024-3205(01)01357-1

Sugiura, M., Shoyama, Y., Saito, H., and Abe, K. (1994). Crocin (Crocetin Di-gentiobiose Ester) Prevents the Inhibitory Effect of Ethanol on Long-Term Potentiation in the Dentate Gyrus *In Vivo*. *J. Pharmacol. Exp. Ther.* 271, 703–707.

Sugiura, M., Shoyama, Y., Saito, H., and Abe, K. (1995a). The Effects of Ethanol and Crocin on the Induction of Long-Term Potentiation in the CA1 Region of Rat Hippocampal Slices. *Jpn. J. Pharmacol.* 67, 395–397. doi:10.1254/jjp.67.395

Sugiura, M., Saito, H., Abe, K., and Shoyama, Y. (1995b). Ethanol Extract ofCrocus Sativus L. Antagonizes the Inhibitory Action of Ethanol on Hippocampal Long-Term Potentiationin Vivo. *Phytother. Res.* 9, 100–104. doi:10.1002/ptr.2650090204

Sugiura, M., Shoyama, Y., Saito, H., and Nishiyama, N. (1995c). Crocin Improves the Ethanol-Induced Impairment of Learning Behaviors of Mice in Passive Avoidance Tasks. *Proc. Jpn. Acad. Ser. B: Phys. Biol. Sci.* 71, 319–324. doi:10.2183/pjab.71.319

Takemoto, Y., Ueyama, T., Saito, H., Horio, S., Sanada, S., Shoji, J., et al. (1984). Potentiation of Nerve Growth Factor-Mediated Nerve Fiber Production in Organ Cultures of Chicken Embryonic Ganglia by Ginseng Saponins: Structure-Aactivity Relationship. *Chem. Pharm. Bull. (Tokyo)* 32, 3128–3133. doi:10.1248/cpb.32.3128

Terasawa, K., Shimada, Y., Kita, T., Yamamoto, T., Tosa, H., Tanaka, N., et al. (1997). Choto-san in the Treatment of Vascular Dementia: A Double-Blind, Placebo-Controlled Study. *Phytomedicine* 4, 15–22. doi:10.1016/S0944-7113(97)80022-0

Tung, N. H., and Shoyama, Y. (2013). New Minor Glycoside Components from Saffron. *J. Nat. Med.* 67, 672–676. doi:10.1007/s11418-012-0721-4

Urata, Y., Yamamoto, H., Goto, S., Tsushima, H., Akazawa, S., Yamashita, S., et al. (1996). Long Exposure to High Glucose Concentration Impairs the Responsive Expression of Gamma-Glutamylcysteine Synthetase by Interleukin-1beta and Tumor Necrosis Factor-Alpha in Mouse Endothelial Cells. *J. Biol. Chem.* 271, 15146–15152. doi:10.1074/jbc.271.25.15146

Uto, T., Morinaga, O., Tanaka, H., and Shoyama, Y. (2012). Analysis of the Synergistic Effect of Glycyrrhizin and Other Constituents in Licorice Extract on Lipopolysaccharide-Induced Nitric Oxide Production Using Knock-Out Extract. *Biochem. Biophys. Res. Commun.* 417, 473–478. doi:10.1016/j.bbrc.2011.11.143

Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., et al. (1996). Requirement for Ceramide-Initiated SAPK/JNK Signalling in Stress-Induced Apoptosis. *Nature* 380, 75–79. doi:10.1038/380075a0

Voutquenne, L., Lavaud, C., Massiot, G., and Men-Olivier, L. L. (2002). Structure-activity Relationships of Haemolytic Saponins. *Pharm. Biol.* 40, 253–262. doi:10.1076/phbi.40.4.253.8470

Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H., Jr. (1991). Inhibition of Sphingolipid Biosynthesis by Fumonisins. Implications for Diseases Associated with Fusarium Moniliforme. *J. Biol. Chem.* 266, 14486–14490. doi:10.1016/S0021-9258(18)98712-0

Wang, W. Y., Tan, M. S., Yu, J. T., and Tan, L. (20152015). Role of Pro-inflammatory Cytokines Released from Microglia in Alzheimer's Disease. *Ann. Transl Med.* 3 (10), 136. doi:10.3978/j.issn.2305-5839.2015.03.49

Xu, G. L., Li, G., Ma, H. P., Zhong, H., Liu, F., and Ao, G. Z. (2009). Preventive Effect of Crocin in Inflamed Animals and in LPS-Challenged RAW 264.7 Cells. *J. Agric. Food Chem.* 57, 8325–8330. doi:10.1021/jf901752f

Xu, G. L., Qian, Z. Y., Yu, S. Q., Gong, Z. N., and Shen, X. C. (2006). Evidence of Crocin against Endothelial Injury Induced by Hydrogen Peroxide *In Vitro*. *J. Asian Nat. Prod. Res.* 8, 79–85. doi:10.1080/10286020500044732

Xu, G. L., Yu, S. Q., Gong, Z. N., and Zhang, S. Q. (2005). [Study of the Effect of Crocin on Rat Experimental Hyperlipemia and the Underlying Mechanisms]. *Zhongguo Zhong Yao Za Zhi* 30, 369–372.

Xuan, L., Tanaka, H., Xu, Y., and Shoyama, Y. (1999). Preparation of Monoclonal Antibody Against Crocin and Its Characterization. *Cytotechnology* 29, 65–70. doi:10.1023/A:1007993615489

Yabe, T., Iizuka, S., Komatsu, Y., and Yamada, H. (1997). Enhancements of Choline Acetyltransferase Activity and Nerve Growth Factor Secretion by Polygalae Radix-Extract Containing Active Ingredients in Kami-Untan-To. *Phytomedicine* 4, 199–205. doi:10.1016/S0944-7113(97)80068-2

Yabe, T., Toriizuka, K., and Yamada, H. (1996). Kami-untan-to (KUT) Improves Cholinergic Deficits in Aged Rats. *Phytomedicine* 2, 253–258. doi:10.1016/S0944-7113(96)80051-1

Yabe, T., and Yamada, H. (1997b). Induction Mechanism of Nerve Growth Factor Synthesis by Kami-Untan-To; Role of Cyclic AMP and C-Fos mRNA Accumulation. *Phytomedicine* 4, 191–198. doi:10.1016/S0944-7113(97)80067-0

Yabe, T., and Yamada, H. (1997a). Kami-Untan-To Enhances Choline Acetyltransferase and Nerve Growth Factor mRNA Levels in Brain Cultured Cells. *Phytomedicine* 3, 361–367. doi:10.1016/S0944-7113(97)80010-4

Yamaguchi, Y., Higashi, M., and Kobayashi, H. (1996). Effects of Ginsenosides on Impaired Performance Caused by Scopolamine in Rats. *Eur. J. Pharmacol.* 312, 149–151. doi:10.1016/0014-2999(96)00597-3

Yang, W. Z., Hu, Y., Wu, W. Y., Ye, M., and Guo, D. A. (2014). Saponins in the Genus Panax L. (Araliaceae): a Systematic Review of Their Chemical Diversity. *Phytochemistry* 106, 7–24. doi:10.1016/j.phytochem.2014.07.012

Yim, S. B., Park, S. E., and Lee, C. S. (2007). Protective Effect of Glycyrrhizin on 1-Methyl-4-Phenylpyridinium-Induced Mitochondrial Damage and Cell Death in Differentiated PC12 Cells. *J. Pharmacol. Exp. Ther.* 321 (2), 816–822. doi:10.1124/jpet.107.119602

Yun, T. K. (2001). Brief Introduction of Panax Ginseng C.A. Meyer. *J. Korean Med. Sci.* 16 (Suppl. l), S3–S5. doi:10.3346/jkms.2001.16.S.S3

Zeinali, F., Anvari, M., Dashti, R. M. H., and Mahmood Hosseini, S. (2009). Evaluating the Effect of Saffron (Crocus sativus) on Prevention and Treatment of Alzheimer's Disease in Mice by the "one Way Active Avoidance Learning and Memory" Tests. *Planta Med.* 75, PI26. doi:10.1055/s-0029-1234790

Zhang, G., Liu, A., Zhou, Y., San, X., Jin, T., and Jin, Y. (2008). Panax Ginseng Ginsenoside-Rg2 Protects Memory Impairment via Anti-apoptosis in a Rat Model with Vascular Dementia. *J. Ethnopharmacol* 115, 441–448. doi:10.1016/j.jep.2007.10.026

Zhang, Y., Shoyama, Y., Sugiura, M., and Saito, H. (1994). Effects of Crocus Sativus L. On the Ethanol-Induced Impairment of Passive Avoidance Performances in Mice. *Biol. Pharm. Bull.* 17, 217–221. doi:10.1248/bpb.17.217

Zou, S., Zhang, M., Feng, L., Zhou, Y., Li, L., and Ban, L. (2017). Protective Effects of Notoginsenoside R1 on Cerebral Ischemia-Reperfusion Injury in Rats. *Exp. Ther. Med.* 14, 6012–6016. doi:10.3892/etm.2017.5268

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

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

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 March 2022
doi: 10.3389/fphar.2022.804103

In vitro Suppression of SARS-CoV-2 Infection by Existing Kampo Formulas and Crude Constituent Drugs Used for Treatment of Common Cold Respiratory Symptoms

*Masaki Kakimoto[1], Toshihito Nomura[2,3], Tanuza Nazmul[3], Hiroki Kitagawa[2], Keishi Kanno[1], Keiko Ogawa-Ochiai[1,4], Hiroki Ohge[2], Masanori Ito[1] and Takemasa Sakaguchi[3]**

[1]Department of General Internal Medicine, Hiroshima University Hospital, Hiroshima, Japan, [2]Department of Infectious Disease, Hiroshima University Hospital, Hiroshima, Japan, [3]Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan, [4]Kampo Clinical Center, Department of General Internal Medicine, Hiroshima University Hospital, Hiroshima, Japan

OPEN ACCESS

Edited by:
Kenji Watanabe, Yokohama College of Pharmacy, Japan

Reviewed by:
Xuepeng Gong, Huazhong University of Science and Technology, China
Theresa Li-Yun Chang, Rutgers, The State University of New Jersey, United States

***Correspondence:**
Takemasa Sakaguchi
tsaka@hiroshima-u.ac.jp

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

Received: *28 October 2021*
Accepted: *07 March 2022*
Published: *29 March 2022*

Citation:
Kakimoto M, Nomura T, Nazmul T, Kitagawa H, Kanno K, Ogawa-Ochiai K, Ohge H, Ito M and Sakaguchi T (2022) In vitro Suppression of SARS-CoV-2 Infection by Existing Kampo Formulas and Crude Constituent Drugs Used for Treatment of Common Cold Respiratory Symptoms. Front. Pharmacol. 13:804103. doi: 10.3389/fphar.2022.804103

Several traditional Japanese Kampo formulas are known to have inhibitory effects on infections with viruses that cause respiratory symptoms. Although some herbs and their components have been reported to suppress SARS-CoV-2 replication *in vitro*, it is difficult to compare effective Kampo formulas because of the different methods used in studies. Thus, we carried out *in vitro* experiments on the suppression of SARS-CoV-2 infection by Kampo formulas and crude drugs used for the common cold to compare their suppressive effects on virus infection. After infecting VeroE6/TMPRSS2 cells with SARS-CoV-2, lysates of the Kampo formulas and crude drugs were added, and after 24 h, the infectious titer in the medium was measured by the $TCID_{50}$ method. Maoto was the most effective among the Kampo formulas, and Ephedrae herba was the most effective among the constituent crude drugs. However, a comparison of the suppressive effects of Ephedrae herba and Kampo formulas containing Ephedrae herba showed that the suppressive effect on virus infection did not depend on the content of Ephedrae herba. Based on the results, we believe that the use of Maoto among Kampo formulas is suitable as a countermeasure against COVID-19.

Keywords: anti-viral, COVID-19, SARS-CoV-2, herbal medicine, kampo medicine, crude drug

INTRODUCTION

In September 2021, the cumulative number of SARS-CoV-2 infection cases worldwide was more than 200 million and the number of deaths from SARS-CoV-2 infection was more than 4.5 million. There has been no apparent reduction in the number of new infections or deaths (World Health Organization, 2021). Although the efficacy of vaccines has been recognized and vaccination has been accelerated in many countries worldwide, there are still many unclear issues about vaccines as the efficacy of vaccines against emerging mutant strains (Liu et al., 2021). On the other hand, there are few drugs that can be expected to have antiviral effects and most of the drugs are expensive (Hsu, 2020). Therefore, there is an urgent need to find new anti-viral drugs or alternative treatments with drug repositioning.

Kampo medicine is a systematized medical system based on traditional East-Asian medicine and unique drugs. Kampo medicine originated in traditional Chinese medicine, which was introduced to Japan around the 5th century and was refined from the 17th century to the currently used Kampo medicine in Japan. Kampo formulas are mixtures of crude drugs, which are extracts of herbs, insects, minerals, fungi, and other substances (Tsumura and Co., 2016). All Kampo formulas were approved as drugs for humans by Japan's Ministry of Health, Labour and Welfare in 1986 and are regulated by the National Institute of Health and Welfare (STORK, 2020). Among the medications certified by the Ministry of Health, Labour and Welfare, medical Kampo formulas are comparatively inexpensive, and many of them have versatility in use. On the other hand, herbal medicines themselves are also used as folk remedies not only in Japan but also in many other countries.

Influenza and respiratory syncytial viruses are RNA viruses and the primary symptoms of infections with these viruses are respiratory symptoms that are similar to the symptoms of SARS-CoV-2 infection. In Japan, two Kampo prescriptions, Maoto and Saikokeishito, have been approved for treatment of influenza infection. There have been *in vitro* studies showing suppression of infection with these viruses by Kampo formulas and crude drug extracts (Mantani et al., 1999; Nomura et al., 2019; Hou et al., 2020). There seems to be a relationship between the clinical effects of Kampo formulas and inhibition of influenza virus growth in cultured cells.

In this study, we examined the suppressive effects of Kampo formulas on SARS-CoV-2 infection using VeroE6/TMPRSS2 cells, which are highly susceptible to SARS-CoV-2 infection (Matsuyama et al., 2020). We selected eight Kampo formulas that have been shown to be effective against influenza virus infections and common cold symptoms and examined their inhibitory effects on SARS-CoV-2 infection. We also investigated the inhibitory effects of six crude constituent drugs constituting those Kampo formulas on SARS-CoV-2 infection.

