FOOD/DIET SUPPLEMENTS FROM NATURAL SOURCES: CURRENT STATUS AND FUTURE CHALLENGES FROM A PHARMACOLOGICAL PERSPECTIVE

EDITED BY: Michał Tomczyk, Marcello Locatelli and Sebastian Granica
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frontiers Research Topics

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FOOD/DIET SUPPLEMENTS FROM NATURAL SOURCES: CURRENT STATUS AND FUTURE CHALLENGES FROM A PHARMACOLOGICAL PERSPECTIVE

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EDITORIAL
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Editorial: Food/Diet Supplements From Natural Sources: Current Status and Future Challenges From a Pharmacological Perspective

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Editorial on the Research Topic

Food/Diet Supplements From Natural Sources: Current Status and Future Challenges From a Pharmacological Perspective

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Nowadays, several natural products are used as food additives even if knowledge on their properties is not complete. Often, several techniques can be employed to improve extractions, workup, and isolation/purification of bioactive materials from natural sources. These findings are reported well and exposed in Dall'Acqua et al., Ahmad et al., and Li et al. These authors report new extraction procedures and chemical profiles able to justify specific biological activities with the phytocomplex. From an industrial point of view, the availability of procedures and knowledge (firstly developed in laboratory scale) is essential to obtain the scale-up and adequate quality control, particularly to the innovative instrument configurations able to improve not only the analytical performances (especially sensitivity and selectivity) but also to reduce solvents consumption, time-per-analysis, and ruggedness, following the GAC (Green Analytical Chemistry) guidelines. Specifically, this research topic focuses on the biological activities of a specific plant-derived material and the discovery of innovative activities and new biological targets. In this field, more interesting are the papers from Zhang et al., Jin et al., Blažević et al., Chang et al., Schreck and Melzig. Furthermore, in Pharmacology and Ethnopharmacology, this collection focuses on specific effects ideally on identifiable targets. Specifically, some papers report interesting approaches/applications of natural products on health protection, such as Wang et al., Ye et al., Jiang et al., and Yong et al. This research topic also includes works on food supplements (Chen and Tsim) and an interesting paper related to a very recent problem related to the naturally derived products that are not fully identified and regulated by current legislation, especially concerning heavy metals (Puścion-Jakubik et al.). Other essential elements in this topic collection are two review papers, the first on the trends of adulterated and illegal food supplements in the EU based on the warnings of the Rapid Alert System for Food and Feed (Koncz et al.) and the second on the potential of edible and herbal plants for the prevention and management of COVID-19 (Li et al.).

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

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REVIEW
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A Review of Edible Jujube, the *Ziziphus jujuba* Fruit: A Heath Food Supplement for Anemia Prevalence

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The fruits of *Ziziphus jujuba*, commonly known as jujube, red date or Chinese date, are taken as fresh or dried food, and as traditional medicine worldwide due to high nutritional and health values. Traditionally in China, jujube is considered as a medicinal fruit that is being used in treating blood deficiency. In this review, the beneficial effects of jujubes on the hematopoietic functions are summarized and discussed. As illustrated in cell and animal models, the application of jujube extract possessed beneficial effects, including regulation of erythropoiesis via activation of hypoxia inducible factor-induced erythropoietin, potential capacity in recycling heme iron during erythrophagocytosis and bi-directional regulation of immune response. Thus, the blood-nourishing function of jujube is being proposed here. Flavonoid, polysaccharide and triterpenoid within jujube could serve as the potential active ingredients accounting for the aforementioned health benefits. Taken together, these findings provide several lines of evidence for further development of jujube as supplementary products for prevention and/or treatment of anemia.

Keywords: *Ziziphus jujuba*, Rhamnaceae, blood deficiency, bio-active ingredient, food supplement

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INTRODUCTION

Jujube is usually called red date or Chinese date, which is the fruit of *Ziziphus jujuba* Mill. that belongs to Rhamnaceae family. Jujube is native to China, and which has been commonly consumed as food supplement and traditional Chinese medicine (TCM) for thousands of years (**Figure 1**). Today, jujube plant is distributed widely not only in China but also in other countries, e.g. Korea, India, Japan, Europe and the United States. In *Huangdi Neijing* (475–221 BC), a classic medical text from ancient China, jujube was recorded as one of extremely valuable fruits. According to *Shennong Bencao Jing* written between 300 BC and 200 AD, one of the earliest books specializing in Chinese medicine, jujube was regarded as one of the top-grade medicinal herbs that could extend one's life expectancy by nourishing blood, increasing sleep quality and improving digestive system. Along with growing number of studies on jujube, various beneficial nutrients within jujube are being proposed, including carbohydrate, mineral, vitamin, sugar and amino acid. Thus, jujube is considered as a popular nutritious food, worldwide (Li et al., 2007; San and Yildirim, 2010; USDA, 2012; Guo et al., 2013; Reche et al., 2019). Being a Chinese herb or health food supplement, recent studies have indicated that jujube possesses a wide range of pharmacological activities in nervous system, cardiovascular system, as well as anti-oxidation and anti-cancer properties (**Table 1**).

Clinically, blood deficiency is usually encountered in women due to the loss of menstrual blood, or in patient who has lost blood or suffered from chronic malnutrition. Jujube is a functional food,

FIGURE 1 | The photo of *Z. jujuba* fruits. The photo of dried jujubes collected from Xinjiang province, one of major production regions of jujubes in China. Bar: 1 cm.

which is believed to possess robust effect in tonifying the blood, in order to prevent blood deficiency in human. According to the theory of TCM, blood deficiency shows similarity to anemia of individual in western medicine (Shi et al., 2019). In line to this notion, pharmacological studies have reported that jujube has potential hematopoietic functions both *in vivo* and *in vitro* (Xu et al., 2004; Chen et al., 2014b). Specific targets supporting the clinical usage of jujube in hematopoietic functions however remain unclear. Here, we are focusing on a discussion of jujube associating with hematopoietic functions, i.e., erythropoiesis, erythrophagocytosis and immune functions (**Table 2**). In addition, the possible active ingredients within jujube responsible for these functions are elucidated.

Potential Bio-Active Ingredients of Jujube in Hematopoietic Function

Jujube has a promising source of flavonoids, polysaccharides, terpenoids, saponins, nucleotides and others (**Figure 2**). Here, the trophic ingredients having potential beneficial effects on hematopoietic function are highlighted. At present, a variety of flavonoids were isolated and identified in jujube (Cheng et al., 2000; Pawlowska et al., 2009; Choi et al., 2011; Gao et al., 2013). Flavonoids from jujube have been found to stimulate the expression of erythropoietin (EPO), a hormone stimulating blood production (Zheng et al., 2011), and therefore we speculated that jujube flavonoid might be one of the active compounds that possessed the ability to induce the expression of EPO. Supporting this notion in the pHRE-Luc (the DNA promoter construct of *EPO* gene, hypoxia response element) transfected cultured HEKT293T cells, the application of kaempferol at 10 μM for 24 h could significantly induce the transcriptional activity of pHRE-Luc with 127% of increase, as compared to control group (Xu et al., 2018). Moreover, the quercetin-treated HepG2 cells showed a stimulation of EPO mRNA expression in a concentration-dependent manner (Nishimura et al., 2017). Similarly, the protein level of HIF-1α was markedly up regulated at the treatment of 10 μM quercetin (Nishimura et al., 2017). Indeed, kaempferol and quercetin derivatives, including kaempferol 3-O-rutinoside, quercetin, quercetin 3-O-rutinoside, quercetin 3-O-galactoside and quercetin 3-O-β-D-glucoside (**Figures 3A**), were identified in jujube (Gao et al., 2012; Chen et al., 2013). Therefore, jujube flavonoid can induce EPO expression, probably, through HIF-α protein accumulation. In addition, the combination of catalpol and puerarin with doses of 65.4 and 32.7 mg/kg, respectively, enhanced the expressions of EPO and EPO receptor in ischemic/reperfusion rats (Xue et al., 2016). In parallel, puerarin was identified in the seeds of jujube (Cheng et al., 2000). Flavonoids are common chemical presented in a wide range of plants. Plant extracts rich in aforesaid flavonoids are considered, therefore, potentially useful as therapeutic agents for anemia.

Polysaccharides from plant have been demonstrated to possess various bio-activities, e.g., anti-oxidation, anti-complementary and immunological activities (Li et al., 2003; Gao et al., 2007; Chen M. et al., 2016). Several polysaccharides have been isolated and purified from the jujube. The polysaccharides extracted from jujube usually consist of five monosaccharides, i.e., galactose, arabinose, rhamnose, glucose and xylose (Zhao et al., 2008) (**Figures 3B**). Fractions named as ZSP1, ZSP2, ZSP3 and ZSP4 with weight ratio of 29.3:17.6:37.2:15.9 have been purified from jujube (Li et al., 2011). The fractions of ZSP3 and ZSP4 at various concentrations (30–200 μg/ml) were applied onto peritoneal macrophages, and the cell proliferation was detected by MTT assay. These two fractions were found to dose-dependently induce proliferation of spleen lymphocyte, having the highest response under the treatment of jujube polysaccharide at 200 μg/ml. This finding suggests the immunological activity of jujube polysaccharide. In line with this, Ju-B-2, a molecular weight of over 2,000 kDa polysaccharide from jujube, was shown to have the immune activity. Application of Ju-B-2 at 10–100 μg/ml onto cultured spleen cells for 3 days induced cell proliferation. Furthermore, the authors proposed the structures of rhamnogalacturonan and its side chains of Ju-B-2 polysaccharide contributing to the immune response (Zhao et al., 2006). Although several reports support the beneficial effects of jujube polysaccharides in preventing anemia, its detail action mechanisms are still rather limit. Hence, possible signaling pathways involved in jujube polysaccharide-treated *in vitro* or *in vivo* models are needed for further investigation.

Triterpenic acids have been isolated and purified from jujube, including ceanothenic acid, zizyberanal acid, zizyberenalic acid, zizyberanalic acid, and ceanothic acid (**Figures 3C**) (Yu et al., 2012). These acids possessed notable inhibitory activity on the activated inflammatory cells, and which could be one of the main ingredients in supporting the anti-inflammatory activity of jujube (Yu et al., 2012). Besides, jujuboside and flavonoid in the fruit were also proposed to be active compounds, and which might responsible for anti-inflammatory effects (Goyal et al., 2011).

TABLE 1 | Health beneficial properties of jujube.