MATERIALS AND METHODS

Cells and Viruses

VeroE6/TMPRSS2 cells [African green monkey kidney-derived cells expressing human TMPRSS2, purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank, JCRB 1819] were propagated in Dulbecco's modified Eagle's minimum essential medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS; Biosera, Kansas City, MO, United States), penicillin G (100 units/ml, Meiji Seika Pharma, Tokyo, Japan), and streptomycin (100 μg/ml, Meiji Seika Pharma). The cells were cultured at 37°C in 5% CO_2. SARS-CoV-2/JP/Hiroshima-46059T/2020 (Yamamotoya et al., 2021; B1.1.1, GISAID accession ID: EPI_ISL_6289932, GenBank/DDBJ/EMBL accession number: MZ853926) was used as the test virus.

To prepare virus suspensions, VeroE6/TMPRSS2 cells were infected with the virus and incubated in DMEM. When cytopathic effects were fully developed, the culture supernatant was harvested and filtered through a 0.45-μm filter after low-speed centrifugation. The virus titer was determined by the standard 50% tissue culture infectious dose ($TCID_{50}$) method. Briefly, a 10-fold serial dilution of the virus was inoculated into cells in a 96-well plate in tetraplicate or octuplicate and incubated for 7 days to check for CPE. Based on this result, infectivity was calculated and expressed as $TCID_{50}$/ml, as described previously (Nomura et al., 2021).

Reagents

Extract powders of Kampo formulas including Maoto, Saikokeishito, Shomakakkonto, Kakkonto, Shoseiryuto, Senkyuchachosan, Bakumondoto, and Hochuekkito were kindly provided by Tsumura & Co. (Tokyo, Japan) (**Table 1**). Crude drugs including *Glycyrrhiza uralensis* Fisch. ex DC [Fabaceae] (Glycyrrhizae radix), *Ephedra sinica* Stapf [Ephedraceae] (Ephedrae herba), *Paeonia lactiflora* Pall [Paeoniaceae] (Paeoniae radix), *Ligusticum officinale* (Makino) Kitag [Apiaceae] (Cnidii rhizoma), *Scutellaria baicalensis* Georgi [Lamiaceae] (Scutellariae radix), and *Bupleurum falcatum* L [Apiaceae] (Bupleuri radix) were purchased from Tsumura & Co. The Kampo formulas used in this study and their constituent crude drugs (generic name, scientific name, and percentage included) are shown in detail in **Table 1**.

Solutions of the Kampo formulas for testing were prepared as described previously (Nomura et al., 2019). The powder of each Kampo formula was mixed with DMEM to a concentration of 20 mg/ml. The powder was dissolved at 50°C for 1 h and the mixture was centrifuged at a low speed and then the supernatant was filter-sterilized through a 0.22-μm filter. The crude drugs were prepared in a similar way. For both the Kampo formulas and the crude drugs, little insoluble material was found after low-speed centrifugation, and the weight of the initial powder was therefore used as the weight of the solute.

Cytotoxicity Assay

VeroE6/TMPRSS2 cells were cultured in DMEM with the specified concentrations of reagents for 24 h, and lactate dehydrogenase (LDH), which was released from the cells into the medium, was assayed with a colorimetric method using the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) by measuring absorbance at 490 nm in the TriStar LB 941 plate reader (Bertohold Technologies, Wildbad, Germany). The cytotoxicity of the reagents was calculated from the absorbance measurements as 100% for the high control (cell lysis by surfactant) and 0% for the low control (culture medium only).

Replication of SARS-CoV-2 *in vitro*

Confluent monolayers of VeroE6/TMPRSS2 cells in a 96-well plate were infected with 50 μl/well of the virus at an input multiplicity of infection (m.o.i.) of 0.05 or 10. After adsorption for 2 h, the inoculated viruses were removed, and the cells were further cultured in 100 μl/well of DMEM containing different concentrations of Kampo formulas or crude drugs. The conditions of m.o.i. and virus adsorption time were based on the conditions used in a previous study for effective infection of

TABLE 1 | The Kampo medicines used in this study and their constituent crude drugs.

Maoto									
Indications	Common cold, Influenza (Acute), Rheumatoid arthritis								
Crude drugs (Latin name)	Armeniacae semen			Ephedrae herba[a]			Cinnamomi cortex	Glycyrrhizae radix*	
Scientific name	*Prunus armeniaca var. armeniaca [Rosaceae] Prunus armeniaca* L. *[Rosaceae]*			*Ephedra sinica* Stapf *[Ephedraceae]*			*Neolitsea cassia* (L.) *Kosterm. [Lauraceae]*	*Glycyrrhiza uralensis* Fisch. ex DC. *[Fabaceae]*	
Rate of crude drugs configuring Kampo extracts (excluding additives)	32.3%			32.3%			25.8%	9.7%	
Saikokeishito									
Indications	Common cold, Influenza, Feverish diseases such as pneumonia and pulmonary tuberculosis Gastric ulcer, Duodenal ulcer, Cholecystitis, Gallstone, Pain in liver dysfunction and pancreatitis								
Crude drugs (Latin name)	Bupleuri radix[a]	Pinelliae Tuber	Scutellariae radix[a]	Glycyrrhizae radix[a]	Cinnamomi cortex	Paeoniae radix[a]	Ziziphi fructus	Ginseng radix	Zingiberis rhizoma
Scientific name	*Bupleurum falcatum* L. *[Apiaceae]*	*Pinellia ternata (*Thunb.) Makino *[Araceae]*	*Scutellaria baicalensis* Georgi *[Lamiaceae]*	*Glycyrrhiza uralensis* Fisch. ex DC. *[Fabaceae]*	*Neolitsea cassia* (L.) Kosterm. *[Lauraceae]*	*Paeonia lactiflora* Pall. *[Paeoniaceae]*	*Ziziphus jujuba* Mill. *[Rhamnaceae]*	*Panax ginseng* C.A.Mey. *[Araliaceae]*	*Zingiber officinale* Roscoe *[Zingiberaceae]* —
Rate of crude drugs configuring Kampo extracts (excluding additives)	22.7%	18.2%	9.1%	9.1%	9.1%	9.1%	9.1%	9.1%	4.6% —
Kakkonto									
Indications	Common cold, Early stage of febrile disease, Inflammatory diseases (conjunctivitis, keratitis, otitis media, tonsillitis, mastitis, lymphadenitis) Stiff shoulders, Neuralgia in the upper body, Urticaria								
Crude drugs (Latin name)	Puerariae Radix	Ziziphi fructus	Ephedrae herba[a]	Glycyrrhizae radix[a]	Cinnamomi cortex	Paeoniae radix[a]	Zingiberis rhizoma		
Scientific name	*Pueraria montana var. lobata* (Willd.) Maesen & S.M.Almeida ex Sanjappa & Predeep *[Fabaceae]*	*Ziziphus jujuba* Mill. *[Rhamnaceae]*	*Ephedra sinica i [Ephedraceae]*	*Glycyrrhiza uralensis* Fisch. ex DC. *[Fabaceae]*	*Neolitsea cassia* (L.) Kosterm. *[Lauraceae]*	*Paeonia lactiflora* Pall. *[Paeoniaceae]*	*Zingiber officinale* Roscoe *[Zingiberaceae]*		
Rate of crude drugs configuring Kampo extracts (excluding additives)	22.2%	16.7%	16.7%	11.1%	11.1%	11.1%	11.1%		
Shomakakkonto									
Indications	Common cold (early stage), Dermatitis								
Crude drugs (Latin name)	Puerariae Radix	Paeoniae radix[a]	Cimicifugae Rhizoma	Glycyrrhizae radix[a]	Zingiberis rhizoma				

(Continued on following page)

TABLE 1 | (*Continued*) The Kampo medicines used in this study and their constituent crude drugs.

Scientific name	*Pueraria montana var. lobata* (Willd.) Maesen & S.M.Almeida ex Sanjappa & Predeep *[Fabaceae]*	*Paeonia* lactiflora Pall. [Paeoniaceae]	*Actaea dahurica (*Turcz. ex Fisch. & C.A.Mey.*) Franch. [Ranunculaceae]*· *Actaea heracleifolia* (Kom.) *J.Compton [Ranunculaceae]*· *Actaea cimicifuga* L. *[Ranunculaceae]*			*Glycyrrhiza uralensis* Fisch. ex DC. *[Fabaceae*]		*Zingiber officinale* Roscoe *[Zingiberaceae]*	
Rate of crude drugs configuring Kampo extracts (excluding additives)	41.7%	25.0%	16.7%			12.5%		4.2%	
Shoseiryuto									
Indications	Common cold, Bronchitis, Bronchial asthma, Rhinitis, Allergic Rhinitis, Allergic conjunctivitis								
Crude drugs (Latin name)	Pinelliae tuber	Paeoniae radix[a]	Zingiberid rhizoma processum	Glycyrrhizae radix[a]	Cinnamomi cortex	Asiasari radix	Schisandrae fructus	Ephedrae herba[a]	
Scientific name	*Pinellia ternata* (Thunb.) Makino *[Araceae]*	*Paeonia* lactiflora Pall. [Paeoniaceae]	*Zingiber officinale* Roscoe *[Zingiberaceae]*	Glycyrrhiza uralensis Fisch. ex DC. [Fabaceae]	*Neolitsea cassia* (L.) Kosterm. [Lauraceae]	*Asarum heterotropoides* F.Schmidt [Aristolochiaceae]	*Schisandra chinensis* (Turcz.) Baill. [Schisandraceae]	*Ephedra sinica* Stapf [Ephedraceae]	
Rate of crude drugs configuring Kampo extracts (excluding additives)	22.2%	11.1%	11.1%	11.1%	11.1%	11.1%	11.1%	11.1%	
Senkyuchachosan									
Indications	Common cold, automatic imbalance syndrome peculiar to women resembling climacteric disturbance, and headache								
Crude drugs (Latin name)	Cyperi rhizoma	Cnidii rhizoma[a]	Notopterygii rhizoma	Schizonepetae spica	Menthae herba	Angelicae dahuricae radix	Saposhnikoviae radix	Glycyrrhizae radix[a]	Camelliae sinensis folium
Scientific name	*Cyperus rotundus* L. *[Cyperaceae]*	*Ligusticum officinale (*Makino) Kitag. *[Apiaceae]*	*Hansenia weberbaueriana (*Fedde ex H.Wolff*)* Pimenov & Kljuykov *[Apiaceae]*	*Nepeta tenuifolia* Benth. *[Lamiaceae]*	*Mentha canadensis* L. *[Lamiaceae]*	*Angelica dahurica* (Hoffm.) Benth. & Hook.*f. ex* Franch*. & Sav. [Apiaceae]*	*Saposhnikovia divaricata (*Turcz. ex Ledeb.) Schischk. *[Apiaceae]*	*Glycyrrhiza uralensis* Fisch. ex DC. *[Fabaceae]*	— *Camellia sinensis (L.) Kuntze [Theaceae]*
Rate of crude drugs configuring Kampo extracts (excluding additives)	20.0%	15.0%	10.0%	10.0%	10.0%	10.0%	10.0%	7.5%	7.5%
Bakumondoto									
Indications	Coughing with a hard, Obstructive sputum, Bronchitis, and bronchial asthma								
Crude drugs (Latin name)	Ophiopogonis radix	Oryzae fructus	Pinelliae tuber	Ziziphi fructus	Glycyrrhizae radix[a]	Ginseng radix			

(Continued on following page)

TABLE 1 | (*Continued*) The Kampo medicines used in this study and their constituent crude drugs.

Scientific name	Ophiopogon japonicus (Thunb.) Ker Gawl. [Asparagaceae]	Oryza sativa L. [Poaceae]	*Pinellia ternata (Thunb.) Makino [Araceae]*	Ziziphus jujuba Mill. [Rhamnaceae]	Glycyrrhiza uralensis Fisch. ex DC. [Fabaceae]	*Panax ginseng C.A.Mey. [Araliaceae]*				
Rate of crude drugs configuring Kampo extracts (excluding additives)	37.0%	18.5%	18.5%	11.1%	7.4%	7.4%				
Hochuekkito										
Indications	Common cold, Summer thinness, Loss of appetite, Gastroptosis, Hemorrhoids, Anal prolapse, Drooping uterus, Pubic atrophy, Hyperhidrosis									
Crude drugs (Latin name)	Astragali radix	Atractylodis Lanceae Rhizoma	Ginseng radix	Angelicae acutilobae radix	Bupleuri radix[a]	Ziziphi fructus	Aurantii nobilis pericarpium	Glycyrrhizae radix[a]	Cimicifugae Rhizoma	Zingiberis rhizoma
Scientific name	*Astragalus mongholicus* Bunge [Fabaceae]	*Atractylodes lancea* (Thunb.) DC. [*Asteraceae*]	*Panax ginseng* C.A.Mey. *[Araliaceae]*	*Angelica acutiloba (Siebold & Zucc.)* Kitag. *[Apiaceae]*	*Bupleurum falcatum* L. *[Apiaceae]*	*Ziziphus jujuba* Mill. *[Rhamnaceae]*	*Citrus deliciosa* Ten. *[Rutaceae]*	*Glycyrrhiza uralensis Fisch.* ex DC. *[Fabaceae]*	*Actaea dahurica (*Turcz. ex Fisch. & C.A.Mey.*) Franch. [Ranunculaceae]· Actaea heracleifolia (*Kom.*) J.Compton [Ranunculaceae]· Actaea cimicifuga* L. *[Ranunculaceae]*	*Zingiber officinale Roscoe [Zingiberaceae]*
Rate of crude drugs configuring Kampo extracts (excluding additives)	16.7%	16.7%	16.7%	12.5%	8.3%	8.3%	8.3%	6.3%	4.2%	2.1%

Kampo medicines used in this study and their constituent crude drugs are shown with indications of the Kampo medicines. The ratio of constituents included in Kampo drugs of extract powder and crude drugs referred to the website of Tsumura & Co.