Biological functions	Findings	References
Nervous system	Oleamide isolated from jujube at 14–16 mg/kg significantly restored memory and/or cognitive impairment in mice induced by scopolamine	Heo et al., 2003
	Jujube hydroalcoholic extract at100, 250, 500, and 1,000 mg/kg ameliorated seizures, oxidative stress, and cognitive impairment in epilepsy rat model	Pahuja et al., 2011
	Jujube at 0.72, 1.8, and 4.5 g/kg improved learning and memory ability in ovariectomized rat model in the Morris water maze experiment	Li et al., 2013
Cardiovascular system	The seeds of Z. jujuba (30, 100, or 300 mg/kg) and its active component jujuboside B (10, 30, or 100 mg/kg) were reported to exhibit anti-platelet aggregation activity	Seo et al., 2013
	Jujube extracts at 25 and 50 μg/ml suppressed lipid accumulation and GPDH[a] activity in 3T3-L1 preadipocytes	Kubota et al., 2009
	Consumption of jujube infusion (10 g/100 ml) three times/day prior to main meals for 12 weeks in patients with type 2 diabetes mellitus showed a robust improvement in lipid profiles and glycaemic index	Yazdanpanah et al., 2017
Anti-oxidative activity	Jujube extracts at various concentrations (0.25–1.0 mg/ml) inhibited DPPH radical, and its DPPH radical scavenging effect was in a dose dependent manner	Li et al., 2005
	Jujube extracts (0–3.0 mg/ml) protected tBHP-induced oxidation insult on PC12 cells via activation of ARE-mediated transcriptional activity[b]	Chen et al., 2013
	Jujube polysaccharides (0–3.0 mg/ml) possessed the potential effect to scavenge hydroxyl radicals, and the scavenging rates increased dose-dependently	Ji et al., 2020
Anti-cancer activity	Dietary jujube for 70 days (5% or 10% w/w) inhibited tumor progression and promoted the tumor apoptosis in CAC cancer mice	Periasamy et al., 2020
	Jujube extracts (0–200 μg/ml) induced dose-dependently effect on apoptosis and a differential cell cycle arrest, i.e., G1 and G2/M arrest in HepG2 cells	Huang et al., 2007
	3OTPCA at 5–80 μM, a triterpenoid isolated from jujube induces apoptotic cell death in human leukemia cells via the generation of reactive oxygen species and activation of UPR[c]	Mitsuhashi et al., 2017
Other medicinal properties	Mice given with 1 and 10% of Z. jujuba essential oil at different concentrations (0.1, 1, and 10%) induced a greater activity on the length of hair	Yoon et al., 2010
	Glucans (10, 20, 50, and 100 μg/ml) from jujube possessed effect on regeneration of damaged skin through prompting cellular survival and cell migration	Fazio et al., 2020
	Jujube extracts at various concentrations (100–400 mg/kg) showed potent anti-asthmatic activity in ovalbumin (OVA) -induced allergic asthma of mice	Ninave and Patil, 2019

[a]*GPDH, glycerol-3-phosphate dehydrogenase.*
[b]*tBHP, tert-butyl hydroperoxide; ARE, Anti-oxidant response element.*
[c]*CAC, colitis-associated colon; UPR, unfolded protein response.*

Another animal study showed that jujube essential oil could inhibit the inflammatory responses of skin (Al-Reza et al., 2010).

In addition, jujube was reported to contain numerous minerals, e.g., iron and vitamin. About 0.48 mg iron and 69 mg vitamin C per 100 g of fresh fruit were reported (Li et al., 2007; USDA, 2012). Thus, the daily intake of jujube could increase our dietary iron and vitamin, as to prevent anemia due to deficiency of iron or vitamin C. Moreover, cAMP was found to have high abundance in jujube, and surprisingly this content was much higher than other horticultural fruits (Hanabusa et al., 1981). It is well accepted that increasing cAMP level can stimulate protein kinase A and, subsequently, which phosphorylates CREB (Argyrousi et al., 2020). Besides, jujube cAMP has been found to possess anti-melancholic effect in animal model of depression (Chi and Zhang, 2009). Thus, it is supposed that the cAMP in the jujube may account for its role on HIF (hypoxia inducible factor)-dependent EPO induction.

TABLE 2 | Hematopoietic properties of jujube.

Findings	Model	Treatment	References
Jujube extract exhibited anti-platelet aggregations effect in a dose dependent manner	Platelet-rich plasma was prepared from SD rats; *in vitro* platelet aggregation study	Pretreatment with jujube extracts at 30, 100, 300 mg/ml for 5 min at 37°C; collagen (2 mg/ml)-, thrombin (0.4 U/ml)-, and AA (100 mM) was employed to induce PLT aggregations[a]	Seo et al., 2013
Dietary jujube increased RBC, Hb, and HCT levels in cancer mice	Mice with CAC [b] were induced by injecting with azoxymethane followed by three cycles of 2% (w/v) DSS; hematological examination	Mice were given with Z. jujuba fruit for 70 days (5 or 10%°w/w)	Periasamy et al., 2020
Jujube water extract stimulated EPO expression via hypoxia inducible factor signaling pathway	Cultured Hep3B cells; mRNA and protein expression	Treatment with jujube extracts at different dosages (0.75–3.0 mg/ml) for 24 h	Chen et al., 2014b
Jujube induced expressions of iron recycling enzymes via Nrf2/ARE pathway	Cultured RAW 264.7 macrophages; mRNA expression; ARE transcriptional activity	Jujube water extract at 0.375, 0.75, 1.5 and 3.0 mg/ml. cells were treated for 24 h	Chen J. et al., 2016
Jujube water extract corrected anemia in iron deficiency rats	Iron-deficient diet to induce anemic rats; hematological analysis	jujube extracts at various concentrations (2.7, 5.4, and 10.8 g/kg/day); rats was treated for 14°weeks	Yang et al., 2016
Jujube extract stimulated thymus and spleen indices to enhance nonspecific immunity of mice model	Kunming mice; relative thymus and spleen weight	Oral administration of jujube extracts (50, 150, and 250 mg/kg/day) for 4°weeks	Li et al., 2011
Jujube extract showed anti-inflammatory effect via inhibition of nitric oxide expression	Chronic inflammatory rat model was induced by interscapular implantation of a sterile cotton pellet (50 mg); nitrite/nitrate estimation	The hydroalcoholic extract of jujube at 200 and 400 mg/kg was given to rats for 7°days	Goyal et al., 2011
Jujube extract regulated pro-inflammatory cytokine expressions under different conditions via NF-kB signaling	Cultured RAW 264.7 cells; mRNA and protein expression; luciferase activity	Jujube extract at different dosages (0–3.0 mg/ml) for 24 h	Chen et al., 2014a
Ju-B-2 from jujube induced spleen cells proliferation, and the structural-activity relationship of which in stimulating immune response were proposed	Cultured spleen cells obtained from Balb/c male mice; immunomodulating activity	Ju-B-2, Ju-B-2-spl, Ju-B-3 at 10, 30, 100 μg/ml; LPS at 2.5 μg/ml served as positive control; cells were treated for 3 days	Zhao et al., 2006

[a]*AA, arachidonic acid; PLT, platelet.*
[b]*DSS, dextran sulfate sodium; CAC, colitis-associated colon cancer.*

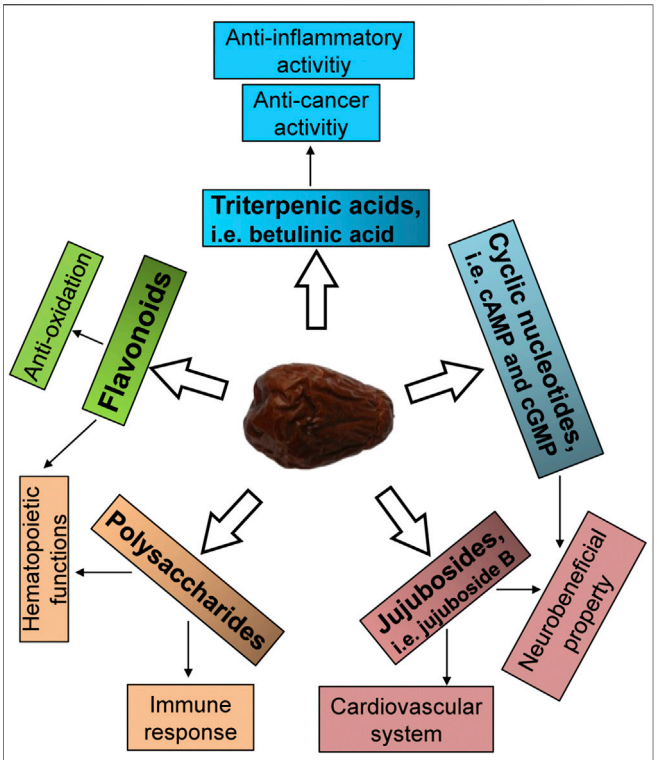

FIGURE 2 | Active ingredients of jujube. The potential active ingredients relating to the activities of jujube are summarized.

Jujube on Erythropoiesis

Erythropoiesis is considered to play critical roles in hematopoiesis, by which production of red blood cells (RBCs) is occurred. In this process, EPO, a RBC-specific hormone, is able to regulate erythropoiesis in bone marrow (Ascensao et al., 1991). EPO gene expression regulates primarily at the level of transcription, and which is further controlled by a number of transcriptional and post-transcriptional factors (Schuster et al., 1989; Goldberg et al., 1991). Failure to up regulate the circulating EPO under hypoxia thereafter leads to anemia (Jelkmann, 1992). Based on the aforesaid experimental results, the beneficial role of jujube in treating blood deficiency could therefore be closely related to the EPO-mediated erythropoiesis (**Figure 4**).

Jujube polysaccharide has been reported to improve hematological parameters in anemic animal models. Mice of blood deficiency model were induced by releasing blood and injection of cyclophosphamide. The levels of RBC, hemoglobin and hematocrit were decreased, and the level of platelet was increased in model mice, as compared to control group. Compared with model mice, the decreased levels of RBC, hemoglobin and hematocrit were reversed by treatment with jujube extract; while the increased level of platelet however was down regulated in jujube-treated mice (Xu et al., 2004). In another investigation, the activities of dietary jujube on the levels of RBC, hemoglobin and hematocrit were analyzed in colitis-associated colon cancer mice. These hematopoietic parameters in cancer mice were significantly decreased, as compared to control group; however, which were markedly increased in the jujube-treated mice (Periasamy et al., 2020), suggesting possible beneficial effects of this fruit on cancer patients suffering from anemia. Besides, the extract of jujube possessed the ability to stimulate the activity of ATPase, i.e., Na+-K+-ATPase, Ca^{2+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase, in erythrocyte, and therefore which was shown to promote bone marrow nuclear proliferation and to inhibit atrophy thymus and spleen in blood deficient animals (Miao et al., 2006; Miao et al., 2010). Moreover, the intake of jujube extract (100–400 mg/kg) was found to increase the level of EPO in blood circulation, which suggested that jujube might promote RBC level through up regulation of EPO production. In support of this notion, the applied jujube extract at concentrations of 0.75–3 mg/ml in cultured Hep3B cells for 48 h increased the expression of EPO transcription, and the increase was shown to be in a dose-dependent manner (Chen et al., 2014b). In parallel, the applied jujube extract was able to stimulate the protein expression of EPO, giving ~50% increase of the total protein (Lam et al., 2016). The circulating EPO is produced by adult kidney cells; while kidney dysfunction contributes to inadequate amount of EPO production and renal anemia. In ibuprofen-induced nephrotoxicity rats, the intake of jujube extract (500 mg/kg) improved kidney function by declining the levels of creatinine and urea, and this treatment could prevent histopathological damages of kidney (Awad et al., 2014). On the other hand, the intake of Jian-Pi-Yi-Shen, a Chinese herbal decoction comprising of Astragali Radix, Atractylodis Macrocephalae Rhizoma, Dioscoreae Rhizoma, Cistanches Herba, and other four herbs, was able to improve renal function and kidney injury in anemia rats suffering from chronic kidney disease (Chen et al., 2019). Jian-Pi-Yi-Shen improved the hematological parameters and stimulated EPO production (Chen et al., 2019). Massive EPO-producing cells are identified in the renal interstitium. The occurrence of renal interstitial fibrosis is accompanied by decrease of fibroblasts, which impairs the production of EPO. Moreover, Jian-Pi-Yi-Shen was believed to ameliorate renal interstitial fibrosis, and the renal recovery might be related to improvement of EPO production. Thus, the function of jujube in promoting EPO expression in renal anemia patients was in line to that of Jian-Pi-Yi-Shen (Chen et al., 2019). This assumption requires further studies as to confirm the ability of jujube to prevent/treat renal anemia through regulation of EPO production.