[a]*The crude drugs investigated are marked.*

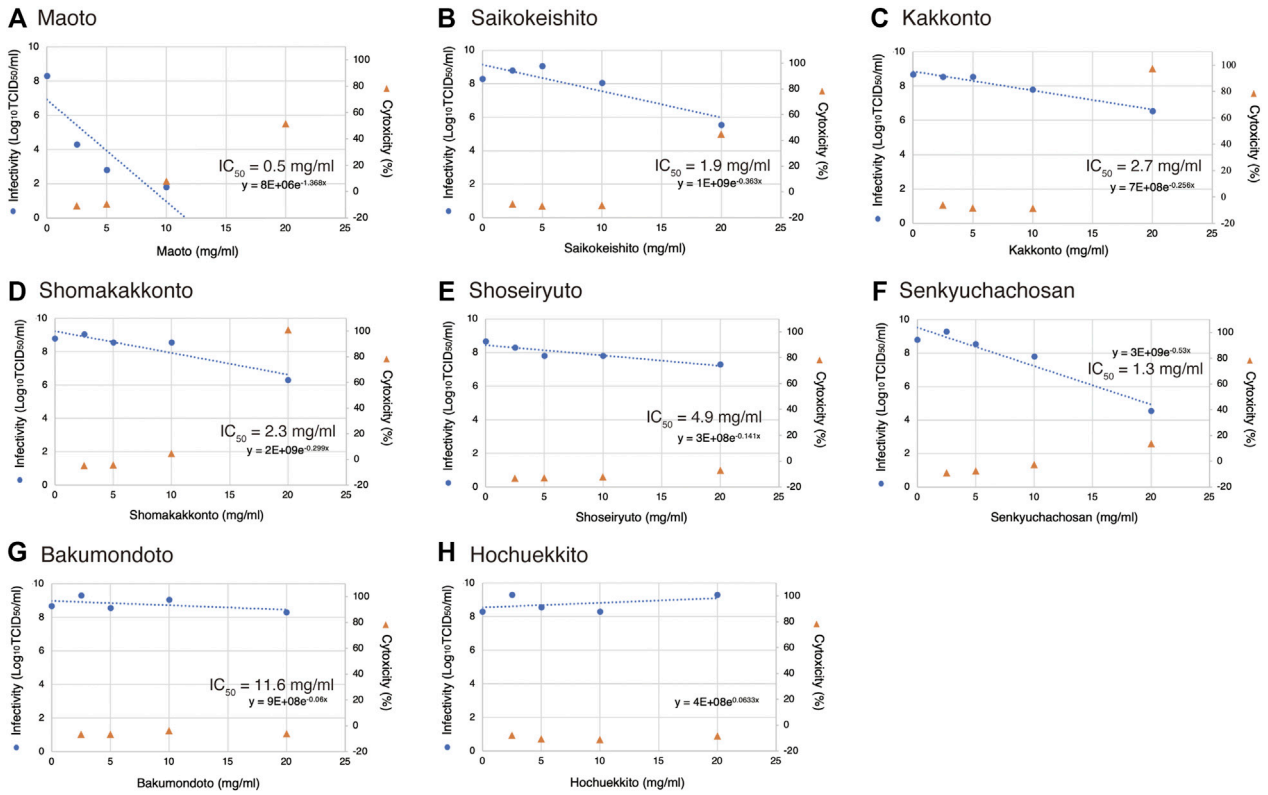

FIGURE 1 | Inhibition of SARS-CoV-2 replication by Kampo formulas. **(A-H)** The Kampo formula used is noted at the top of each panel. VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 at an m.o.i. of 0.05. After 2-h adsorption, the inoculum was removed and the cells were cultured in DMEM containing different concentrations of Kampo formulas for 24 h. The viral infectivity in the medium was assayed by the $TCID_{50}$ method. The left y-axis of the graph is the infectivity for each concentration. An exponential approximation was made by Excel to calculate the drug concentration that reduces the infectivity in the absence of a drug to 50% (IC_{50}). The IC_{50} values and approximation equations are shown in the graph. If there is no decrease, the IC_{50} is not shown (Panel 1). VeroE6/TMPRSS2 cells were incubated in DMEM supplemented with the designated concentrations of a Kampo formula for 24 h, and LDH values in the media were then measured to evaluate cytotoxicity. The LDH value from detergent-treated cells was set at 100%, and the right y-axis is the percent inhibition of cytotoxicity for each concentration.

cells (Wang et al., 2020). The medium was harvested after 24 h, and viral infectivity was assayed by the $TCID_{50}$ method. The logarithm of infectivity titer and reagent concentration in the medium were plotted and an approximation straight-line was drawn to calculate the 50% inhibitory concentration (IC_{50}), as described previously (Nomura et al., 2021).

We conducted infection experiments under two conditions: m o.i. of 0.05 and m.o.i. of 10. At m.o.i. of 10, all cells are infected at once, allowing us to observe the process of virus entry and replication (one-step replication). When m.o.i. of 0.05, on the other hand, 20 cells are infected with a single virus. In addition to the entry and replication of the virus into the cell, the progeny virus is released from the cell and further infects the surrounding cells (multi-step replication).

Assay for Inactivation of Viral Particles

For the Kampo formulas, the solution was mixed with 90 µl of the drug at a concentration of 20 mg/ml and 10 µl of the virus solution at 2.0×10^9 $TCID_{50}$/ml and incubated for 3 min at room temperature. The mixture was then serially diluted 10-fold in DMEM, and adsorbed on VeroE6/TMPRSS cells for 1 h. The inoculum was removed, and cell culture medium was added and incubated for 7 days. The infectivity of the solution was determined by the $TCID_{50}$ method. For crude drugs, reagents at 1.25–10 mg/ml concentrations were used considering their cytotoxicity. Phosphate-buffered saline (PBS) was used as an untreated control, and 70% (w/w) ethanol was used as an inactivation control as described previously (Nomura et al., 2021).

RESULTS

Suppressive Effects of Kampo Formulas on SARS-CoV-2 Infection

Eight Kampo formulas used to treat respiratory symptoms, including respiratory symptoms of influenza and common cold infections, were investigated for their inhibitory effects on SARS-CoV-2 infection *in vitro*. **Table 1** shows the clinical indications of each Kampo formula, the crude drugs included, and the proportions of the crude drugs.

Initially, the cytotoxicity of each Kampo formula was examined by an LDH assay, and the values were plotted on a graph (**Figure 1**, △ marker, right Y-axis). Twenty mg/ml of each Kampo formula of Maoto (**Figure 1A**), Saikokeishito (**Figure 1B**), Kakkonto (**Figure 1C**), and Shomakakkonto (**Figure 1D**) showed more than

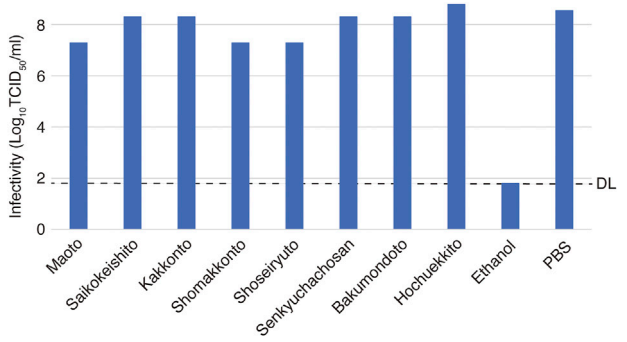

FIGURE 2 | Effects of Kampo formulas on virus particle inactivation. A solution of the Kampo formula (20 mg/ml, 90 µl) and 10 µl of the virus solution at 2.0×10^9 $TCID_{50}$/ml was incubated for 3 min at room temperature. The mixture was then serially diluted 10-fold in DMEM, and the infectivity was determined by the $TCID_{50}$ method. Phosphate-buffered saline (PBS) was used as an untreated control, and ethanol [70% (w/w)] was used as an inactivation control. The dotted line indicates the detection limit (DL) of the infectivity assay.

30% cytotoxicity. However, at concentrations below 10 mg/ml, none of the Kampo formulas showed apparent cytotoxicity. The results at concentrations below 10 mg/ml could be interpreted without considering the effect of cytotoxicity, while at 20 mg/ml, the results should be interpreted with caution.

VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 at an m.o.i. of 0.05, and each Kampo formula was added to the medium. After 24 h, the infection titer was measured by the $TCID_{50}$ method and plotted on a graph against the concentration of the Kampo formula (**Figure 1**, ○ marker, left Y-axis). In the case of Maoto, viral replication was inhibited as the concentration was increased and was almost completely inhibited at a concentration of 10 mg/ml (**Figure 1A**). An approximate line of these points was drawn, and the equation is shown in the graph. The 50% inhibitory concentration (IC_{50}) that was calculated from the equation is also shown in the graph (**Figure 1A**). In the case of Hochuekkito, there was little change even when the drug concentration was increased (**Figure 1H**). According to the IC_{50} data shown in **Figure 1**, both Maoto and Senkyuchachosan had strong suppressive effects on SARS-CoV-2 infection. On the other hand, no suppressive effects of Bakumondo and Hochuekkito on virus infection were observed.

The cytotoxicity of Saikokeishito at 20 mg/ml was 44.7%, and we cannot deny the possibility that its suppressive effect on virus infection is due to its cytotoxicity. Since Kakkonto and Maoto showed even higher cytotoxicity at a concentration of 20 mg/ml, it was difficult to clearly determine their suppressive effects on virus infection at that concentration. Still, it can be considered that they have clear suppressive effects on virus infection at concentrations below 10 mg/ml.

When the highest concentration (20 mg/ml) of each Kampo formula was mixed with the virus and the infectious titer was measured, none of the Kampo formulas decreased the infectious titer. This suggests that there was no direct inactivating effect of each Kampo formula on the virus particles (**Figure 2**).

In this experiment, the virus was inoculated into cells at an m.o.i. of 0.05. If the virus is inhibited by the Kampo formula, then the virus may be suppressed at one of the following stages: intracellular multiplication, release from the cell, or reinfection of neighboring cells. From the results of the experiment described above (**Figure 2**), it is unlikely that the viral particles are directly inactivated, and intracellular proliferation or release from the cell may therefore be impaired.

Suppressive Effects of Crude Drugs on SARS-CoV-2 Infection

We investigated the suppressive effects on virus infection of the available crude drugs with high percentages of composition among the crude drugs constituting Maoto, Senkyuchachosan, and Saikokeishito, which had strong inhibitory effects on SARS-CoV-2 infection. The crude drugs tested were Ephedrae herba, Glycyrrhizae radix, Scutellariae radix, Paeoniae radix, Bupleuri radix, and Cnidii rhizoma (**Figure 3**). Ephedrae herba showed cytotoxicity at concentration of 2.5 mg/ml and above, Bupleuri radix showed cytotoxicity at concentrations of 10 mg/ml and above, and Scutellariae radix showed cytotoxicity at concentrations of 5 mg/ml and above. Therefore, the effects of these crude drugs at lower concentrations were evaluated.

The cells were infected with the virus at an m.o.i. of 0.05, and the infectious titer was measured by the $TCID_{50}$ method (**Figure 3**). Ephedrae herba showed a strong inhibitory effect on virus infection, while Paeoniae radix, Scutellariae radix, and Glycyrrhizae radix showed weaker inhibitory effects (**Figure 3**).

To investigate the mechanisms by which the crude drugs suppress virus infection, we conducted infection experiments using crude drugs (Ephedrae herba, Scutellariae radix, Paeoniae radix, Glycyrrhizae radix) at an m.o.i. of 10 so that all of the cells would be infected (**Figure 3**). Ephedrae herba showed a strong suppressive effect on virus infection even at an m.o.i. of 10, and Paeoniae radix and Scutellariae radix also showed suppressive effects (**Figure 3**). These results suggest that Ephedrae herba, Paeoniae radix, and Scutellariae radix act on virus-infected cells to inhibit viral replication in the cells. On the other hand, Glycyrrhizae radix showed little inhibitory effect in the infection experiment with an m.o.i. of 10 (**Figure 3**), suggesting that the antiviral effect of Glycyrrhizae radix is due to its inhibition of spread of the virus to neighboring uninfected cells.

The inactivating effect of each of the crude drugs on virus particles was investigated by mixing the crude drug at the highest concentration used in the virus infection experiment and measuring the infectivity titer (**Figure 4**). Ephedrae herba decreased the infections titer by 2.5 Log_{10} ($TCID_{50}$/ml) compared to the control, suggesting that Ephedrae herba may act directly on the virus particles to inactivate them. The other crude drugs did not reduce the infectious titer, indicating that they did not inactivate the virus particles (**Figure 4**).

Contribution of Epherae Herba in Kampo Formulas

In this study, we found that Ephedrae herba has a strong inhibitory effect on SARS-CoV-2 infection. Ephedrae herba is found in several

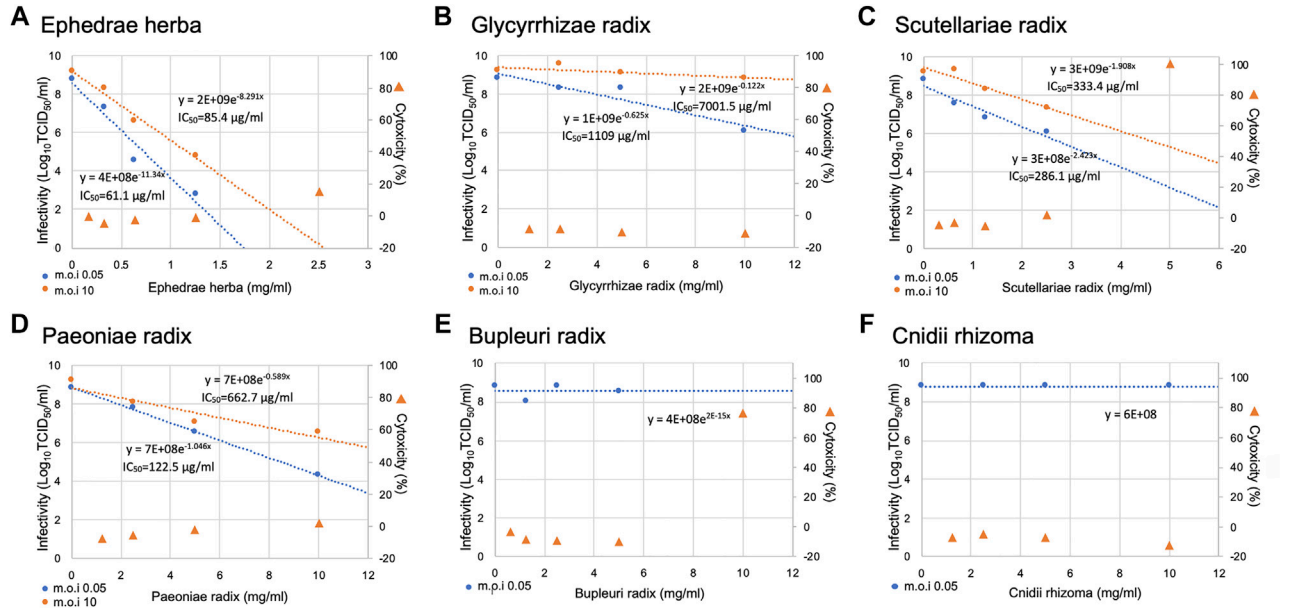

FIGURE 3 | Inhibition of SARS-CoV-2 replication by crude drugs. **(A-F)** The crude drug used is noted at the top of each panel. VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 at an m.o.i. of 0.05 or 10. After 2-h adsorption, the inoculum was removed and the cells were cultured in DMEM containing different concentrations of crude drugs for 24 h. The viral infectivity in the medium was assayed by the $TCID_{50}$ method. The left y-axis of the graph is the infectivity for each concentration. An exponential or linear approximation was made by Excel to calculate the drug concentration that reduces the infectivity in the absence of a drug to 50% (IC_{50}). The IC_{50} values and approximation equations are shown in the graph. If there is little or no decrease, the IC_{50} is not shown **(E,F)**. VeroE6/TMPRSS2 cells were incubated in DMEM supplemented with the designated concentrations of a Kampo fomula for 24 h, and LDH values in the media were then measured to evaluate cytotoxicity. The LDH value from detergent-treated cells was set at 100%, and the right y-axis is the percent inhibition of cytotoxicity for each concentration.