The promoter of *EPO* gene contains HRE, and thus the activation of hypoxia-mediated signaling pathway is leading to activation of EPO expression (Post and Van Meir, 2001). Cultured Hep3B cells were transfected with HRE promoter fragment (i.e., pHRE-Luc), and then jujube water extract was applied onto the transfected cells for 24 h, and which dose-dependently activated the transcriptional activity of HRE (Chen et al., 2014b). To account for the possible mechanism of HIF signaling in jujube-induced HRE activation, the expression of HIF-1α was determined. The jujube extract at various concentrations (0.75–3 mg/ml) was applied onto cultured Hep3B cells for 6 h, and then total RNA was

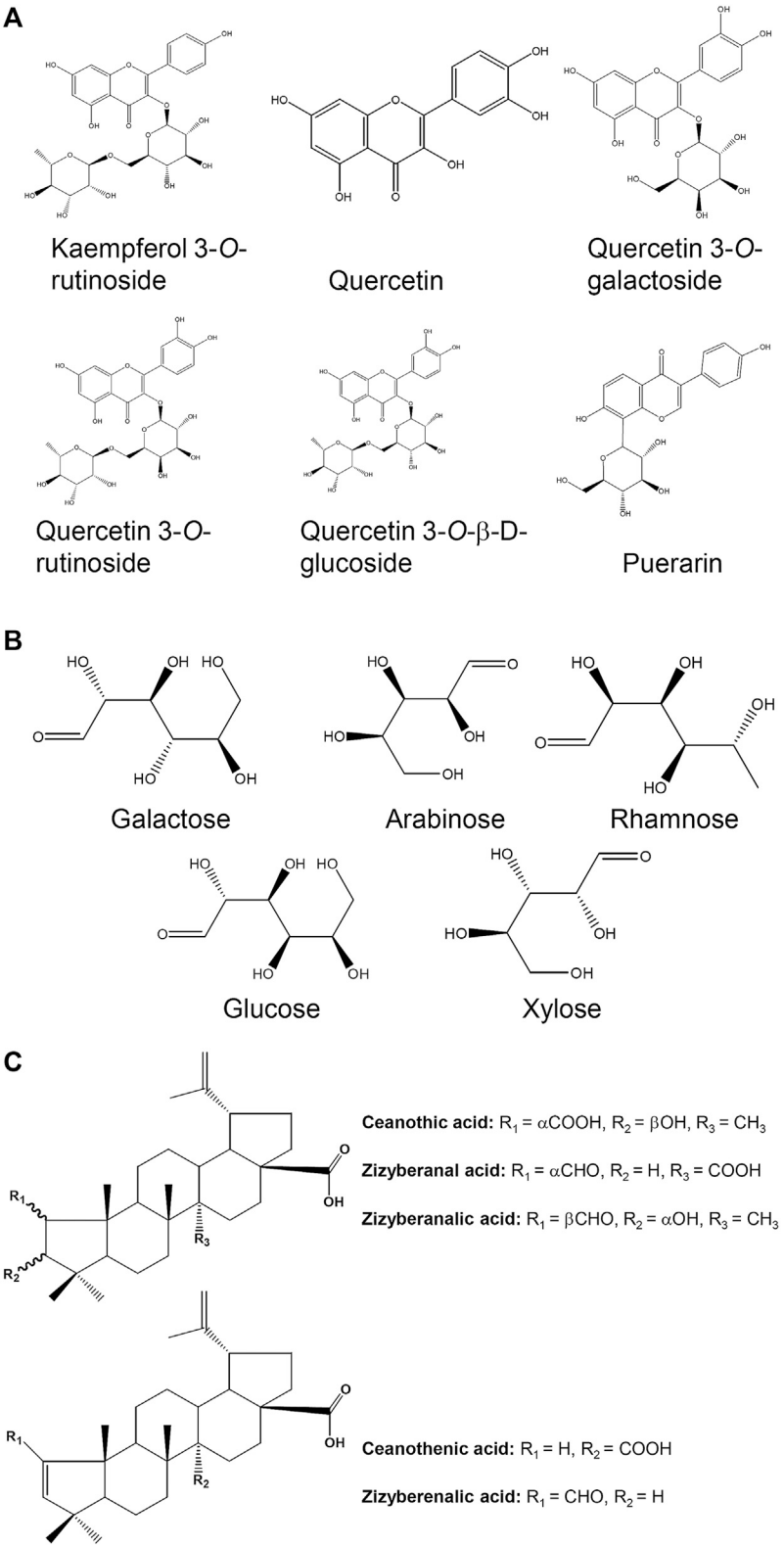

FIGURE 3 | Chemical structures for compounds in jujube possessing potential hematopoietic activity. **(A)** The chemical structures of six flavonoids found in the fruits. **(B)** The composition in monosaccharide purified from jujube. **(C)** The chemical structures of varieties of triterpenic acids found in the fruit.

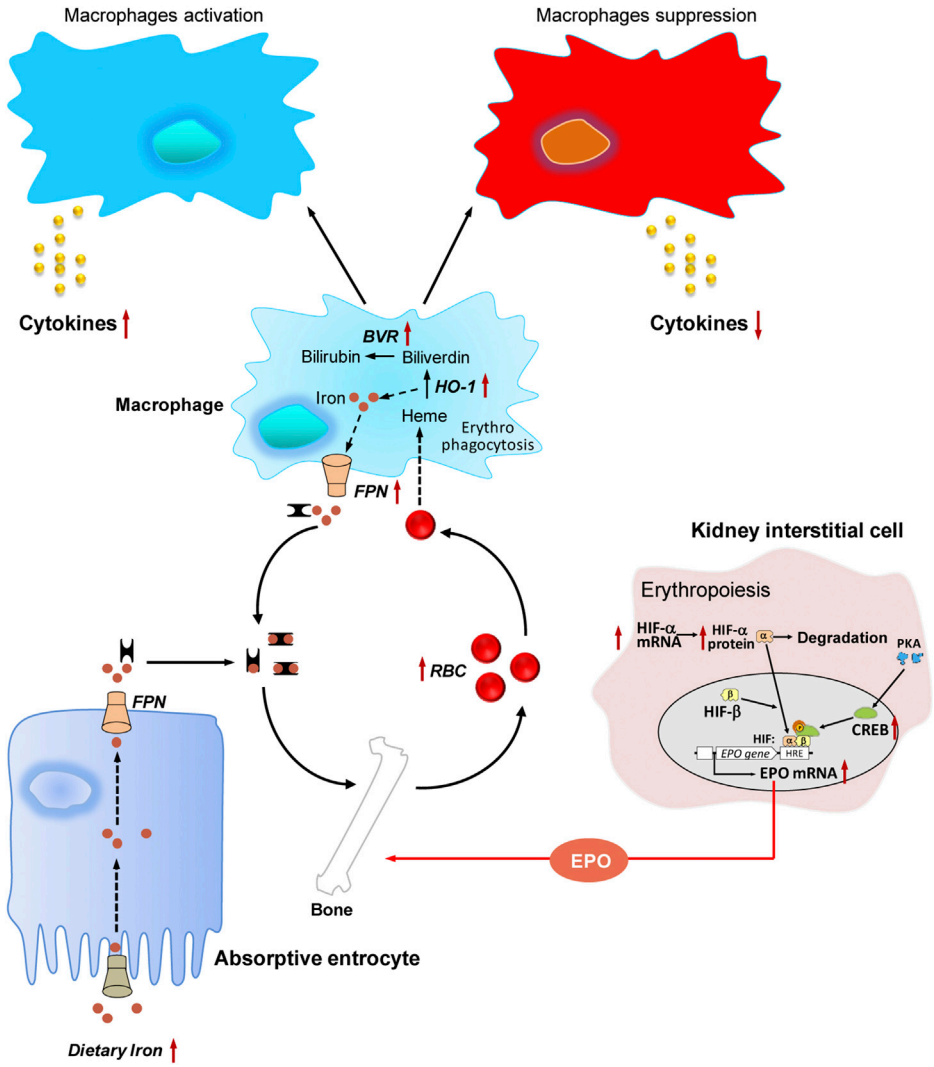

FIGURE 4 | The hematopoietic functions of jujube. Jujube promotes erythropoiesis via activation of hypoxia inducible factor-induced erythropoietin, possesses potential capacity in recycling heme iron during erythrophagocytosis, exhibits bidirectional role in regulating immune response under different conditions, contains numerous minerals including iron. Red dots indicate iron, and yellow dots indicate cytokine. The arrow in red indicates the targets for jujube action. RBC, red blood cell; EPO, erythropoietin; HIF, hypoxia inducible factor; HRE, hypoxia response element; PKA, protein kinase A; CREB, cAMP response element-binding protein; FPN, ferroportin; BVR, biliverdin reductase; HO-1, heme oxygenase-1.

harvested from the cultures for PCR analysis. Jujube extract stimulated the expression of HIF-1α mRNA, and the induced expression was demonstrated to be in a dose-dependent manner, having the highest effect by ~80% of increase. In parallel, the protein level of HIF-1α in the cultures turned to increase after 2 h, and subsequently HIF-1α protein was induced by ~150% at 6 h after the treatment (Chen et al., 2014b). These results support the effect of jujube on HIF-1α expression in both mRNA and protein levels. In addition, Xu et al. (2014) reported that CREB-binding protein was required for HIF-α acetylation and efficient HIF-mediated EPO production during hypoxic stress. In consistent with this, Chen et al. (2014c) found that jujube extract (2 mg/ml) induced CREB phosphorylation in cultured cells, and this effect was fully blocked by H89, a cyclic AMP-dependent protein kinase A inhibitor. These results indicate that CREB-binding protein/HIF signaling can be involved in jujube-induced EPO production.

Jujube on Erythrophagocytosis

Erythrophagocytosis is a process, where the senescent RBCs are phagocytosed by macrophage (Klei et al., 2017). Within the macrophage, the senescent RBC undergoes hemolysis, and the components, such as heme iron, are being recycled. The reused iron will be carried back to bone marrow for erythropoiesis (Gottlieb et al., 2012). Thus, the disorders in iron recycling can result in anemia (Batchelor et al., 2020). Here, the potential effects of jujube on erythrophagocytosis were summarized (**Figure 4**).

Heme oxygenase-1 (HO-1) has a vital role in metabolizing heme to biliverdin, carbon monoxide and free iron. Biliverdin is immediately conversed to bilirubin, as catalyzed by biliverdin reductase containing two isozymes, i.e., biliverdin reductase A and B. Free iron is released to blood circulation by ferroportin and further carried to bone marrow (Kovtunovych et al., 2010). Therefore, HO-1, biliverdin reductase and ferropotin are considered as the main target enzymes in determining the iron recycling in macrophages. Jujube extract at different concentration (0–3.0 mg/ml) was applied onto cultured macrophages for 24 h. The applied jujube extract stimulated the mRNA expressions of HO-1, biliverdin reductase A and B, and ferropotin in dose-dependent manners, giving the highest response by ~2.0, 2.0, 3.0, and 4.0 folds, respectively (Chen J. et al., 2016). In good agreement with this finding, Yang et al. (2016) reported that the intake of jujube extract showed an improvement in iron deficiency anemia rats. In parallel, the extract of jujube significantly increased serum iron, iron saturation, total iron binding capacity in anemia rats, indicating the supply of circulation iron for erythropoiesis. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor, was found to regulate HO-1 expression (Motohashi and Yamamoto, 2004). Nrf2 directly binds to anti-oxidant response element (ARE) in the promoter region of *HO-1* gene resulting in the transcription. In pARE-Luc-expressed cells treated with jujube water extract, the luciferase assay was activated in a dose-dependent manner (Chen J. et al., 2016). The activation by two folds was confirmed under application of jujube extract at 3.0 mg/ml (Chen J. et al., 2016). This result suggests the involvement of Nrf2/HO-1 signaling in jujube-treated cells. In support of this notion, Almeer et al. (2018) revealed the effects of jujube extract on gene expressions of Nrf2 and HO-1 in colitis rats, as induced by treating intrarectally with acetic acid. Compared with model group, the pre-treatment with jujube extract at different doses (100, 200, and 400 mg/kg/day) in colon of rats for 5 days by oral gavage significantly induced mRNA expressions of Nrf2 and HO-1, giving the highest response by seven and two folds, respectively (Almeer et al., 2018). These studies however need further confirmation as no observation has been found in a physiology model of iron recycling. For instance, a cellular model of erythrophagocytosis using artificially-aged RBCs and macrophages may be designed to investigate mRNA and protein expressions of target enzymes relating to iron recycling.

Jujube on Immune Functions

The immune response is impaired in anemia condition. Here, the immune-modulatory properties of jujube under different scenarios are summarized (**Figure 4**). The intake of jujube extracts at concentrations of 150 and 250 mg/kg/day significantly stimulated thymus and spleen indices in mice, which indicated obviously strengthening the non-specific immunity in jujube-treated mice (Li et al., 2011). Furthermore, the authors described that jujube at various concentrations (30–200 μg/ml) showed a significant dose-dependent promotion of splenocyte proliferation, with the highest response at ~100% increase. The effect of jujube extract on anti-complementary activity was also reported. Jujube extracts (25 and 125 μg/ml) exhibited ability to interact with the complement cascade (Li et al., 2011). This finding indicates the activation of innate immune system in jujube-treated cells. In support of this notion, the water-soluble polysaccharide isolated from jujube stimulated proliferation of lymphocyte. In particular, the application of jujube polysaccharide at various concentrations (10–100 μg/ml) onto cultured lymphocytes for 3 days demonstrated an enlarging cell volume and an increasing cell number (Zhao et al., 2008). Moreover, jujube extracts at different concentrations of 0–3 mg/ml were applied onto cultured RAW 264.7 cells for 24 h. The extract dose-dependently stimulated the expressions of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, and the highest effect was induced at ~7-fold, ~9-fold, and 4-fold, respectively (Chen et al., 2014a).

The hydroalcoholic extract of jujube at 200–400 mg/kg was applied onto acute and chronic rat models of inflammation. The study revealed that jujube extract markedly declined granuloma tissue formation, as compared with model rat. Serum nitrite/nitrate level was notably up regulated in inflammatory rat; while pre-treatment with jujube significantly reduced the increased level of nitrite/nitrate (Goyal et al., 2011). These findings suggest the anti-inflammatory effects of jujube. In line with this, the applied jujube extract at 100–500 μg/ml inhibited nitric oxide production and splenocyte proliferation on the inflammatory activated cells (Yu et al., 2012). The excessive induction of pro-inflammatory cytokines, i.e., IL-1β and IL-6, also contributes to chronic inflammation. In the lipopolysaccharide (LPS)-induced macrophages, the pre-treatment with jujube water extract repressed the expressions of IL-1β and IL-6 (Chen et al., 2014a). Besides, the triterpene acid fraction of jujube at a dose higher than 10 μg/ml was able to inhibit the production TNF-α (Yu et al., 2012). NF-κB is one of main transcription factors that has vital role in controlling pro-inflammatory cytokine production (Du et al., 2013). The treatment with jujube extract slightly inhibited NF-κB activity. On the other hand, the pre-treatment with jujube water extract (0–3 mg/ml) for 3 h in cultured macrophages, before the addition of LPS at 1 μg/ml for 24 h, dose-dependently repressed the activation of NF-κB activity. The reduction at 60% was observed under the pre-treatment of extract at 3 mg/ml (Chen et al., 2014a). In parallel, the anti-inflammatory property of jujube was shown in colitis-associated colon cancer mice (Periasamy et al., 2020). In this study, mice were injected with azoxymethane followed by three cycles of dextran sulfate sodium, as to induce colitis-associated colon, before the intake of jujube extract for 70 days. The results showed that dietary jujube intake could attenuate inflammation in model mice. Moreover, the extract of jujube markedly inhibited the protein expressions of IL-6, NF-κB, JAK1, and STAT3 in colon tissues, as compared with model group. This result suggests that jujube can suppress the stimulation of NF-κB/IL-6/JAK1/STAT3 signaling (Periasamy et al., 2020).

Jujube in Blood Circulation

Blood is circulating within the blood vessels, and the effects of jujube in blood circulation are summarized here. The

pre-incubation of jujube extract (30, 100, and 300 mg/ml) for 5 min at 37°C in platelet-rich plasma was reported to suppress collagen (2 mg/ml)-, thrombin (0.4 U/ml)-, and arachidonic acid (100 mM)-induced aggregation of platelets (Seo et al., 2013). Jujuboside B from jujube markedly inhibited platelet aggregation, and which was considered as one of the active ingredients in processing anti-platelet effect (Seo et al., 2013). In animal model, angiotensin II was intravenously injected into rats to induce acute hypertension, which characterized by cardiovascular parameters, i.e., notably elevated systolic blood pressure and mean arterial pressure, as well as the decline of heart rate (HR) compared with control group. Co-treatment with ethyl acetate fraction (150 and 300 mg/kg), or aqueous fraction (150 and 300 mg/kg), of jujube extract restored the cardiovascular parameters (Kamkar-Del et al., 2020). In line with this notion, Mohebbati et al. (2018) reported protective effect of jujube extract on hypertensive rats. The rats were treated with hydroalcoholic extracts of jujube at various concentrations (from 100 to 400 mg/kg) for four weeks, and then L-NAME (10 mg/kg) was injected intravenously into rats to induce hypertension. The results showed that jujube extract attenuated blood pressure and mean arterial pressure in L-NAME-induced hypertensive rats (Mohebbati et al., 2018). Betulinic acid, found in Zizyphi Spinosi Semen and jujube, possessed combined properties of inducing endothelial nitric oxide synthase and decreasing nicotinamide adenine dinucleotide phosphate oxidase. In human endothelial cells treated with betulinic acid, the endothelial nitric oxide synthase expression and nitric oxide production were robustly increased (Steinkamp-Fenske et al., 2007). In addition, a triple-masked randomized controlled clinical trial revealed that jujube was well tolerated in general, and which might possess potential beneficial roles on serum lipid profile (Sabzghabaee et al., 2013).

Jujube in Cocktail Recipes

Jujube is not only consumed as daily food, but also prescribed as a tonic TCM for blood nourishment in a formulated decoction. Among these jujube-containing mixtures, Guizhi Tang (GZT), written by Zhang Zhongjing, a great Chinese medicine practitioner in Han Dynasty (~200 AD), composed of jujube and other four medicinal herbs is still popularly used today to deal with common cold, fever and headaches in Asian countries, including China, Japan and Korea (Yoo et al., 2016). GZT belongs to exterior-releasing formula that is able to dispel pathogenic factors from superficies of body, and the effect of jujube within this formula is believed to tonify "Qi" and to replenish "Blood" of the body resulting from corresponding pathological changes. Yoo et al. (2016) reported that application of GZT extract in cultured RAW264.7 macrophages showed anti-inflammatory activity, which involved in blocking ERK and NF-κB signaling pathways. The cells were pre-treated with GZT (31.25–1,000 μg/ml) for 4 h prior to application of LPS for an additional 20 h. The treatment with GZT extract enhanced the expression of HO-1 and significantly inhibited pro-inflammatory cytokines, e.g., TNF-α and IL-6, in LPS-induced macrophages. Besides, GZT extract prevented ERK phosphorylation and NF-κB translocation in LPS-treated macrophages (Yoo et al., 2016). In another experiment, Lam et al. (2016) investigated the inductive roles of GZT on EPO expression in cultures. Cultured Hep3B cells were treated with GZT extracts (0.5–4.0 mg/ml) for 24 h. Applied GZT was able to stimulate the mRNA and protein expressions of EPO. In addition, GZT stimulated the transcriptional activity of HRE in a dose-dependent manner (Lam et al., 2016). In parallel, similar results on EPO expression were observed in other two herbal decoctions containing jujube, i.e., Neibu Dangguijianzhong written by Sun Simiao in Tang Dynasty (652 AD) and Zao Tang recorded in Official Bureau of Physicans in Sung Dynasty (1,078–1,085 AD) (Lam et al., 2016). In Zhigancao Tang, jujube combining with ginseng was used to tonify the "Qi." Besides, jujube was proposed to regulate the relationship between protective and nutritive "Qi." Nevertheless, jujube showed similar function with other herbal formulae in treating "Qi" and "Blood" deficiency, e.g., Renshen Yangying Tang, Bazhen Tang and Xiangbei Yangying Tang.

In addition to formulated decoction, jujube is also commonly supplemented with other foods to achieve health benefits. Jeong and Kim (2019) investigated the effect of jujube and chokeberry diet in high-fat and high-fructose diet-induced dyslipidemia in animal studies. Jujube (0.5%) and chokeberry powder (0.5%) were mixed to animal diet. After 10 weeks of dietary treatment, jujube and chokeberry significantly ameliorated high-fat and high-fructose diet-induced dyslipidemia and improved insulin resistance (Jeong and Kim, 2019). In support of this finding, the consumption of mixed jujube, almond and rice in healthy human showed a significantly lower glucose level, as compared to those with rice as reference (Zhu et al., 2018).

Future Opportunities

Herbal cuisine is a practice in achieving the therapeutic functions by using natural herbs, especially the edible and medicinal dual-purpose herbs as materials during cooking processes. Jujube has been considered as a favorite fruit in daily life for its health properties spanning thousands of years. In practice of herbal cuisine, jujube is one of common materials that is considered as boosting or nourishing type of food. It can be taken into decoction for daily consumption, or which can be taken together with other foods to prepare delicious soup. According to the aforesaid cellular and animal findings, jujube has a promising potential in developing medicinal food and supplement for prevention against anemia, cancer, inflammation and iron/vitamin deficiency.

Jujube-containing herbal decoctions are routinely recorded in *Jingui Yaolue* by Zhang Zhongjing, which are prescribed to address various ailments. One-sixth of prescriptions described in this ancient classic book contain jujube, in which jujube is commonly served as assistant or courier medicinal herb with a herbal formulated decoction, according to the theory of TCM. The intake of jujube is believed to increase blood supply to the spleen meridian that further improves nutrient uptake and strengthens the immune system. Clinically, several controlled trials have revealed that jujube is a safe and effective herb for human consumption (Naftali et al., 2008; Ebrahimimd et al., 2011; Sabzghabaee et al., 2013); however, there is currently no human study on the blood deficiency effects. In addition, there

are no known toxicity and drug interaction being reported clinically for consumption of jujube.

Apart from the medicinal application, fresh immature jujubes are widely consumed as fruits, and the dried fruits are also eaten as a snack, or with tea. In China, jujube has been made into a wide range of products, i.e., juice, vinegar and wine. In southern part of India, jujube is mixed with tamarind, jaggery, salt and chilies, and then pounded into cakes. In Lebanon and Persia, jujube is used as digestive aid being consumed with the desserts. In Morocco, the honey obtained from jujube extract is believed to be beneficial to sore throats (Lim, 2013). Additional of natural herbal extracts to dairy products has increasingly popular because of their health benefits. Feng et al. (2019) demonstrated a new goat dairy product adding of jujube pulp as ingredients with satisfactory nutritional quality and sensory property, which provided alternative approach in developing a unique goat dairy product with high nutritional properties. The water extract of immature jujube extract showed better activity in stimulating transcriptional activity of HRE than that of mature jujube (Chen et al., 2015). These findings indicate the maturity of jujube should therefore be taken into consideration when it is being prepared for health food supplements.

In folk medicine, three pieces of jujube are recommended to be consumed daily, about 15 g of dried weight in total. The content of benefit ingredients within jujube could be changed robustly between fresh jujubes and dried ones (Guo et al., 2015; USDA, 2012; Chen et al., 2013). The dietary nutrient facts of main ingredients in fresh and dried jujubes are summarized in **Supplementary Table S1**, which provides an appropriate recommendation for selection of different forms of jujube for certain health benefits. In addition, it has been reported that the content of bio-active ingredients, including nucleotide, flavonoid and polysaccharide, varied among different jujube cultivars (Chen et al., 2013), as indicated in **Supplementary Table S2**. Jujubes from Shanxi, Shaanxi, Hebei, Xinjiang, Shandong, Ningxia provinces of China had higher chemical amounts, which might contribute better biological functions and could be a good choice of selection.