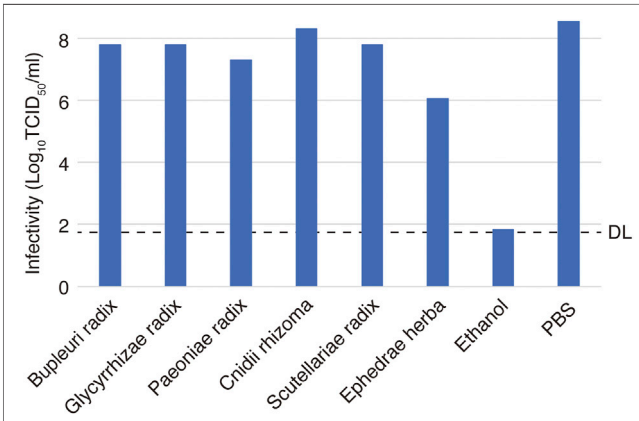

FIGURE 4 | Effects of crude drugs on virus particle inactivation. A solution of a crude drug (90 μl) and 10 μl of the virus solution at 2.0×10^9 $TCID_{50}$/ml was incubated for 3 min at room temperature. Reagents at 1.25–10 mg/ml concentrations were used considering their cytotoxicity. The mixture was then serially diluted 10-fold in DMEM, and the infectivity was determined by the $TCID_{50}$ method. Phosphate-buffered saline (PBS) was used as an untreated control, and ethanol [70% (w/w)] was used as an inactivation control. The dotted line indicates the detection limit (DL) of the infectivity assay.

Kampo formulas, and to verify the role of Ephedrae herba in inactivation of SARS-CoV-2 by the herbal medicines, we recalculated the effect of Ephedrae herba on SARS-CoV-2 based on the amount of Ephedrae herba in each Kampo formula.

The IC_{50} value of Ephedrae herba alone was 61.1 μg/ml (**Figures 3A**, **5A**). Maoto contains 32.3% of Ephedrae herba (**Table 1**), and the IC_{50} value was calculated by plotting the amount of Ephedrae herba in Maoto on the horizontal axis of the graph (**Figure 5B**) to be 163.6 μg/ml (**Figure 5B**). Thus, although it should be the same amount of Ephedrae herba, Ephedrae herba in the form of Maoto was weakened, suggesting that other components of Maoto may be inhibiting the effect Ephedrae herba. The IC_{50} value of Maoto itself was calculated to be 0.5 mg/ml (**Figure 1A**), being consistent with this hypothesis.

Similarly, in the case of Kakkonto containing 16.7% of Ephedrae herba (**Table 1**), the IC_{50} value was calculated to be as high as 517.3 μg/ml based on the amount of Ephedrae herba (**Figure 5C**). The IC_{50} value of Kakkonto itself was 2.7 mg/ml (**Figure 1C**). Shoseiryuto contained 11.1% of Ephedrae herba (**Table 1**), and the IC_{50} value of Ephedrae herba was as high as 450.4 μg/ml based on Ephedrae herba (**Figure 5D**). The IC_{50} value of Shoseiryuto itself was 4.9 mg/ml (**Figure 1E**). These results suggest that the inhibitory effect of Ephedrae herba in the Kampo formulas on SARS-CoV-2 infection is weakened by other components.

DISCUSSION

Kampo formulas consist of various combinations of crude drugs, traditionally regarded as units, and are often used on the base of ancient experience (Tanaka et al., 1995; Odaguchi et al., 2019). In Japan, Kampo formulas are covered by the national health

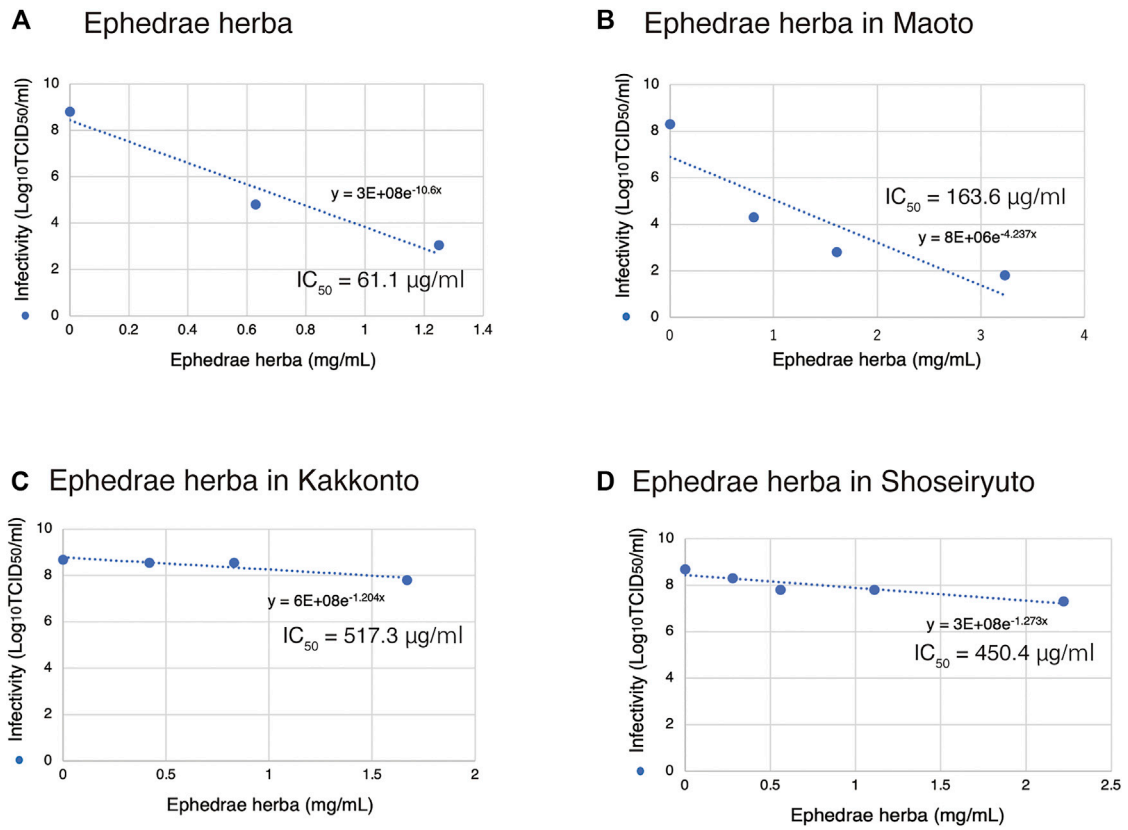

FIGURE 5 | Inhibition of SARS-CoV-2 replication based on the amount of Ephedrae herba contained. **(A-D)** The origin of Ephedra herba is noted at the top of each panel. The results for growth inhibition of SARS-CoV-2 by Ephedrae herba and Kampo formulas containing Ephedrae herba (**Figure 1**) are re-plotted in a graph based on the amount of Ephedrae herba contained. An exponential approximation was made by Excel to calculate the drug concentration that reduces the infectivity in the absence of a drug to 50% (IC_{50}). The IC_{50} values and approximation equations are shown in the graph.

insurance system and are prescribed by doctors under the condition of government subsidies. Some Kampo formulas are also available as over-the-counter drugs that can be purchased at pharmacies without a doctor's prescription. The extensive experience of using Kampo formulas in Japan has led to their safe and easy use in medical practice. In recent years, scientific analysis of the effects of Kampo formulas has provided evidence of their clinical benefits, and their clinical usefulness for the treatment of COVID-19 has also been suggested (Motoo et al., 2014; Takayama et al., 2021).

Some advantages of Kampo formulas are that they can be taken orally, are relatively inexpensive, and can be taken at an early stage. For example, Maoto, one of the Kampo formulas examined in this study, can be used to treat common colds in infants even before the onset of cold symptoms such as fever and nasal discharge (Kubo and Nishimura, 2007; Nagai et al., 2014). On the other hand, antivirals, as shown in clinical trials for influenza (Muthuri et al., 2014; Dobson et al., 2015), need to be taken after the onset of illness and before the peak of viral replication. The clinical course of COVID-19 is longer than that of influenza, and viral replication in patients with COVID-19 likely to continue for a long time. Although existing antivirals such as remdesivir may be effective if treatment with the antivirals started after the diagnosis of COVID-19, consideration should be given to the use of Kampo formulas, which can be administered earlier.

Kampo formulas that are used for the treatment of influenza have been reported to inhibit the growth of influenza viruses in cultured cells (Mantani et al., 1999; Nomura et al., 2019; Hou et al., 2020). Although the relationship between inhibition of influenza virus replication in cultured cells and clinical efficacy is not entirely clear, the therapeutic effect of Kampo formulas may be elicited by suppression of the ability of the virus of replicate in the human respiratory tract. In addition, Lian hua qing wen, which contains Ephedrae herba and is one of the therapeutic agents used for influenza viruses in traditional Chinese medicine, has been shown i*n vitro* experiments to have an inhibitory effect on SARS-CoV-2 infection and has also been sown to be effective for COVID-19 in a clinical setting, (Su et al., 2020; Hu et al., 2021). Therefore, we investigated the effects of Kampo formulas on SARS-CoV-2 infection *in vitro* experiments.

Since commercial Kampo formulas contain additives and the types and amounts of additives are not uniform, we used the same concentrations of Kampo formulas without additives in this study. We found that virus infection was inhibited by

the Kampo formulas Maoto, Saikokeishito, and Senkyuchachosan but not by the Kampo formulas Bakumondoto and Hochuekkito. We also examined the viral inhibitory effects of crude drugs as constituents of the Kampo formulas that had strong inhibitory effects on SARS-CoV-2 infection. Ephedrae herba had the strongest inhibitory effect on SARS-CoV-2 infection. Among the six crude drugs examined, Ephedrae herba, Paeoniae radix, Scutellariae radix, and Glycyrrhizae radix had the strongest inhibitory effects in that order.

Ephedrae herba showed direct inactivating effects on virus particles as well as on infected cells. Previous studies have shown that tannins, which are components of Ephedrae herba, inhibit influenza virus infection (Mantani et al., 1999) and that tannins extracted from plants such as persimmon have inactivating effects on various viruses (Ueda et al., 2013). In the present study, it was also thought that tannins in Ephedrae herba may have direct inhibitory effects on virus infection. However, in the infection experiments to investigate the suppressive effects of crude drugs, on virus infection, it was found that Ephedra herba had a suppressive effect even under the condition of an m.o.i. of 10, in which all cells were infected at once, and no virus particle formation process was involved. Therefore, Ephedrae herba may have both a direct inactivation effect on virus particles and a suppressive effect on infection of cells, suggesting a combined virus suppression mechanism.

However, it was found that Kampo formulas containing Ephedrae herba had little direct inactivation effect on virus particles. The contents of Ephedrae herba in the Kampo formulas used in the virus direct inactivation test (20 mg/ml) were 6.45 mg/ml in Maoto, 3.33 mg/ml in Kakkonto, and 2.22 mg/ml in Shoseiryuto. These concentrations were higher than the concentration of Ephedrae herba tested as a crude drug (1.25 mg/ml), which was found to be effective in the virus particle direct inactivation test. Furthermore, when the IC_{50} values of Kampo formulas containing Ephedrae herba and Ephedrae herba alone were compared on the basis of the content of Ephedrae herba, the effects of all Kampo formulas were weaker than the effect of Ephedrae herba alone. These results suggest that the combination of Ephedrae herba with other crude drugs in Kampo formulas has a weaker antiviral effect than that of Ephedrae herba. Ephedra herba has various side effects, and Kampo formulas are shown to contain crude drugs that alleviate the side effects of the strong Ephedra herba (Odaguchi et al., 2019). The reduced suppressive effect of Ephedra herba on virus infection may mean that its side effects on the human body are reduced.

Among the Kampo formulas that do not contain Ephedrae herba, Saikokeishito and Senkyuchachosan showed efficacy. These Kampo formulas have been reported to inhibit influenza virus *in vitro* (Shirayama et al., 2016; Nomura et al., 2019), and the Ministry of Health, Labour, and Welfare has approved Saikokeishito for treatment of influenza. Therefore, it would not be surprising if these Kampo formulas also have inhibitory effects on SARS-CoV-2 infection. Furthermore, viral inhibitory effects of Glycyrrhizae radix, Paeoniae radix, and Scutellariae radix as crude drugs contained in these Ephedrae herba-free Kampo formulas were confirmed. It was shown in an *in vitro* study that rhizoma had an inhibitory effect on influenza virus infecion (Nomura et al., 2019), but it had no inhibitory effect on SARS-CoV-2 infection.