AUTHOR CONTRIBUTIONS

JC and KT: Concept, design, literature search and manuscript review. JC: acquisition of data, drafting the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.593655/full#supplementary-material

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Short Chain (≤C4) Esterification Increases Bioavailability of Rosmarinic Acid and Its Potency to Inhibit Vascular Smooth Muscle Cell Proliferation

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Rosmarinic acid is a natural phenolic acid and active compound found in many culinary plants, such as rosemary, mint, basil and perilla. Aiming to improve the pharmacokinetic profile of rosmarinic acid and its activity on vascular smooth muscle cell proliferation, we generated a series of rosmarinic acid esters with increasing alkyl chain length ranging from C1 to C12. UHPLC-MS/MS analysis of rat blood samples revealed the highest increase in bioavailability of rosmarinic acid, up to 10.52%, after oral administration of its butyl ester, compared to only 1.57% after rosmarinic acid had been administered in its original form. When added to vascular smooth muscle cells *in vitro*, all rosmarinic acid esters were taken up, remained esterified and inhibited vascular smooth muscle cell proliferation with IC_{50} values declining as the length of alkyl chains increased up to C4, with an IC_{50} of 2.84 μM for rosmarinic acid butyl ester, as evident in a resazurin assay. Vascular smooth muscle cells were arrested in the G_0/G_1 phase of the cell cycle and the retinoblastoma protein phosphorylation was blocked. Esterification with longer alkyl chains did not improve absorption and resulted in cytotoxicity in *in vitro* settings. In this study, we proved that esterification with proper length of alkyl chains (C1–C4) is a promising way to improve *in vivo* bioavailability of rosmarinic acid in rats and *in vitro* biological activity in rat vascular smooth muscle cells.

Keywords: vascular smooth muscle cells, rosmarinic acid, proliferation, pharmacokinetics, rosmarinic acid esters

Abbreviations: AUC, area under the curve; Cmax, maximum plasma concentration, CL, clearance; CDK, cyclin-dependent kinase; DMAP, 4-dimethylamino-pyridine; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; F, bioavailability; IS, internal standard; LDH, lactate dehydrogenase; MRM, multiple reaction monitoring; PDGF, platelet-derived growth factor; PI, propidium iodide; VSMC, vascular smooth muscle cell; RA, rosmarinic acid; RABU, rosmarinic acid butyl ester; RAET, rosmarinic acid ethyl ester; RAME, rosmarinic acid methyl ester; RADOD, rosmarinic acid dodecyl ester; RAOCT, rosmarinic acid octyl ester; Rb, retinoblastoma; T_{max}, time to reach $C_{max;}$ $T_{1/2}$, plasma half-life; V_d, apparent volume of distribution.

INTRODUCTION

Blood vessel wall disorders like atherosclerosis and restenosis are characterized by a switch of quiescent vascular smooth muscle cells (VSMC) into a proliferative and synthetic phenotype. Platelet-derived growth factor (PDGF) is a powerful stimulator of VSMC migration and proliferation. Identification of compounds counteracting the PDGF-induced VSMC proliferation would be an effective approach for ameliorating these vessel wall disorders (Levitzki, 2004).

Aside from having nutritional value, food and spices are excellent sources for lead structure identification. We previously found that polyphenols from Mediterranean spices could inhibit VSMC proliferation, especially rosmarinic acid (RA) and its congeners. Among the 12 tested constituents, rosmarinic acid methyl ester (RAME) showed the best anti-proliferative activity in VSMC, even more potent than RA, and it inhibited neointima formation *in vivo* (Liu et al., 2018).

RA is an ester of caffeic (1) and 3,4-dihydroxyphenyl lactic acid (5). Besides chlorogenic acid, it represents one of the most frequently occurring caffeic acid esters in the whole plant kingdom (Petersen, 2013). The most prominent plant families containing RA are *Boraginaceae* and *Lamiaceae* (sub-family *Nepetoideae*). RA is an integral part of the daily human diet, found as well in food supplements to act preventive or therapeutic against various diseases. It shows extensive pharmacological activities, e.g. antioxidant, anti-inflammatory, antiviral and cardioprotective (Nunes et al., 2017). However, poor oral bioavailability and marked metabolism impeded exploitation of RA as a therapeutic agent. Different pharmacokinetic studies in rats and humans showed that only a small amount of orally ingested RA, up to 1.69%, is absorbed, presumably in the upper intestine (Nakazawa and Ohsawa, 1998; Baba et al., 2005; Wang et al., 2017), and in part in its conjugated form (Baba et al., 2005; Noguchi-Shinohara et al., 2015). Similar reports on low bioavailability of RA were obtained from absorption studies using the Caco-2 cell model (Qiang et al., 2011; Villalva et al., 2018). Unabsorbed RA seems to reach the colon, where it is hydrolyzed by gut bacteria into metabolites, like caffeic acid (1) and 3,4-dihydroxyphenyl lactic acid (2) (Nakamura et al., 1998; Bel-Rhlid et al., 2009; Zoric et al., 2016) as depicted in **Figure 1**. Other metabolites were also reported after ingestion of RA in rat and human studies, like intact and conjugated forms of methylated RA (Baba et al., 2005), *m*-coumaric acid (3), ferulic acid (4) (Nakazawa and Ohsawa, 1998; Baba et al., 2005) and *m*-hydroxyphenylpropionic acid (5) (Nakazawa and Ohsawa, 1998; Mosele et al., 2014). Cumulative proportions of all RA metabolites detected in urine corresponded to about 32% and 6% of the administered dose in rat (Nakazawa and Ohsawa, 1998) and human (Baba et al., 2005) studies, respectively. However, to what extent so far identified metabolites account for the biological effects of RA has been largely underexplored.

The high hydrophilicity is one of the main reasons for RA's poor bioavailability. Ester-containing small molecules are common prodrugs, as exemplified by acetylsalicylic acid (aspirin). Esterification of plant polyphenols can ameliorate their strong resistance to 1st-pass effects during absorption, as shown in the case of quercetin (Biasutto et al., 2007; Hu et al., 2016).

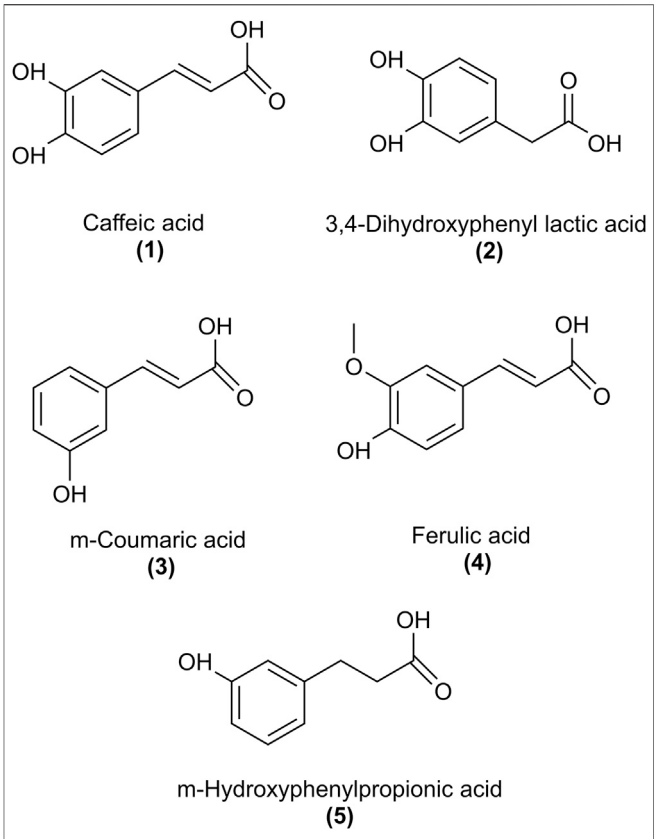

FIGURE 1 | Structures of rosmarinic acid metabolites that were examined for the inhibition of PDGF-BB-induced VSMC proliferation.

Prompted by our previous findings that the methyl-ester of RA was more potent against PDGF-induced VSMC proliferation than RA (Liu et al., 2018), we hypothesized that esters of RA may serve as prodrugs with increased cell permeability *in vitro*, higher bioavailability *in vivo* and subsequent higher intracellular levels of bioactive RA. A series of RA (6) alkyl esters was synthesized: methyl (7), ethyl (8), butyl (9), octyl (10) and dodecyl (11), as shown in **Figure 2**, and the effect of the alkyl chain length on bioavailability of RA *in vivo* as well as cell permeability and antiproliferative activity in VSMC *in vitro* were investigated. Moreover, known RA metabolites were tested for inhibition of VSMC growth *in vitro*.

MATERIALS AND METHODS

Materials

Rosmarinic acid, caffeic acid, ferulic acid, *m*-coumaric acid and 3,4-dihydroxyphenyl lactic acid were purchased from Victory Biological Technologies (Sichuan, China, purity of compounds as stated by the company was >95%), whereas *m*-hydroxyphenylpropionic acid (purity obtained by HPLC was 99.8%, as stated by the company's COA) was from Sigma-

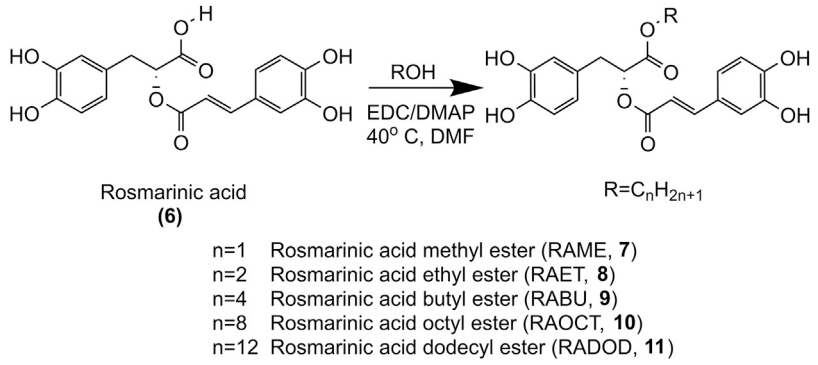

FIGURE 2 | Synthesis of methyl, ethyl, butyl, octyl and dodecyl RA esters.

Aldrich (MO, United States). Methyl, ethyl, butyl, octyl and dodecyl RA esters were synthesized in our laboratory (Key Laboratory of Molecular Pharmacology and Drug Evaluation, University of Yantai, China), and their structures were confirmed by high-resolution MS data and NMR spectroscopic data (see **Supplementary Material**). The purity of synthesized compounds was analyzed on Waters Acquity I-Class UHPLC system, using chromatographic column Waters BEH C18 (2.1 × 50 mm, 1.7 μm) and water (V:V; 0.1% formic acid) as mobile phase A and acetonitrile (V:V; 0.1% formic acid) as mobile phase B. Elution was conducted under a flow rate of 0.3 ml/min and conditions as follows: 0–1 min, 5%–15% B; 1–3 min, 15% B; 3–3.5 min, 15%–25% B; 3.5–4 min, 25% B; 4–4.5 min, 25%–40% B; 4.5–6 min, 40%–70% B; 6–7 min, 70% B; 7–7.5 min, 70%–95% B; 7.5–8 min, 95% B; 8–8.1 min, 95%–5% B; 8.1–13 min, 5% B. The absorption was detected at 330 nm. The determined purities of RA esters were: 98.68% for methyl-, 96.82% for ethyl-, 95.46% for butyl-, 96.46% for octyl- and 97.86% for dodecyl-ester, and the respective chromatograms are depicted in **Supplementary Figure S1**.

Silibinin, used as an internal standard (IS), was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all reference standards were determined to be over 95% by HPLC-UV. HPLC-grade reagents were obtained from Fisher Scientific (Fairlawn, NJ).

Primary rat aortic VSMC, growth media, and cell culture supplements were purchased from Lonza (Basel, Switzerland). Serum for cell culture was supplied from Gibco Life Technologies (Darmstadt, Germany), and PDGF-BB was obtained from Bachem (Weilheim, Germany).

All other used reagents and chemicals were of analytical grade and obtained from Sigma–Aldrich (MO, United States). The monoclonal anti-phospho-Rb (Ser807/811), the anti-α/β-tubulin and the secondary horseradish-peroxidase-coupled antibody were all from Cell Signaling (Leiden, Netherlands).

Synthesis of Rosmarinic Acid Alkyl Esters

The synthesis of RAME (7), RA ethyl ester (RAET, 8), RA butyl ester (RABU, 9), RA octyl ester (RAOCT, 10) and RA dodecyl ester (RADOD, 11) were carried out by esterification of RA (6) with corresponding alcohols (methanol, ethanol, n-butanol, n-octanol, n-dodecanol) respectively, as shown in **Figure 2**. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 4-dimethylamino-pyridine (DMAP) were used as catalysts, DMF was the solvent. The products were purified by liquid-liquid extraction and column chromatography. The structures of RA alkyl esters were confirmed by high-resolution MS data and NMR spectroscopic data. High-resolution MS data was recorded on a Q Exactive Orbitrap MS system at a resolution of 70,000 FWHM (Thermo scientific, Waltham, MA, United States). 1H NMR data was measured with a Bruker AVANCE 400 NMR spectrometer at 400 MHz (Bruker, Fallanden, Switzerland). Mass errors obtained from high-resolution MS data of these RA esters were all within 5 ppm (**Supplementary Figure S2**). 1H NMR data (**Supplementary Figures S3–S7**) obtained from methyl, ethyl, butyl, octyl and dodecyl RA esters was consistent with those previously reported (Hamada and Abdo, 2015; Wicha et al., 2015; Thammason et al., 2018).

Bioavailability and Pharmacokinetics of Rosmarinic Acid and Its Esters in Rats

Animal Experiment

Male Sprague–Dawley rats (220–250 g) were purchased from Jinan Peng Yue Experimental Animal Breeding Co., Ltd (Shandong, China). Rats were housed under standard conditions with free access to food and deionized water. All animal experimental protocols were approved by the Ethics Committee of Yantai University (IACUC No. 2018-DA-12) and conducted according to the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). Rats were fasted overnight with free access to water before animal experiments. Animals were randomly divided into twelve groups (three rats per group). All compounds were administered as a single dose orally (80 μmol/kg) and as an intravenous injection via tail vein (1 μmol/kg). Compounds were suspended in DMSO: 1% CMC-Na (5:95, v/v) for oral administration and dissolved in DMSO: 0.5% tween 80 (5:95,

v/v) as a clear solution for intravenous administration, respectively. Approximately 250 μL blood samples were collected from the orbital veins in heparinized tubes before and 0.033, 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 8 h after drug administration. Blood samples were centrifuged at 8,000 rpm, 4°C for 10 min, and plasma was collected and stored at −20°C until analysis.

Sample Preparation

Plasma sample (50 μl) was mixed with 5 μl internal standard solution (1 μg/ml silibinin in methanol) and 145 μl 0.05% formic acid-methanol for protein precipitation. After vortexing for 30 s, the mixture was centrifuged at 13,000 rpm, 4°C for 10 min, and 5 μl of supernatant were injected into UHPLC-MS/MS for analysis.

Preparation of Calibration Standards, and Quality Control (QC) Solutions

The stock solution of RA (5 mg/mL) was prepared in methanol and further diluted to 20 μg/ml with water: methanol (1:1, v/v, 0.1% formic acid). The stock solution of IS (silibinin, 2 mg/mL) was prepared in DMSO and further diluted to 1 μg/mL with methanol. Both stock solutions were stored at −20°C before analysis. The working solution of RA was obtained by serial dilutions of stock solution with water: methanol (1:1, v/v, 0.1% formic acid). Then, 5 μl working solutions were added to 45 μl blank plasma and acquired final plasma concentrations at 1, 5, 10, 50, 100, 500, 1,000, 2000 ng/mL for the calibration standards and 2, 40, 800 ng/mL for QC solutions. Finally, solutions were treated in the same manner as sample preparation.

UHPLC-MS/MS Conditions

The UHPLC-MS/MS analysis was performed on a Shimadzu LC-30AD system (Shimadzu Corporation, Kyoto, Japan) coupled with an Applied Biosystems Sciex 4,500 triple quadrupole MS/MS system (AB Sciex, Foster City, CA, United States). The separation was performed on an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters) with a Van Guard pre-column at 40°C. The mobile phase consisted of water (0.1% formic acid, v/v) as solvent A and acetonitrile (0.1% formic acid, v/v) as solvent B. The linear gradient elution steps were as follows: 0–0.5 min, 15–65% B; 0.5–1.4 min, 65% B; 1.4–1.41 min, 65–90% B; 1.41–1.80 min, 90% B; 1.80–1.81 min, 90–15% B; 1.81–2.3 min, 15% B. During 0–0.8 min, the flow was moved into waste. The flow rate and the autosampler temperature were set at 0.3 ml/min and 4°C, respectively. Biological samples were analysed in negative ion mode using an ESI source. The optimized multiple reaction monitoring (MRM) transitions were m/z 359.0–161.0 for RA, and m/z 481.0–301.0 for IS. Other relevant mass spectrometer parameters were as follows: ion spray voltage, −4500 V; source temperature, 550°C; curtain gas, 10 psi; ion source gas1, 55 psi; ion source gas2, 55 psi; collision gas, 8 psi; entrance potential, −10 V; collision cell exit potential, −11 V. Quantification of analytes was conducted using Analyst 1.6.3 and MultiQuant 3.0.2 softwares (AB Sciex, Foster City, CA, United States).

Method Validation

In order to prove the authenticity, reliability and reproducibility of the experimental data, the full bio-analytical method validation was carried out according to the US Food and Drug Administration (US-FDA) and European Medicines Agency guidance on bio-analytical method validation.

Data Analyses

Pharmacokinetic parameters of RA and its esters were calculated using the non-compartmental method of Phoenix WinNonlin 8.1 software (Pharsight, Mountain View, CA, United States). The absolute oral bioavailability was calculated using the following equation:

$$F\ (\%) = \frac{(AUC\ p.o.\ \times\ Dose\ i.v.)}{(AUC\ i.v.\ \times\ Dose\ p.o.)} \times\ 100\%$$

Biological Evaluation of Rosmarinic Acid Esters as Inhibitors of Vascular Smooth Muscle Cell Proliferation

Cell Culture

Primary VSMC were cultivated in DMEM-F12 (1:1) supplemented with 20% fetal bovine serum, 30 μg/ml gentamicin, and 15 ng/mL amphotericin B at 37°C in an incubator with 5% CO_2 flow in a humidified atmosphere. Passages 4 to 12 were used in experiments.

Proliferation Assay

Resazurin conversion to fluorescent resorufin was used as a measure for cell proliferation. Primary VSMC were seeded at 5×10^3 cells/well in a 96-well plate to grow for the next 24 h. Cells were then synchronized into G_0 phase by serum deprivation for 24 h, pretreated with compounds for 30 min and stimulated with 20 ng/mL PDGF-BB for the next 48 h. After one washing step, cells were incubated in starvation medium containing 10 μg/mL resazurin for 2 h, and changes in fluorescence were monitored in a Tecan Spark (Tecan Group, Männedorf, Switzerland) plate reader at an excitation wavelength of 535 nm and an emission wavelength of 580 nm.

Cytotoxicity Assay

Increased lactate dehydrogenase (LDH) release is an indicator for the loss of cell membrane integrity, associated with cell death. Determination of LDH release in response to increasing concentrations of RAME (7), RAET (8), RABU (9), RAOCT (10) and RADOD (11) was performed as previously described (Liu et al., 2018).

Cell Cycle Analysis

Flow cytometry was used to analyze cell cycle progression. VSMC were seeded at a density of 10^5 cells per well in 12-well plates. After rendered quiescent, cells were treated with increasing concentrations of RAME (**7**), RAET (**8**), RABU (**9**) and RAOCT (**10**) or with 0.1% DMSO for 30 min followed by stimulation with 20 ng/mL PDGF-BB for 16 h. Cells were

collected by trypsinization, washed and resuspended in a hypotonic propidium iodide (PI) solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/mL PI. PI-stained nuclei were analyzed on a FACSCalibur (BD Biosciences, Vienna, Austria) flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 585 nm.

SDS-PAGE and Immunoblot Analysis

VSMC were seeded in 6-well plates at 2.5×10^5 cells per well and cultivated for 24 h. Cells were serum-deprived for another 24 h, then pretreated for 30 min with RAET, RABU, RAOCT (all at 10 μmol/L) or vehicle (0.1% DMSO) and subsequently incubated with or without PDGF (20 ng/mL) for the indicated time. Afterward, cells were lysed with an ice-cold lysis buffer (50 mmol/L HEPES, 50 mmol/L NaCl, 10 mmol/L DTT, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7 \times 10\ H_2O$, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4), supplemented with 1 mmol/L PMSF, 1 × Complete™ (Roche Applied Science), and 1% (v/v) TritonX-100. Lysates were centrifuged at 5,600 × g at 4°C for 20 min, and supernatants were used for protein denaturation in 3 × SDS sample buffer for 8–10 min at 95°C. Protein concentrations were determined using Rotiquant reagent according to the manufacturer's instructions (Carl Roth). Protein extracts (10 μg) were subjected to SDS-PAGE and immunoblot analysis. All antibodies were diluted as recommended by the providing company. Proteins were visualized using enhanced chemiluminescence reagent and quantified using a LAS-3000 luminescent image analyzer (Fujifilm) with AIDA software (Raytest).

Determination of Intracellular Bioavailability of RA Esters

After cells had been lysed and membranes had been removed by centrifugation, as described in the previous section, half of the cytoplasmic fractions were collected in 1.5 mL eppendorf tubes, diluted with methanol in 1:3 ratios and centrifuged at 5,600 × g for 20 min to remove proteins. Supernatants were then subjected to LC-MS/MS analysis to determine the cytoplasmic concentrations of RA esters at each treatment time point up to 16 h PDGF-BB stimulation. Cell culture media were also collected prior to the lysis procedure, diluted with methanol in a manner described above and subjected to LC-MS/MS. It was monitored whether the sum of cell lysate and culture medium concentrations of RA esters at the beginning of the experiment do not extensively deviate from treatment concentrations. Intracellular concentrations of RA esters were normalized to protein concentrations at each treatment time point. Details of the quantification method are shown in the **Supplementary Material**.

Statistical Analysis

Statistical analysis was performed using ANOVA/Bonferroni test. Data in the figures represent mean ± SD, and the number of experiments is given in the figure legends. All statistical analysis was performed using GraphPad PRISM 6 software, and a probability value <0.05 was considered significant.

RESULTS

In vivo Bioavailability of Rosmarinic Acid and Its Esters

First, chemical stability and plasma stability of RA esters were assessed in solution (50% methanol with 0.1% formic acid) and in fresh rat plasma. The alkyl esters of RA remained stable during 5 h at room temperature in solution, while they converted to RA instantly in fresh plasma due to enzymatic hydrolysis (data not shown). As shown by others for ester-prodrugs of indomethacin and fusidic acid (Takahashi et al., 2018; Strydom et al., 2020), the alkyl esters of RA in our study were also hydrolyzed to form RA *in vivo*. Therefore, we developed and validated an UHPLC-MS/MS method to analyze the plasma concentration of RA after intravenous and oral administration of RA, RAME, RAET, RABU, RAOCT, RADOD in rats.

The method for determining RA in plasma was validated for selectivity, linearity, low limit of quantification (LLOQ), precision, accuracy, recovery, matrix effect, and stability. Details of the method validation are presented in the **Supplementary Material**. Typical chromatograms are shown in **Supplementary Figure S8**, with no endogenous interfering peaks in the chromatograms of blank plasma at retention times of RA and IS. The method displayed good linearity in the range of 1–2000 ng/mL with the correlation coefficient greater than 0.999. The LLOQ was 1 ng/mL with the signal-to-noise ratio of >10:1. Precision, accuracy, recovery and matrix effect of RA in rat plasma are shown in **Supplementary Table S1**. Stability of RA under different storage conditions is shown in **Supplementary Table S2**.