Glycyrrhizae radix is a crude drug in many Kampo formulas. All of the Kampo formulas used in this study contained Glycyrrhizae radix (Maoto: 9.7%, Saikokeishito: 9.1%, Kakkonto: 11%, Shomakakkonto: 12.5%, Shoseiryuto: 11%, Senkyuchachosan: 7.5%, Bakumondoto: 7.4%, Hochuekkito: 6.3%; **Table 1**). Although a suppressive effect of Glycyrrhizae radix alone on SARS-CoV-2 infection was shown in a previous study (van de Sand et al., 2021), not all of the Kampo formulas containing Glycyrrhizae radix showed suppressive effects on virus infection, and some Kampo formulas showed no effect at all. Therefore, other crude drug components in the Kampo formulas may weaken the effect of Glycyrrhizae radix. Alternatively, the amount of Glycyrrhizae radix in the Kampo formulas may have been too small to reach the threshold for suppression of virus infection.

Paeoniae radix was suggested to be effective against SARS-CoV-2 infection by molecular modeling predictions (Ma et al., 2020). It was also shown to be effective against influenza virus infection in cell infection experiments (Ho et al., 2014). Scutellariae radix is one of the crude drugs contained in Saikokeishito, which was effective against SARS-CoV-2 infection. In addition, Baicalin, a component of Scutellariae radix, has been shown to have an inhibitory effect on SARS-CoV-2 infection in cell and animal experiments (Song et al., 2021). In the present study, Glycyrrhizae radix, Paeoniae radix, and Scutellariae radix showed inhibitory effects on SARS-CoV-2 infection, but their effects were inferior to the effect of Ephedrae herba.

CONCLUSION

We performed *in vitro* experiments to determine the inhibitory effects on SARS-CoV-2 infection of Kampo formulas and their constituent crude drugs that are used to treat respiratory symptoms including influenza and common cold symptoms. We found that Maoto among the Kampo formulas and Ephedrae herba among the crude drugs had the strongest inhibitory effects on SARS-CoV-2 infection. Some other Kampo formulas and crude drugs also showed suppressive effects on virus infection. Although further analysis and evidence are needed, Kampo formulas might contribute to the treatment of COVID-19.

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

MK, TNo and TNa. developed the study design, performed the experiments, and analyzed the data. HK, KO-O, HO and TS provided assistance in developing the study design and experiments. MK, TNo, TNa and TS wrote the manuscript. KK, KO-O, HO and MI provided advice on interpretation of data and revised the manuscript. All authors read and approved the manuscript.

FUNDING

This study was supported by the Research Grants for COVID-19 from AMED, Japan (to HO and TS) and a grant from the Government-Academia Collaboration of Hiroshima Prefecture (to HO and TS). This study also received funding from the Sumitomo Mitsui Trust Bank COVID-19 Research Fund (to TS) and the Otsuka Toshimi Scholarship Foundation (to TNa.). The funders were not involved in the study design, collection, analysis, and interpretation of data, writing of this article, or the decision to submit it for publication. All authors declare no other competing interests.

ACKNOWLEDGMENTS

We thank Rie Hirakawa and Chika Masaki for manuscript preparation. We also thank the staff of the Analysis Center of Life Science, Hiroshima University for the use of their facilities.

REFERENCES

Dobson, J., Whitley, R. J., Pocock, S., and Monto, A. S. (2015). Oseltamivir Treatment for Influenza in Adults: a Meta-Analysis of Randomised Controlled Trials. *Lancet* 385, 1729–1737. doi:10.1016/S0140-6736(14)62449-1

Ho, J. Y., Chang, H. W., Lin, C. F., Liu, C. J., Hsieh, C. F., and Horng, J. T. (2014). Characterization of the Anti-influenza Activity of the Chinese Herbal Plant Paeonia Lactiflora. *Viruses* 6, 1861–1875. doi:10.3390/v6041861

Hou, S., Xu, X., Wang, Y., and Yang, Y. (2020). Ephedrannin B Exerts Anti-viral and Anti-inflammatory Properties in BEAS-2B Cells Infected with Respiratory Syncytial Virus. *J. Biosci.* 45, 46. doi:10.1007/s12038-020-0016-y

Hsu, J. (2020). Covid-19: What Now for Remdesivir? *BMJ* 371, m4457. doi:10.1136/bmj.m4457

Hu, K., Guan, W. J., Bi, Y., Zhang, W., Li, L., Zhang, B., et al. (2021). Efficacy and Safety of Lianhuaqingwen Capsules, a Repurposed Chinese Herb, in Patients with Coronavirus Disease 2019: A Multicenter, Prospective, Randomized Controlled Trial. *Phytomedicine* 85, 153242. doi:10.1016/j.phymed.2020.153242

Kubo, T., and Nishimura, H. (2007). Antipyretic Effect of Mao-To, a Japanese Herbal Medicine, for Treatment of Type A Influenza Infection in Children. *Phytomedicine* 14, 96–101. doi:10.1016/j.phymed.2006.09.015

Liu, Y. Arase, N. Kishikawa, J. Hirose, M. Lie, S. Tada, A., et al. (2021). The SARS-CoV-2 Delta Variant Is Poised to Acquire Complete Resistance to Wild-type Spike Vaccines. bioRxiv [Preprint]. Available at: https://www.biorxiv.org/content/10.1101/2021.08.22.457114v1 (Accessed September 8, 2021).

Ma, J., Huo, X. Q., Chen, X., Zhu, W. X., Yao, M. C., Qiao, Y. J., et al. (2020). Study on Screening Potential Traditional Chinese Medicines against 2019-nCoV Based on Mpro and PLP. *Zhongguo Zhong Yao Za Zhi* 45, 1219–1224. (in Chinese). doi:10.19540/j.cnki.cjcmm.20200216.401

Mantani, N., Andoh, T., Kawamata, H., Terasawa, K., and Ochiai, H. (1999). Inhibitory Effect of Ephedrae Herba, an oriental Traditional Medicine, on the Growth of Influenza A/PR/8 Virus in MDCK Cells. *Antivir. Res* 44, 193–200. doi:10.1016/s0166-3542(99)00067-4

Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., et al. (2020). Enhanced Isolation of SARS-CoV-2 by TMPRSS2-Expressing Cells. *Proc. Natl. Acad. Sci. U S A.* 117, 7001–7003. doi:10.1073/pnas.2002589117

Motoo, Y., Arai, I., and Tsutani, K. (2014). Use of Kampo Diagnosis in Randomized Controlled Trials of Kampo Products in Japan: a Systematic Review. *PLoS One* 9, e104422. doi:10.1371/journal.pone.0104422

Muthuri, S. G., Venkatesan, S., Myles, P. R., Leonardi-Bee, J., Al Khuwaitir, T. S., Al Mamun, A., et al. (2014). Effectiveness of Neuraminidase Inhibitors in Reducing Mortality in Patients Admitted to Hospital with Influenza A H1N1pdm09 Virus Infection: a Meta-Analysis of Individual Participant Data. *Lancet Respir. Med.* 2, 395–404. doi:10.1016/S2213-2600(14)70041-4

Nagai, T., Kataoka, E., Aoki, Y., Hokari, R., Kiyohara, H., and Yamada, H. (2014). Alleviative Effects of a Kampo (A Japanese Herbal) Medicine "Maoto (Ma-Huang-Tang)" on the Early Phase of Influenza Virus Infection and its Possible Mode of Action. *Evid. Based Complement. Alternat Med.* 2014, 187036. doi:10.1155/2014/187036

Nomura, T., Fukushi, M., Oda, K., Higashiura, A., Irie, T., and Sakaguchi, T. (2019). Effects of Traditional Kampo Drugs and Their Constituent Crude Drugs on Influenza Virus Replication *In Vitro*: Suppression of Viral Protein Synthesis by Glycyrrhizae Radix. *Evid. Based Complement. Alternat Med.* 2019, 3230906. doi:10.1155/2019/3230906

Nomura, T., Nazmul, T., Yoshimoto, R., Higashiura, A., Oda, K., and Sakaguchi, T. (2021). Ethanol Susceptibility of SARS-CoV-2 and Other Enveloped Viruses. *Biocontrol Sci.* 26, 177–180. doi:10.4265/bio.26.177

Odaguchi, H., Hyuga, S., Sekine, M., Nakamori, S., Takemoto, H., Huang, X., et al. (2019). The Adverse Effects of Ephedra Herb and the Safety of Ephedrine Alkaloids-free Ephedra Herb Extract (EFE). *Yakugaku zasshi* 139, 1417–1425. (in Japanese). doi:10.1248/yakushi.19-00122

Shirayama, R., Shoji, M., Sriwilaijaroen, N., Hiramatsu, H., Suzuki, Y., and Kuzuhara, T. (2016). Inhibition of PA Endonuclease Activity of Influenza Virus RNA Polymerase by Kampo Medicines. *Drug Discov. Ther.* 10, 109–113. doi:10.5582/ddt.2016.01010

Song, J., Zhang, L., Xu, Y., Yang, D., Zhang, L., Yang, S., et al. (2021). The Comprehensive Study on the Therapeutic Effects of Baicalein for the Treatment of COVID-19 *In Vivo* and *In Vitro*. *Biochem. Pharmacol.* 183, 114302. doi:10.1016/j.bcp.2020.114302

STORK (2020). *Standards of Reporting Kampo Products, ver. 4.3*. Department of Pharmacognosy, Phytochemistry and Narcotics (DPPN), National Institute of Health Sciences (NIHS) of Japan and National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN). Available at: http://mpdb.nibiohn.go.jp/stork/index.html.(Accessed September 1, 2021)

Su, H. X., Yao, S., Zhao, W. F., Li, M. J., Liu, J., Shang, W. J., et al. (2020). Anti-SARS-CoV-2 Activities *In Vitro* of Shuanghuanglian Preparations and Bioactive Ingredients. *Acta Pharmacol. Sin* 41 (9), 1167–1177. doi:10.1038/s41401-020-0483-6

Takayama, S., Namiki, T., Odaguchi, H., Arita, R., Hisanaga, A., Mitani, K., et al. (2021). Prevention and Recovery of COVID-19 Patients with Kampo Medicine: Review of Case Reports and Ongoing Clinical Trials. *Front. Pharmacol.* 12, 656246. doi:10.3389/fphar.2021.656246

Tanaka, T., Obha, K., Lawaahara, K., and Sakai, E. (1995). Comparison of the Constituents of Ephedra Herbs from Various Countries on Ephedrine Type Alkaloids. *Nat. Med* 49, 418–424.

Tsumura & Co. (2016). About Kampo. Available at: https://www.tsumura.co.jp/english/kampo/02.htm (Accessed September 1, 2021).

Ueda, K., Kawabata, R., Irie, T., Nakai, Y., Tohya, Y., and Sakaguchi, T. (2013). Inactivation of Pathogenic Viruses by Plant-Derived Tannins: strong Effects of Extracts from Persimmon (Diospyros Kaki) on a Broad Range of Viruses. *PLoS One* 8, e55343. doi:10.1371/journal.pone.0055343

van de Sand, L., Bormann, M., Alt, M., Schipper, L., Heilingloh, C. S., Steinmann, E., et al. (2021). Glycyrrhizin Effectively Inhibits SARS-CoV-2 Replication by Inhibiting the Viral Main Protease. *Viruses* 13, 609. doi:10.3390/v13040609

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

World Health Organization. (2021). WHO Coronavirus (COVID-19) Dashboard. Available at: https://covid19.who.int (Accessed September 1, 2021).

Yamamotoya, T., Nakatsu, Y., Kanna, M., Hasei, S., Ohata, Y., Encinas, J., et al. (2021). Prolyl Isomerase Pin1 Plays an Essential Role in SARS-CoV-2 Proliferation, Indicating its Possibility as a Novel Therapeutic Target. *Sci. Rep.* 11, 18581. doi:10.1038/s41598-021-97972-3

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

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

frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 April 2022
doi: 10.3389/fphar.2022.688074

Kampo Formula-Pattern Models: The Development of 13 New Clinically Useful Standard Abdominal Pattern Models in the Fukushin Simulator

Shuji Yakubo[1], Masaki Baba[1], Hiroshi Odaguchi[2], Akino Wakasugi[2], Mariko Sekine[2], Toshihiko Hanawa[2], Tadamichi Mitsuma[3], Takao Namiki[4], Makoto Arai[5], Shin-Ichi Muramatsu[6], Yutaka Shimada[7] and Naotoshi Shibahara[8]*

[1]Department of Clinical Kampo Medicine, Meiji Pharmaceutical University, Tokyo, Japan, [2]Oriental Medicine Research Center, Kitasato University, Tokyo, Japan, [3]Department of Kampo Medicine, Aizu Medical Center, Fukushima Medical University, Fukushima, Japan, [4]Department of Japanese-Oriental (Kampo) Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan, [5]Department of Kampo Medicine, Tokai University School of Medicine, Kanagawa, Japan, [6]Division of Oriental Medicine, Jichi Medical University, Tochigi, Japan, [7]Department of Japanese Oriental Medicine, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan, [8]Division of Kampo Diagnostics, Institute of Natural Medicine, University of Toyama, Toyama, Japan

OPEN ACCESS

Edited by:
Kenny Kuchta, University Medical Center Göttingen, Germany

Reviewed by:
Ning Wang, The University of Hong Kong, Hong Kong SAR, China
Jong Hoon Ryu, Kyung Hee University, South Korea

***Correspondence:**
Shuji Yakubo
yakubo@my-pharm.ac.jp

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

Received: *30 March 2021*
Accepted: *07 April 2022*
Published: *29 April 2022*

Citation:
Yakubo S, Baba M, Odaguchi H, Wakasugi A, Sekine M, Hanawa T, Mitsuma T, Namiki T, Arai M, Muramatsu S-I, Shimada Y and Shibahara N (2022) Kampo Formula-Pattern Models: The Development of 13 New Clinically Useful Standard Abdominal Pattern Models in the Fukushin Simulator. Front. Pharmacol. 13:688074. doi: 10.3389/fphar.2022.688074

Aim: In Kampo medicine, there exists an important system of diagnosis called Fukushin, or abdominal diagnosis or palpation. By applying pressure to the abdomen of the patient, the physician can gain important information on the patient's physical state and use those indications to choose a suitable Kampo formulation. We have previously developed a Fukushin simulator, a teaching tool that reproduces the important abdominal patterns that doctors will encounter in clinical practice and that has received favourable feedback for students and practitioners. In order to make diagnosis and prescription easier, it is desirable to have matched formula–pattern pairings. The present study aims to develop such pairings.

Methods: With the previously developed models as a foundation, in the present study the production team (two members) used materials such as urethane foam and silicone rubber to build an additional 13 standard abdominal pattern models matched to Kampo herbal formulas commonly used by practitioners in Japan. Subsequently, the evaluation team (the remaining 10 authors) investigated the viability of these models.