The plasma concentrations of RA after the intravenous (1 μmol/kg) and oral (80 μmol/kg) administrations of RA and its esters were determined by the validated LC-MS/MS method. All mean plasma concentration-time profiles are shown in **Figure 3**. The main pharmacokinetic parameters including area under the curve (AUC), maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), plasma half-life ($T_{1/2}$), apparent volume of distribution (V_d), clearance (CL), and bioavailability (F) are shown in **Table 1**. After oral administration of RA (**6**), RAME (**7**), RAET (**8**), RABU (**9**), RAOCT (**10**), RADOD (**11**) at 80 μmol/kg, the C_{max} of RA were 0.55 ± 0.16, 9.81 ± 1.18, 8.51 ± 1.53, 10.98 ± 1.13, 0.84 ± 0.30 and 0.04 ± 0.01 μmol/L, respectively. The absolute bioavailability of RA after RA, RAME, RAET, RABU, RAOCT and RADOD administration were 1.57%, 3.30%, 9.65%, 10.52%, 1.93% and 0.22%, respectively. With the increase of the alkyl chain length, T_{max} and $T_{1/2}$ did not show obvious changes, while the C_{max} and bioavailability values increased only up to C4 alkyl chain. When applied in the form of the butyl-ester (RABU), the C_{max} and the bioavailability of RA increased around 20- and 7-fold, respectively.

Rosmarinic Acid Esters, Not Metabolites, Suppress the PDGF-Induced Vascular Smooth Muscle Cell Proliferation

The antiproliferative effect of RA and, in particular, of its methyl ester (RAME) in VSMC was described in an earlier study (Liu

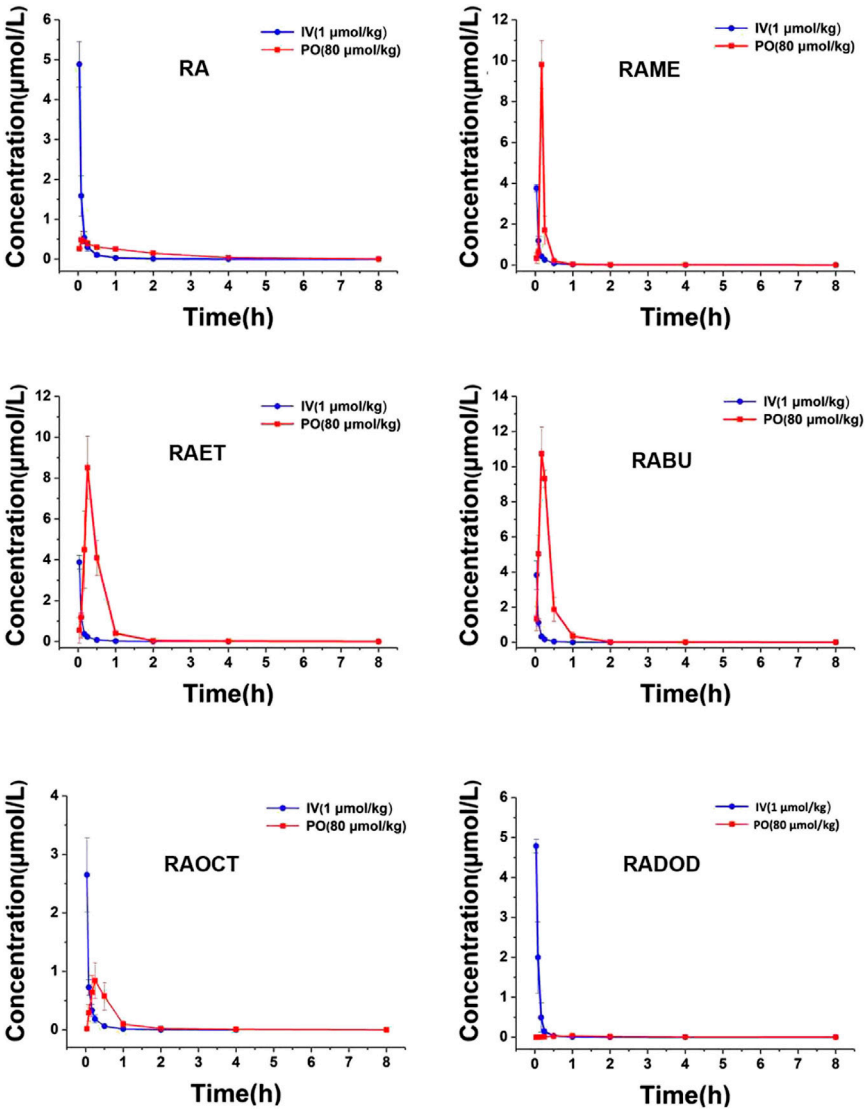

FIGURE 3 | Mean rat plasma concentration-time curves of RA after intravenous (1 μmol/kg) and oral (80 μmol/kg) administration of RA, RAME, RAET, RABU, RAOCT, RADOD, respectively. Data are presented as mean ± SD, n = 3.

et al., 2018). We, therefore, decided to examine: 1) whether an increase in the length of an alkyl chain could enhance the potency of RA esters in VSMC and 2) whether other known RA metabolites (Baba et al., 2005) (**Figure 1**) might have an effect on VSMC proliferation as well. RA (6) and its esters RAME (7), RAET (8), RABU (9), RAOCT (10) and RADOD (11), as well as RA metabolites: 2, 3, 4 and 5 were all tested at 10 μmol/L in PDGF-BB-activated VSMC using the resazurin assay. RA metabolites, including 1 at 50 μmol/L, did not render active at tested concentrations, whereas all the tested esters of RA completely blocked the PDGF-triggered increase of VSMC metabolic activity, as surrogate indicator for proliferation (**Table 2**). A consistent reduction in VSMC biomass by the RA esters was observed in the crystal violet assay (data not shown). Determining the IC_{50} values of RA and of each of the tested RA esters revealed a decrease in IC_{50} from 7.9 μmol/L in RAME-treated to 3.37 μmol/L in RAET-treated VSMC. A further slight decrease in IC_{50} was observed by extending the alkyl chain of the ester group up to C8-ester (**Table 2**). In the case of the RADOD, VSMC proliferation was unaffected at 1 μmol/L, but completely abrogated at 3 μmol/L of concentration, which indicated a possible toxic effect of this compound in VSMC (data not shown). Therefore, we examined the LDH release in VSMC treated with all five RA esters. RAME (7), RAET (8) and RABU (9) did not induce cell death up to 30 μmol/L, whereas RAOCT (10)-treated VSMC showed a marked but not significant increase in LDH production at 30 μmol/L (**Figure 4**). RADOD (11)-induced cell death at 30 μmol/L was comparable to that of the positive control, digitonin. At lower concentrations, 3 and 10 μmol/L, RAOCT also induced a distinct LDH release, which however did not reach significance (**Figure 4**). Due to the

TABLE 1 | Pharmacokinetic parameters of RA after intravenous (1 μmol/kg) and oral (80 μmol/kg) administration of RA or its alkyl esters.

Parameters[a]		AUC_{0-t} (h*μmol/L)	$AUC_{0-\infty}$ (h*μmol/L)	C_{max} (μM)	T_{max} (h)	$T_{1/2}$ (h)	V_d (L/kg)	CL (L/h/kg)	F (%)
RA (6)	iv	0.64 ± 0.09	0.64 ± 0.09	–[b]	–	0.22 ± 0.07	0.49 ± 0.08	1.58 ± 0.21	–
	po	0.78 ± 0.09	0.80 ± 0.07	0.55 ± 0.16	0.17 ± 0.08	1.31 ± 0.23			1.57
RAME (7)	iv	0.50 ± 0.04	0.50 ± 0.04	–	–	0.33 ± 0.01	0.97 ± 0.09	2.01 ± 0.15	–
	po	1.30 ± 0.15	1.32 ± 0.15	9.81 ± 1.18	0.17 ± 0	1.04 ± 0.11			3.30
RAET (8)	iv	0.49 ± 0.01	0.49 ± 0.01	–	–	0.30 ± 0.08	0.87 ± 0.25	2.02 ± 0.03	–
	po	3.81 ± 0.36	3.82 ± 0.37	8.51 ± 1.53	0.25 ± 0	0.51 ± 0.09			9.65
RABU (9)	iv	0.46 ± 0.10	0.46 ± 0.10	–	–	0.25 ± 0.07	0.80 ± 0.16	2.27 ± 0.58	–
	po	3.86 ± 0.28	3.86 ± 0.27	10.98 ± 1.13	0.19 ± 0.05	0.53 ± 0.10			10.52
RAOCT (10)	iv	0.36 ± 0.09	0.36 ± 0.09	–	–	0.49 ± 0.06	1.99 ± 0.32	2.91 ± 0.87	–
	po	0.55 ± 0.20	0.56 ± 0.19	0.84 ± 0.30	0.25 ± 0	0.62 ± 0.15			1.93
RADOD (11)	iv	0.56 ± 0.07	0.57 ± 0.07	–	–	0.22 ± 0.02	0.56 ± 0.09	1.79 ± 0.22	–
	po	0.09 ± 0.02	0.10 ± 0.02	0.04 ± 0.01	1.00 ± 0	1.78 ± 0.20			0.22

Data are presented as mean ± SD.

[a]AUC0-t, area under the curve from zero to the last measurable time; AUC0-∞, area under the curve from zero to time infinity; Cmax, maximum plasma concentration; Tmax, time to reach Cmax; T1/2, plasma half-life; Vd, apparent volume of distribution; CL, clearance; F, bioavailability.

[b]not available.

TABLE 2 | Antiproliferative effects of RA esters and metabolites on VSMCs quantified by the resazurin conversion assay.

Compound	VSMC proliferation at 10 μmol/L (relative to vehicle control, RU)	IC50 (μmol/L)
RA (6)	0.781 ± 0.167[n.s.]	n.d.
RAME (7)	0.415 ± 0.109[a]	7.90
RAET (8)	0.328 ± 0.081[a]	3.37
RABU (9)	0.308 ± 0.052[a]	2.84
RAOCT (10)	0.338 ± 0.147[a]	2.22
RADOD (11)	0.054 ± 0.081[a]	n.d.
1 (50 μmol/L)	0.922 ± 0.124[n.s.]	–
2	0.940 ± 0.220[n.s.]	–
3	1.072 ± 0.152[n.s.]	–
4	1.068 ± 0.173[n.s.]	–
5	1.205 ± 0.278[n.s.]	–

Results are presented as mean ± SD, relative to the PDGF-BB-stimulated vehicle control, and IC_{50} values were determined if the compound (10 μmol/L) showed a relative inhibitory action lower than 0.75 RU. All values were obtained from a minimum of three independent experiments.

[a]p < 0.001; n.s. not significant compared to vehicle control.

–not tested; n.d.: unable to calculate.

exhibited cytotoxic effect, RADOD (11) was excluded from further *in vitro* experiments.

Ethyl-, Butyl and Octyl Esters Have the Same Mode of Action as the Methyl Ester of Rosmarinic Acid in Vascular Smooth Muscle Cells

We reported previously that RAME induces a G_0/G_1 cell cycle arrest in PDGF-induced VSMC and suppresses retinoblastoma protein (Rb) phosphorylation, presumably by inhibiting the activity of the cyclin-dependent kinase 2 (CDK2) (Liu et al., 2018). To examine in what manner esters of RA with longer alkyl chains affect cell cycle progression, we subjected the PDGF-BB-induced VSMC treated with increasing concentrations of RAME (**7**), RAET (**8**), RABU (**9**) and RAOCT (**10**) to PI staining and flow cytometry analysis. While control VSMC markedly progressed into G_2/M phase of the cell cycle after 16 h PDGF stimulation, all tested RA esters applied at 10 μmol/L arrested VSMC in G_0/G_1 phase (**Supplementary Figure S9** and **Figure 5A**). Furthermore, treatment with RABU and RAOCT at 3 μmol/L resulted in a significantly higher percentage of cells in G_0/G_1 compared to vehicle control (**Figure 5A**). Rb protein phosphorylation at $Ser^{807/811}$ primes the protein for further phosphorylation steps, which are a prerequisite for S-phase entry (Rubin, 2013). To examine whether the esters of RA affect the phosphorylation at $Ser^{807/811}$, we performed a time-course experiment with ethyl-, butyl- and octyl ester in PDGF-BB-stimulated VSMC and a subsequent western blot analysis. All of the three tested RA esters prevented Rb protein phosphorylation at $Ser^{807/811}$ that had been markedly increased after 8 and 16 h of PDGF-BB stimulation (**Figure 5B**). These data strongly indicate that ethyl-, butyl and octyl esters exhibit the same mode of action as previously reported for the methyl ester of RA (Liu et al., 2018).

Rosmarinic Acid Esters Are Absorbed in Cultured Vascular Smooth Muscle Cells in Their Original Esterified Form

Next, we examined whether RA esters exert their intracellular effects as esters or whether they become hydrolyzed into RA. This was achieved by analyzing the contents of RA and its esters in cytoplasmic fractions of VSMC treated with 10 μmol/L concentration of RAME (**7**), RAET (**8**), RABU (**9**) and RAOCT (**10**) for 0.5, 6.5, 8.5 and 16.5 h by LC-MS/MS. Since only the respective RA esters but no free RA could be successfully identified and quantified both in cell lysates and in culture media supernatants at any treatment time point, we conclude that the active principle of RA esters in an *in vitro* setting of cultured VSMC lies in their esterified form. Intracellularly accumulated concentrations after 0.5 h

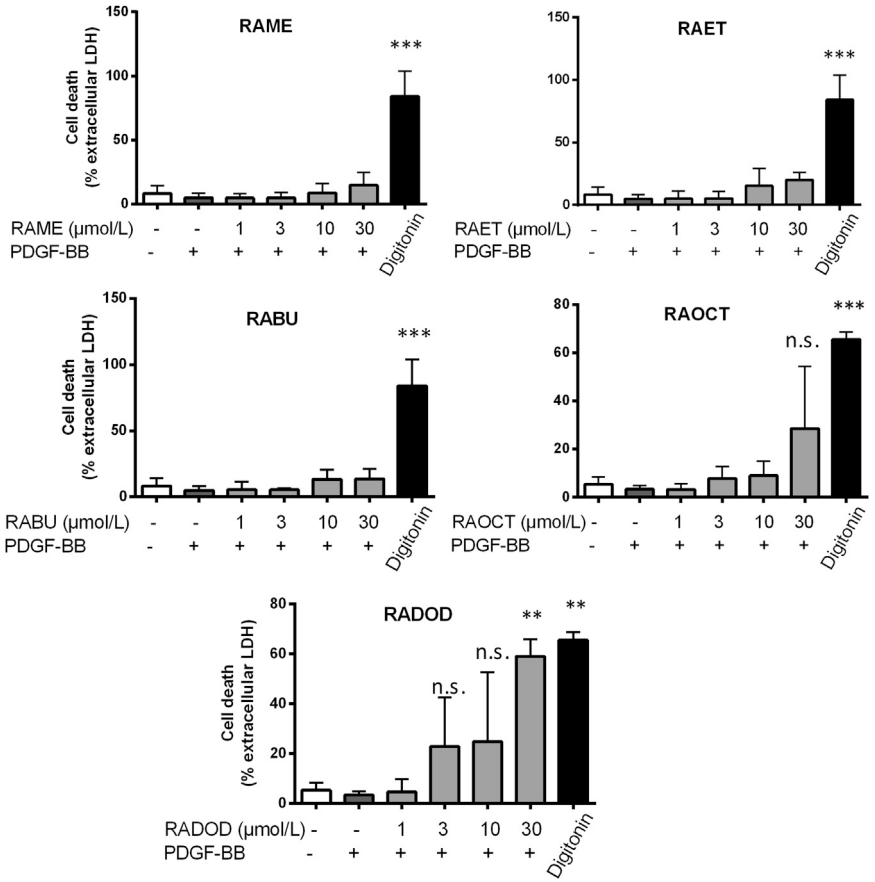

FIGURE 4 | Cytotoxicity of RA esters in PDGF-induced VSMC. VSMC were pretreated with 0.1% DMSO or increasing concentrations of RA esters as indicated for 30 min, stimulated with 20 ng/mL PDGF-BB for 24 h, and released LDH was determined. Digitonin (80 µmol/L) was used as a positive control. Graphs show means ± SD out of 3 (RAME, RAET and RABU) and 4 (RAOCT and RADOD) independent experiments (n.s., not significant, $p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA/Bonferroni, "vehicle with PDGF-BB" vs. "RA ester with PDGF-BB").

exposure to 10 µmol/L of RABU and of RAOCT were about 6 µmol/L, and 3-fold higher than those of RAME and RAET (**Figure 6A**). We then normalized the detected cytoplasmic concentration of each RA ester to the protein content of the respective cell lysate to account for possible changes in cell number (**Figure 6B**). This normalization step did not tremendously affect the trend of intracellular RA ester bioavailability over time. However, it produced a more obvious correlation between increased alkyl chain length and initial intracellular bioavailability (**Figure 6B**). The intracellular contents of RAME, RAET and RABU gradually decreased over time, reaching around 2 pmol per µg of protein at 8.5 h. The concentration of RAOCT within VSMC also slightly declined over time but remained relatively high, even at the 16.5 h time point (**Figures 6A,B**). The inability of VSMC to efficiently eliminate RAOCT points to an increase in intracellular accumulation of this compound when administered at concentrations higher than 10 µmol/L. This, in turn, might lead to cytotoxicity, as evident by a slight increase in LDH release upon treatment with this compound at 30 µmol/L (**Figure 4**). Our results strongly indicate that, of all tested RA esters, RABU possesses the best intracellular bioavailability.

DISCUSSION

RA esters, especially RAME, widely exist in medicinal plants (e.g. thyme, garden and red sage), but in much lower amounts compared to RA (Li et al., 1993; Fecka and Turek, 2008; Putnik et al., 2016). In recent years, both plant-derived and synthetic RA esters have been studied and showed potent biological activities (Abedini et al., 2013; Kang et al., 2016; Thammason et al., 2018). Out of four isolated active compounds from the hydromethanolic stem extract of *Hyptis atrorubens* (*Lamiaceae*), RAME showed the most potent antimicrobial activity (Abedini et al., 2013). Synthetic RAET acted vasorelaxant in rat aorta (Wicha et al., 2015) and anti-inflammatory in LPS-induced MH-S cells (Thammason et al., 2018). RABU was isolated from *Isodon oresbius* in 1999 (Huang

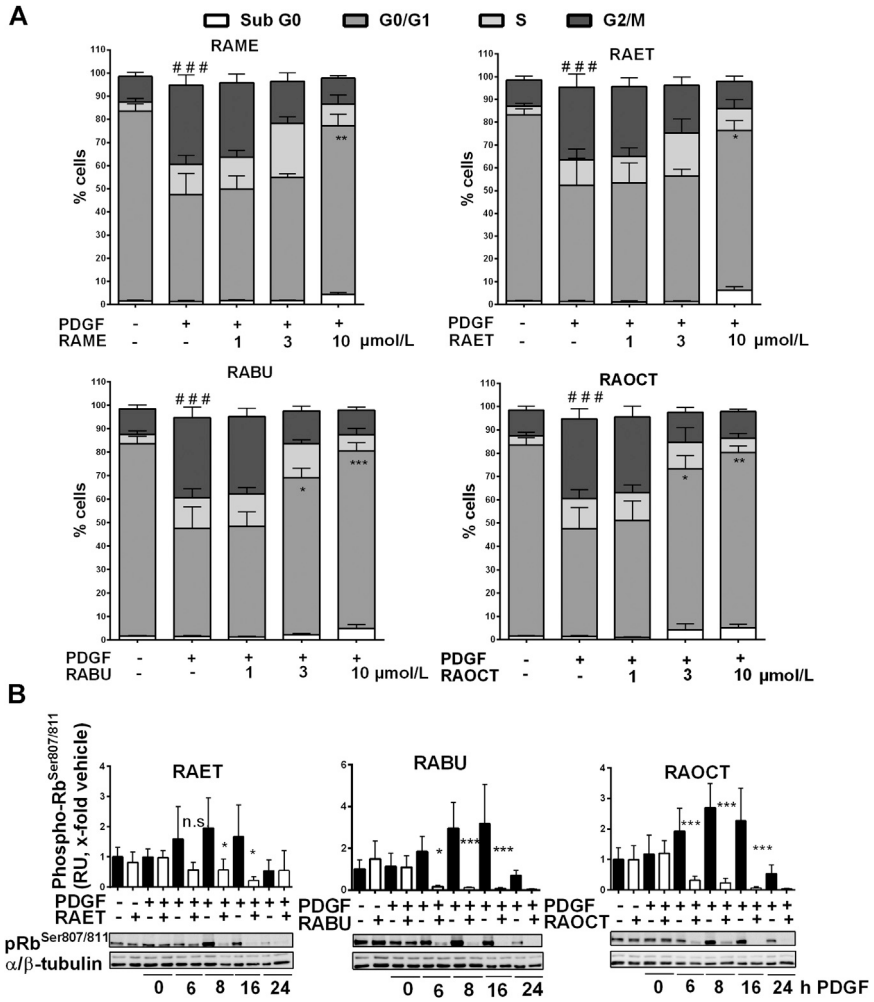

FIGURE 5 | Ethyl, butyl and octyl esters of rosmarinic acid (RA) inhibit the proliferation of VSMC by exerting the same mechanism of action as the methyl ester **(A)** Quiescent VSMC were treated with 0.1% DMSO or increasing concentrations of RA esters as indicated for 30 min, stimulated with 20 ng/mL PDGF-BB for 16 h and the cell cycle progression was analyzed using PI staining and flow cytometry. Graphs show means ± SD out of three independent experiments (G_2/M phase: ###$p < 0.001$, ANOVA/Bonferroni, "vehicle" vs. "vehicle with PDGF-BB"; G_0/G_1 phase: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA/Bonferroni, "vehicle with PDGF-BB" vs. "compound with PDGF-BB") **(B)** Quiescent VSMC were treated with 10 µmol/L of indicated RA esters or 0.1% DMSO for 30 min and stimulated with 20 ng/mL PDGF-BB for indicated amounts of time. Lyzed cells were subjected to western blot analysis for phospho-$Rb^{Ser807/811}$ detection. α/β-tubulin was used as loading control. Representative blots together with compiled results of densitometric analyses out of four independent experiments are shown (mean ± SD, n.s. not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA/Bonferroni, "vehicle" vs. "RA ester").

et al., 1999), and was found cytotoxic against colon, ovary and melanin cancer cell lines (Huang et al., 2006). It was reported that antioxidant, anti-allergic, and antimicrobial activities of RA esters tend to be parabolic with the ester chain increasing (Laguerre et al., 2010; Suriyarak et al., 2013; Zhu et al., 2015). However, to the best of our knowledge, there are no studies examining the oral bioavailability of RA alkyl esters or their effect on VSMC proliferation.

In this study we explored the possibility of improving the bioavailability of RA and its biological activity in proliferating VSMC by esterification with alkyl chains of increasing length. RA esters are rapidly hydrolyzed in fresh rat plasma into RA and the corresponding alcohols. Esterification of RA with up to 4C-alkyl chains increased the *in vivo* bioavailable fraction of RA from 2 to 7-fold, whereas further increase in alkyl chain length resulted in low bioavailability of RA. Regarding the antiproliferative effect in VSMC, derivatization of RA with methyl-, ethyl-, butyl- and octyl-esters resulted in a decrease of the IC_{50} values, whereas RA dodecyl ester was found to be cytotoxic. Contrary to the observed hydrolysis in blood plasma, RA esters were detected in cells in their original form. Treatment with butyl- and octyl-esters achieved the highest intracellular concentrations.

Caffeic (**1**), 3,4-dihydroxyphenyl lactic (**2**), *m*-coumaric (**3**), ferulic (**4**) and *m*-hydroxyphenylpropionic (**5**) acids were found to account for the bioavailable fraction of ingested RA to a larger extent than RA itself (Nakazawa and Ohsawa, 1998). The biological activity of certain polyphenols could in fact be attributed to their bioavailable metabolites, as exemplified by the well-known metabolite of the isoflavone daidzein, S (-)-equol