Results: The evaluation team determined that abdominal pattern models matched to the following typical Kampo formulas were created successfully: Dai-saiko-To (大柴胡湯), Dai-joki-To (大承気湯), Shigyaku-San (四逆散), Saiko-ka-ryukotsu-borei-To (柴胡加竜骨牡蛎湯), Keishi-bukuryo-Gan (桂枝茯苓丸), Hachimi-jio-Gan (八味地黄丸), Hange-shashin-To (半夏瀉心湯), Sho-saiko-To (小柴胡湯), Hochu-ekki-To (補中益気湯), Sho-kenchu-To (小建中湯), Toki-shakuyaku-San (当帰芍薬散), Ninjin-To (人参湯), and Dai-kenchu-To (大建中湯).

Conclusion: We suggest that these new formula-pattern models can make an important contribution to the standardization of abdominal diagnosis and prescription and to Kampo education.

Keywords: Kampo (traditional Japanese herbal medicine), simulator, abdominal diagnosis, medical education, training, standardisation, abdominal palpation

INTRODUCTION

A diagnostic method favored in Kampo medicine, one that originated in ancient China but has been developed independently in Japan, is called Fukushin, or abdominal palpation (**Figure 1**). It is used in clinical practice with all kinds of conditions. The physician applies gentle pressure to the abdomen of the patient, gauging the resistance presented by the patient both overall and at specific sites, and using that information to derive an abdominal pattern, which the physician uses to decide on a suitable Kampo formulation (Terasawa, 1993; The Japan Society for Oriental Medicine, 2005; Ushiroyama, 2005; Protnikoff and WatanabeYashiro, 2008; Arai et al., 2017).

There have been attempts to evaluate abdominal palpation through various medical tests, and some researchers have even attempted to develop equipment especially for that purpose (Tosa et al., 1982; Arichi et al., 1983; Shintani et al., 1989; Yasaka, 1994; Miyamoto and Okita, 2005; Nishida et al., 2012), but these attempts have generally foundered.

Because it is not possible to communicate with precision the sensations in an experienced practitioner's hands when performing abdominal palpation, it has been established that education in abdominal palpation is challenging. To help that cause, as previously reported, we have developed a Fukushin simulator consisting of a set of models that reproduce the most important abdominal patterns that students will encounter in their future clinical practice (**Figure 2**) (Yakubo et al., 2008).

The simulator includes five models representing overall abdominal strength: the obvious excess model, the slight excess model, the intermediate model, the slight deficiency model, and the obvious deficiency model (**Table 1**) (**Figure 3**) (Yakubo et al., 2013).

Based on the intermediate abdominal strength model, we also developed the Fullness in the chest and hypochondrium model, the Stuffiness and rigidity below the heart model, the Rectus muscle tension model, the Lower abdominal fullness model, and the Lower abdominal numbness model (Yakubo et al., 2015). To the deficiency models, we also added models incorporating an Abdominal fluid congestion system and an Abdominal palpitation system (**Table 2**) (Yakubo et al., 2014a).

Because the abdominal patterns uncovered through abdominal palpation leads to prescriptions of standardized Kampo formulas, we have added new models, which we might call formula-pattern models, targeted at popular formulas, such as the Keishi-bukuryo-Gan (桂枝茯苓丸) and Toki-shakuyaku-San (当帰芍薬散) formula-pattern models, both corresponding to formulas frequently prescribed to women. In this spirit, a new model representing a woman's body incorporating an Oketsu tenderness system was developed (**Figure 4**) (Baba et al., 2019).

The present paper describes recent development work conducted on the foundation of the work described above. We have developed a new version of the simulator that consists of 13 abdominal models, including the recently developed Keishi-bukryo-Gan and Toki-shakuyaku-San formula-pattern models, all of female bodies, representing standard abdominal patterns corresponding to well-known formulas (**Table 3**).

MATERIALS AND METHODS

As shown in **Table 3**, Yakubo and Baba, the production team, created 13 formula-pattern models. These are all female models corresponding to 13 standard formulas, based on the female models previously mentioned.

They first created two models representing obvious excess. For the Dai-saiko-To (大柴胡湯) formula-pattern model, they inserted urethane foam and silicone rubber widely in the hypochondriac and epigastric regions (**Figure 5A**). For the Dai-joki-To (大承気湯) pattern model, they further increased the amount of urethane in the abdomen centered around the umbilical region (**Figure 5B**).

The next three models all represent patterns of slight excess. For the Shigyaku-San (四逆散) formula-pattern model, they inserted urethane foam and silicone rubber in the lower hypochondrial and epigastric regions and to represent the abdominal rectus muscle (**Figure 6A**). For the Saiko-ka-

FIGURE 1 | The Fukushin method. In Fukushin, the physician applies gentle pressure to the abdomen of the patient with knees stretched, guaging the resistance presented by the patient both overall and at specific sites, and using the information to derive an abdominal pattern. Written informed consent was obtained from the individual(s) for the publication of any identifiable image or data included this article.

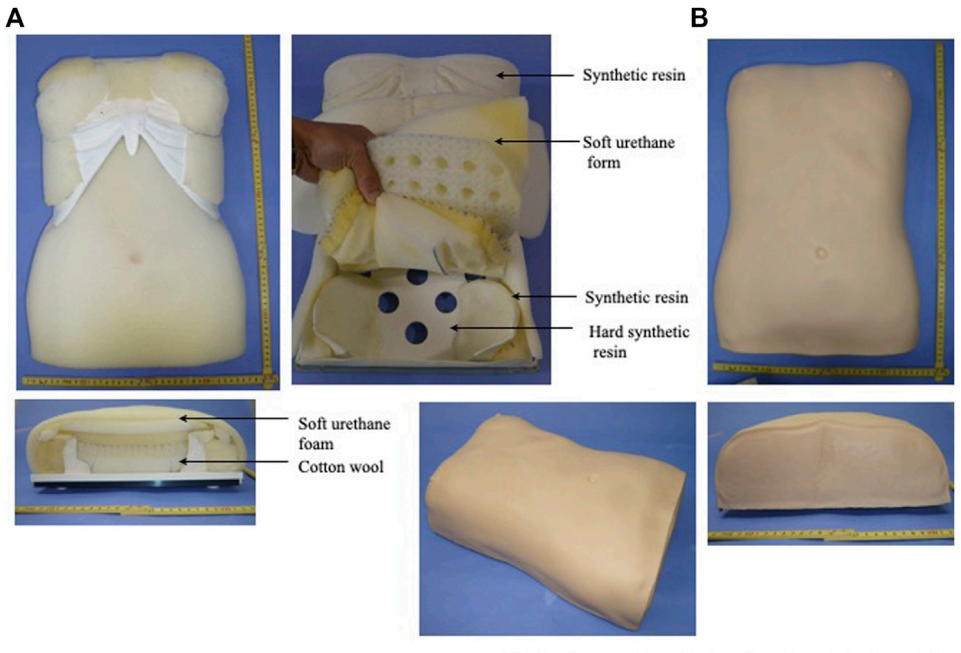

FIGURE 2 | Fukushin simulator female body model. **(A)** Interior of Fukushin simulator female body model. **(B)** Exterior of Fukushin simulator female body model.

TABLE 1 | Abdominal strength pattern models.

Obvious excess abdominal strength pattern model: strongest resistance of abdominal wall and obvious abdominal distension
Slight excess abdominal strength pattern model: somewhat strong resistance of abdominal wall
Intermediate abdominal strength pattern model: neither strong nor weak resistance of abdominal wall
Slight deficiency abdominal strength pattern model: somewhat weak resistance of abdominal wall
Obvious deficiency abdominal strength pattern model: weakest resistance of abdominal wall and retraction of abdomen

ryukotsu-borei-To (柴胡加竜骨牡蛎湯) formula-pattern model, they also inserted urethane foam and silicone rubber in the lower hypochondrial and epigastric regions and added the Abdominal palpitation model previously mentioned (**Figure 6B**). For the Keishi-bukuryo-Gan formula-pattern model, they added urethane foam parts to the lower abdomen and also the previously mentioned Oketsu tenderness system (**Figure 6C**).

The next three models represent intermediate abdominal strength patterns. For the Hachimi-jio-Gan (八味地黄丸) formula-pattern model, they decreased the amount of urethane foam in the midline of the lower abdomen (**Figure 7A**). For the Hange-shashin-To (半夏瀉心湯) formula-pattern model, they added silicone rubber to the urethane foam in the epigastric region (**Figures 7B**, **8A**). For the Sho-saiko-To (小柴胡湯) formula-pattern model, they added silicone rubber to the urethane foam in the lower hypochondrium and the epigastrium (**Figure 7C**).

The next three models represent slight deficiency. For the Hochu-ekki-To (補中益気湯) formula-pattern model, they added silicone rubber to the urethane rubber in the lower hypochondriac region (**Figures 8B**, **9A**). For the Sho-kenchu-To (小建中湯) formula-pattern model, they added silicone rubber to the urethane foam in the area representing the abdominal rectus muscle (**Figures 8C**, **9B**. For the Toki-shakuyaku-San formula-pattern model, they added an Oketsu tenderness system as with the Keishi-bukuryo-Gan formula-pattern model (**Figure 9C**).

Finally, they created two obvious deficiency models. For the Ninjin-To (人参湯) formula-pattern model (**Figure 10A**), they added silicone rubber to the urethane foam in the epigastric region. For the Dai-kenchu-To (大建中湯) formula-pattern model, they packed cotton into an elastic tubular bag 5 cm in diameter to realize the expanded area of tension in the intestinal tract area characteristic of this abdominal pattern (**Figure 10B**).

Subsequently, the 10 members of the evaluation team investigated the new models by palpating them, judging whether the models were representations of the 13 standard Kampo formula abdominal patterns.

RESULTS

In the present research, the production team worked on 13 female abdominal models corresponding to 13 clinically significant Kampo formulations, and then the evaluation team investigated the models by palpating them.

With the two obvious excess models, the evaluators found that the Dai-saiko-To formula-pattern model featured

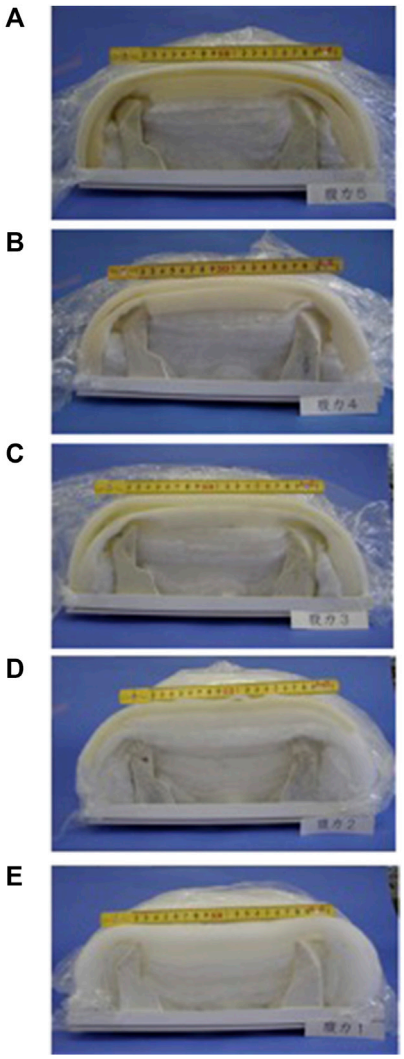

FIGURE 3 | Interior of abdominal strength models. **(A)** An obvious excess abdominal pattern model. **(B)** A slight excess abdominal pattern model. **(C)** An intermediate excess abdominal pattern model. **(D)** A slight deficiency abdominal pattern model. **(E)** An obvious deficiency abdominal pattern model.

TABLE 2 | Specific abdominal pattern models.

Stuffiness and rigidity below the heart model: Increased resistance in the epigastric region
Fullness in the chest and hypochondrium model: Increased resistance on both sides of the hypochondriac region
Rectus muscle tension model: Increased resistance in the area corresponding to the abdominal rectus muscle
Lower abdominal fullness model: A horseshoe-shaped area of markedly increased resistance in the lower abdomen
Lower abdominal numbness mode: Diminished resistance in the center of the lower abdomen
Abdominal fluid congestion model: A splashing sound is heard on tapping the abdomen
Oketsu tenderness model: Increased resistance and tenderness in the lower abdomen and peri-umbilical region
Abdominal palpitation model: A pulsating sensation can be felt in the abdomen

increased resistance over a wide area in the hypochondrium and epigastrium, and the Dai-joki-To formula-pattern model featured swelling and increased resistance in the umbilical area.

With the three slight excess models, the evaluators found that: the Shigyaku-San formula-pattern model featured increased resistance to the touch in the lower hypochondrium, the epigastrium, and the abdominal rectus muscle; the Saiko-ka-ryukotsu-borei-To formula-pattern model featured increased resistance in the hypochondriac and epigastric regions; and the Keishi-bukuryo Gan formula-pattern model featured increased resistance in the lower abdomen and Oketsu tenderness.

With the intermediate strength models, the evaluators found that: the Hachimi-jio-Gan formula-pattern model featured decreased

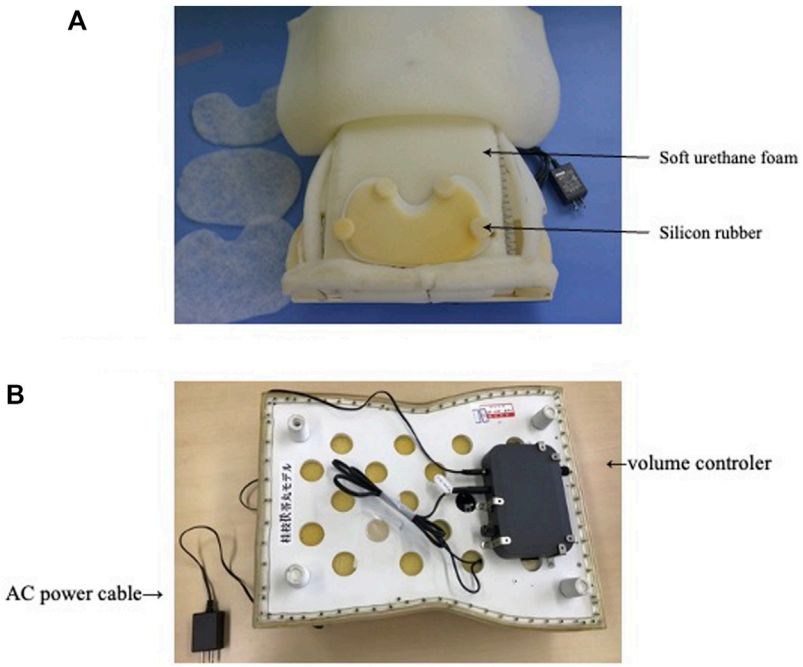

FIGURE 4 | Keishinbukuryo-Gan pattern model. **(A)** The interior of the Keishi-bukuryo-Gan pattern model. **(B)** The reverse of the Keishi-bukuryo-Gan pattern model.

resistance in the medial lower abdomen; the Hange-shashin-To formula-pattern model featured increased resistance in the epigastric region; and the Sho-saiko-To formula-pattern model featured increased resistance in the lower hypochondrium and the epigastrium.

In the case of the slight deficiency models, the evaluators found that: the Hochu-ekki-To formula-pattern model featured increased resistance in the lower hypochondrium; the Sho-kenchu-To formula-pattern model featured increased resistance in the abdominal rectus muscle area; and that the Toki-shakuyaku-San formula-pattern model was similar to the Sho-kenchu-To formula-pattern model but also featured Oketsu tenderness in the lower abdomen.

With the two obvious deficiency models, the Ninjin-To formula-pattern model was fond to feature increased resistance in the epigastric region, and the Dai-kenchu-To formula-pattern model featured an extended area of resistance over the intestinal tract.

The evaluators determined that the producers were successful in creating the above 13 formula-pattern models, corresponding to standard Kampo formulations.

TABLE 3 | 13 Important formula abdominal pattern models.

1. Obvious excess abdominal strength models
   (a) Dai-saiko-To formula-pattern model
   (b) Dai-joki-To formula-pattern model
2. Slight excess abdominal strength models
   (a) Shigyaku-San formula-pattern model
   (b) Saiko-ka-ryukotsu-borei-To formula-pattern model
   (c) Keishi-bukuryo-Gan formula-pattern model
3. Intermediate abdominal strength models
   (a) Hachimi-jio-Gan formula-pattern model
   (b) Hange-shashin-To formula-pattern model
   (c) Sho-saiko-To formula-pattern model
4. Slight deficiency abdominal strength models
   (a) Hochu-ekki-To formula-pattern model
   (b) Sho-kenchu-To formula-pattern model
   (c) Toki-shakuyaku-San formula-pattern model
5. Obvious deficiency abdominal strength models
   (a) Ninjin-To formula-pattern model
   (b) Dai-kenchu-To formula-pattern model

DISCUSSION

It does not appear to be feasible to use laboratory testing equipment to substitute for abdominal diagnoses performed by physicians; therefore, our approach is to create standard models that correspond to the patterns that physicians will encounter in clinical practice. In an earlier study, when clinical practitioners were asked to evaluate our abdominal strength models, 96.1% found them useful, which suggests that this approach has promise (Baba et al., 2018).

Reports of the usefulness of simulators in medical training exist (Ewy et al., 1987; Woolliscroft et al., 1987; Butter et al., 2010; Schubart et al., 2012). The models in the first generation of our Fukushin simulator were inferior to the present ones and gave a far from perfect reproduction of abdominal patterns, yet 149 practitioners taking our workshop on abdominal palpation said that the models were useful and had helped deepen their

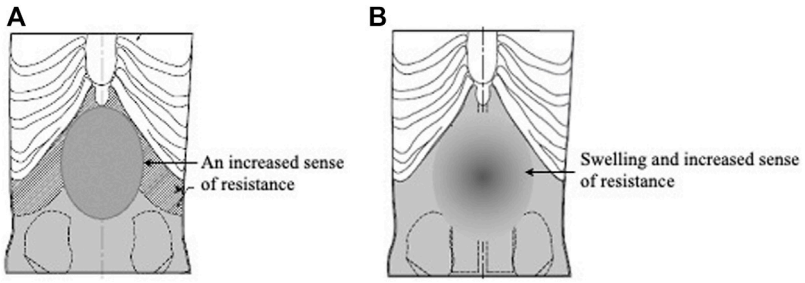

FIGURE 5 | Obvious excess abdominal strength models. **(A)** Dai-saiko-To formula-pattern model. **(B)** Dai-joki-To pattern model.

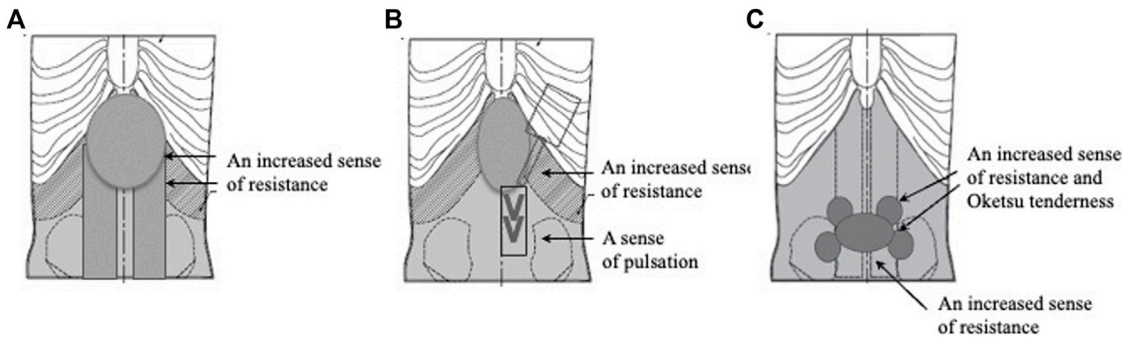

FIGURE 6 | Slight excess abdominal strength models. **(A)** Shigyaku-San pattern model. **(B)** Saiko-ka-ryukotsu-borei-To pattern model. **(C)** Keishi-bukuryo-Gan pattern model.

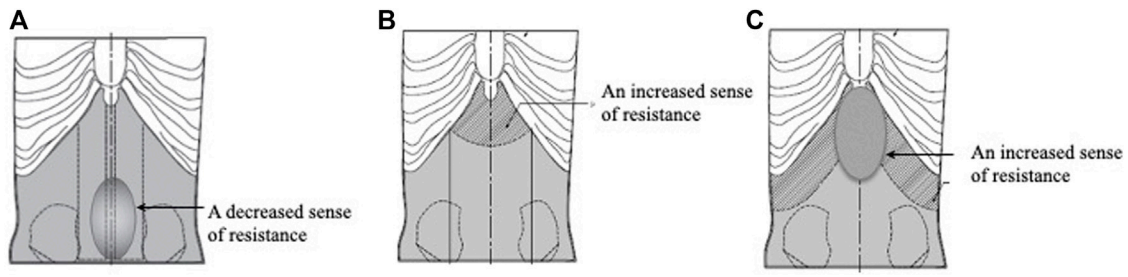

FIGURE 7 | Intermediate abdominal strength models. **(A)** Hachimi-jio-Gan pattern model. **(B)** Hange-shashin-To pattern model. **(C)** Sho-saiko-To pattern model.

understanding of abdominal patterns (Yakubo et al., 2009a). With regard to the various static models included in the current Fukushin Simulator, 78.6% of educators judged them to be very useful or useful (Yakubo et al., 2009b). In general, the simulator is judged to be useful by both trainers and trainees (Yakubo et al., 2012). More recently, we conducted a practical session on abdominal diagnosis using the current version of the Fukushin simulator with medical students, and 98.4% of the students gave positive feedback (Yakubo et al., 2014b).

Arita et al. developed an education program including a general lecture on physical examination in Kampo medicine, followed by a pre-test assessment involving palpation of the simulators, a specific lecture about abdominal palpation, and finally a post-test assessment (Arita et al., 2019). Their education

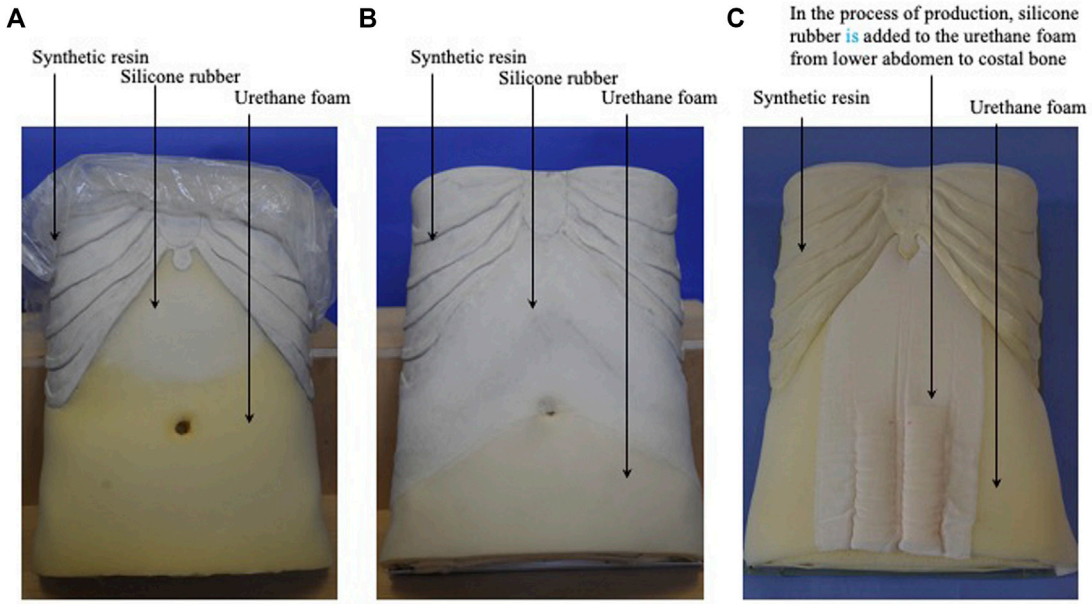

FIGURE 8 | The interior of the Fukushin simulators. **(A)** The interior of the Hange-shashin-To formula-pattern model. **(B)** The interior of the Hochu-ekki-To formula-pattern model. **(A)** The interior of the Sho-kenchu-To formula-pattern model in the process production.

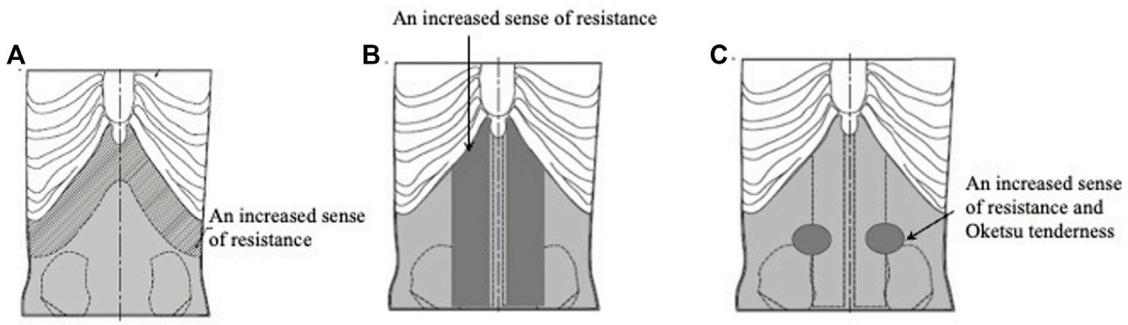

FIGURE 9 | Slight deficiency abdominal strength models. **(A)** Hochu-ekki-To pattern model. **(B)** Sho-kenchu-To pattern model. **(C)** Toki-shakuyaku-San pattern model.

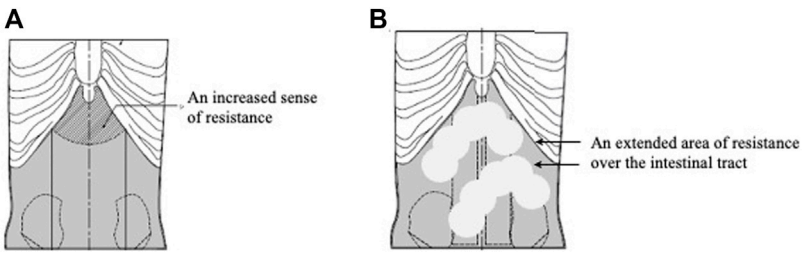

FIGURE 10 | Obvious deficiency abdominal strength models. **(A)** Ninjin-To pattern model. **(B)** Dai-kenchu-To pattern model.

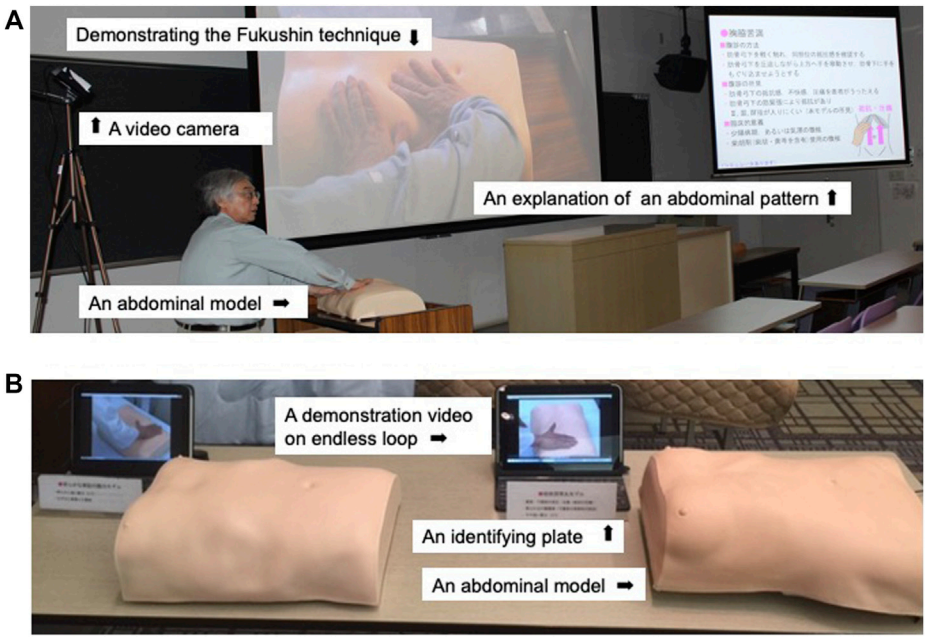

FIGURE 11 | The abdominal palpation learning system in Kampo style (Abpalle Kampo). **(A)** Using an abdominal model in a lecture on Fukushin. **(B)** Placement of abdominal models and monitors.

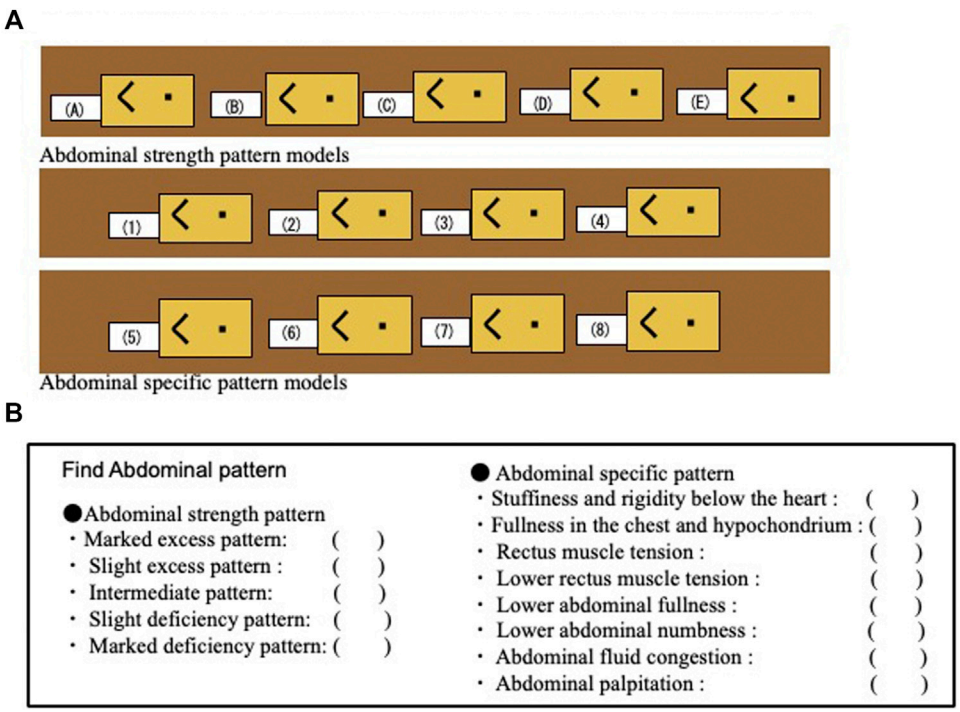

FIGURE 12 | A system of Abpalle KAMPO for students. **(A)** Arrangement of abdominal strength pattern models and abdominal specific pattern models. **(B)** Student's quiz paper.

program using simulators for Kampo abdominal palpation can be effective and useful for beginners in Kampo medicine.

Also recently, we have developed a teaching system for abdominal diagnosis featuring our abdominal models, called the Abdominal palpation learning system in Kampo style (Abpalle Kampo) (Yakubo et al., 2021a), which appears to have benefit in making it easier for participants to understand abdominal patterns and the corresponding herbal formulas and to learn the technique of abdominal palpation. **Figure 11A** demonstrates the use of an abdominal model in a lecture on Fukushin and **Figure 11B** shows the placement of abdominal models and monitors.

For medical students, some of whom are reluctant to take an active part, we prepared Abdominal strength pattern models (five gradations) and eight Abdominal specific pattern models with the Fukushin simulator, labeled not with the name of the model but a number or letter and arranged at random around the room (**Figure 12A**) (Yakubo et al., 2021b). Students are required to go around the room and perform abdominal diagnosis on the different models, attempting to write the correct diagnosis for each one (**Figure 12B**). We think that it is desirable to incorporate this type of training (Abpalle KAMPO for students) as a standard part of Fukushin education for students.

For the present research, we created 13 formula-pattern models representing 13 abdominal patterns each matched to a standard Kampo formula. Using these models, it should become possible for participants to really understand the explanations of the abdominal patterns as well as the required technique. The participants can then perform diagnosis themselves on the models, which should lead to deepening their practical understanding.

Although we do not currently have case reports that list the chief complaint, history of present illness, and medical history alongside the results of abdominal diagnosis, one idea we are exploring currently is to prepare case reports featuring the results of tongue and pulse diagnosis and other available information and give them to workshop participants with an abdominal model whose name has been obscured. Participants would attempt to perform abdominal palpation and prescribe the Kampo formula that best matches the case. We can expect that this will contribute to educating practitioners with greater practical expertise. Accordingly, we believe that these 13 new models have a major contribution to make.

The World Health Organization, in its International Classification of Diseases 11th Revision, now includes mention of the standardization of traditional medical practices (Yakubo et al., 2019; World Health Organization, 2020). Abdominal diagnosis is one of the traditional medical practices mentioned, and its standardization has become an important issue. It is our hope that the models in the Fukushin simulator described in this paper will make a significant contribution to this effort.

CONCLUSION

The diagnostic method known as abdominal palpation is of great importance in Kampo medicine, enabling a Kampo practitioner to determine the most suitable formulation for a patient. Based on our previous work developing a Fukushin simulator consisting of a set of abdominal models recreating important abdominal patterns, the present research describes 13 new standard female models reproducing clinically important abdominal patterns matched to Kampo formulations, which we have called formula-pattern models. These are expected to make a significant contribution to the education and standardization of abdominal palpation.

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

Conception and design of the work: SY. Data/materials collection: MB. Data/materials analysis and interpretation: HO, AW, MS, TH, TM, TN, MA, S-IM, YS, and NS. Drafting the article: SY. Critical revision of the article: MB, HO, AW, MS, TH, TM, TN, MA, S-IM, YS, and NS. Final approval of the manuscript: SY, MB, HO, AW, MS, TH, TM, TN, MA, S-IM, YS, and NS. All authors have contributed significantly to the manuscript, and all authors are in agreement with the content of the manuscript.

FUNDING

TM, MA, HO, S-IM, Ueda, Hattori, and SY have received research funding from Tsumura & Co. TM has received speaker fees from Tsumura & Co. and from Kotaro Pharmaceutical Co. SY has received research funding from the Japan Kampo Medicine Education Foundation.

REFERENCES

Arichi, S., Akamaru, S., and Tani, T. (1983). An Application of Kampo Abdominal Palpation to the Modern Medicine-By thermal Heart Video System (1). *Igaku-to-Yakugaku* 13, 667–674. (In Japanese).

Arita, R., Numata, T., Saito, N., Takayama, S., Togashi, T., Kaneko, S., et al. (2019). Development of a Medical Education Program with Abdominal Palpation Simulators to Support the Understanding of Traditional Japanese (Kampo) Medicine in Beginners. *Traditional Kampo Med.* 6 (3), 148–155. doi:10.1002/tkm2.1230

Arai, M., Hioki, C., and Kosoto, H. (2017).Textbook of Traditional Japanese Medicine, Part 1: Kampo. Available at: http://kampotextbook.sakura.ne.jp/pdf/Part1_Kampo_Textbook_of_Traditional_Japanese_Medicine_en.pdf.

Baba, M., Fukuda, E., and Yakubo, S. (2018). Evaluation of Standard Abdominal Strength Pattern Models in an Abdominal Palpation Simulator and of the Standardization Project Itself. *Int. Med. J.* 25 (1), 1–3.

Baba, M., Fukuda, E., and Yakubo, S. (2019). Modification to an Abdominal Diagnosis Simulator to Educate Standard Abdominal Patterns of Toki-Shakuyaku-San or Keishi-Bukuryo-Gan in Kampo Medicine. *Int. Med. J.* 26 (1), 39–42.

Butter, J., McGaghie, W. C., Cohen, E. R., Kaye, M., and Wayne, D. B. (2010). Simulation-Based Mastery Learning Improves Cardiac Auscultation Skills in Medical Students. *J. Gen. Intern. Med.* 25, 780–785. doi:10.1007/s11606-010-1309-x

Ewy, G. A., Felner, J. M., Juul, D., Mayer, J. W., Sajid, A. W., and Waugh, R. A. (1987). Test of a Cardiology Patient Simulator with Students in Fourth-Year Electives. *J. Med. Educ.* 62 (9), 738–743. doi:10.1097/00001888-198709000-00005

Miyamoto, K., and Okita, K. (2005). Reappearance and Changes of Sub-navel Hyposthenia (SNH) in Evaluation of SNH by Digital Abdominal Diagnometer (DAD). *Kampo Newest Ther.* 13, 185–191. (In Japanese).

Nishida, Y., Narahara, H., and Oribe, K. (2012). Anatomical Evaluation of Shofukukyuketsu by 3D Image Analysis. *Kampo Med.* 61, 856–859. Japanese (Summary in English). doi:10.3937/kampomed.61.856

Protnikoff, G. A., and WatanabeYashiro, K. K. (2008). Kampo, from Old Wisdom Comes New Knowledge. *Herbal Gram* 78, 46–57.

Schubart, J. R., Erdahl, L., Smith, J. S., Jr, Purichia, H., Kauffman, G. L., and Kass, R. B. (2012). Use of Breast Simulators Compared with Standardized Patients in Teaching the Clinical Breast Examination to Medical Students. *J. Surg. Educ.* 69, 416–422. doi:10.1016/j.jsurg.2011.10.005

Shintani, T., Tosa, H., Yamamoto, T., Imadaya, A., and Terasawa, K. (1989). On the Relationship between X-ray Findings of Barium Enema, Abdominal Palpation Signs of Kampoh Medicine and Effective Kampoh Formulas. *Kampo Med. Nihon Toyo Igaku Zasshi* 39, 245–252. (Summary in English). doi:10.3937/kampomed.39.245

Terasawa, K. (1993). *Kampo, Japanese-Oriental Medicine, Insights from Clinical Cases*. Tokyo: Standard McIntyre.

The Japan Society for Oriental Medicine (2005). *Introduction to Kampo Japanese Traditional Medicine*. Tokyo: Elsevier Japan K.K.

Tosa, H., Terasawa, K., Imadaya, A., Mitsuma, T., and Matsumoto, M. (1982). A Study of the Mechanism of “INAI-TEISUI” (Water-Imbalance Syndrome in Kampoh Medicine) -The First Report-. *Nihon Toyo igaku zasshi* 33, 53–58. (Summary in English). doi:10.3937/kampomed.33.53

Ushiroyama, T. (2005). Japanese Kampo Medicine for Women: Historical Perspectives of Koho-Ha School and Current Concerns in Menopausal Medicine. *Adv. Obst. Gynecol.* 57, 131–149. doi:10.11437/sanpunosinpo.57.131

Woolliscroft, J. O., Calhoun, J. G., Tenhaken, J. D., and Judge, R. D. (1987). Harvey: the Impact of a Cardiovascular Teaching Simulator on Student Skill Acquisition. *Med. Teach.* 9, 53–57. doi:10.3109/01421598709028980

World Health Organization (2020). *The Supplementary Chapter 26, Traditional Medicine Conditions-Module I, International Classification of Diseases 11th Revision*. (Geneva, Switzerland: WHO) Available at: https://icd.who.int/browse11/l-m/en#/http%3a%2f%2fid.who.int%2ficd%2fentity%2f718687701.

Yakubo, S., Baba, M., and Fukuda, E. (2021a). A New Method for Training Medical Students in Abdominal Diagnosis in Kampo Style through Use of a Simulator. *Int. Med.* 28 (5), 539–541.

Yakubo, S., Baba, M., and Fukuda, E. (2021b). Developing an Abdominal Palpation Learning System in Kampo Style (Abpalle KAMPO) for Doctors. *Int. Med. J.* 28 (2), 243–245.

Yakubo, S., Kinoshita, Y., and Ota, H. (2009a). Evaluation by Clinicians Learning Kampo Medicine of a Simulator for Learning Abdominal Palpation. *J. Med. Educ. Jpn.* 40, 55–60. Japanese (Summary in English). doi:10.11307/mededjapan.40.55

Yakubo, S., Kinoshita, Y., and Ueda, Y. (2009b). Evaluation by Kampo Medical Faculty of a Simulator for Teaching Abdominal Palpation. *J. Trad. Med.* 26, 104–109.

Yakubo, S., Ueda, Y., and Ishino, S. (2014a). The Development of an Abdominal Palpitation Model for the Fukushin Simulator: towards Improvement and Standardization of Kampo Abdominal Diagnosis. *Int. Med. J.* 21 (2), 1–4.

Yakubo, S., Ueda, Y., and Muroga, K. (2014b). Students' Impressions of an Abdominal Diagnosis Workshop Using the Fukushin Simulator. *Int. Med. J.* 21 (4), 358–361.

Yakubo, S., Ueda, Y., and Ishino, S. (2013). Towards the Standardization of Abdominal Strength in the Abdominal Palpation Diagnostic System of Kampo Medicine: Development of an Abdominal Strength Model in the Fukushin Simulator. *Int. Med. J.* 20 (6), 696–698.

Yakubo, S., Ueda, Y., and Kinoshita, Y. (2012). Making and Evaluation of a Simulator for the Teaching or Learning of Abdominal Pattern in the Japanese Kampo Style by Clinical Doctors and Educational Faculty. *Int. Med. J.* 19 (2), 112–114.

Yakubo, S., Kinoshita, Y., Aki, T., and Ota, H. (2008). Improvement of A Simulator Production Project for Abdominal Palpation in Kampo Medical Training. *Kampo Med.* 59, 595–600. Japanese (Summary in English). doi:10.3937/kampomed.59.595

Yakubo, S., Namiki, T., and Ito, M. (2019). Chapter 26 Traditional Medicine Included in ICD-11 Has Been Released, till Now and from Now on!. *Nihon Toyo igaku zasshi* 70 (2), 167–174. (Summary in Japanese). doi:10.3937/kampomed.70.167

Yakubo, S., Ueda, Y., Muroga, K., Tanekura, N., Okudaira, T., Sasanuma, T., et al. (2015). Modifications to an Abdominal Diagnosis Simulator to Reproduce Patterns Characterized by Local Variations in Resistance to Pressure. *Traditional Kampo Med.* 2 (2), 31–34. doi:10.1002/tkm2.1015

Yasaka, T. (1994). Analitical Use of Ultrasonography in the Signs of "Saikafujin". *Kampo Med. Nihon Toyo Igaku Zasshi* 45, 331–337. Japanese (Summary in English). doi:10.3937/kampomed.45.331

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership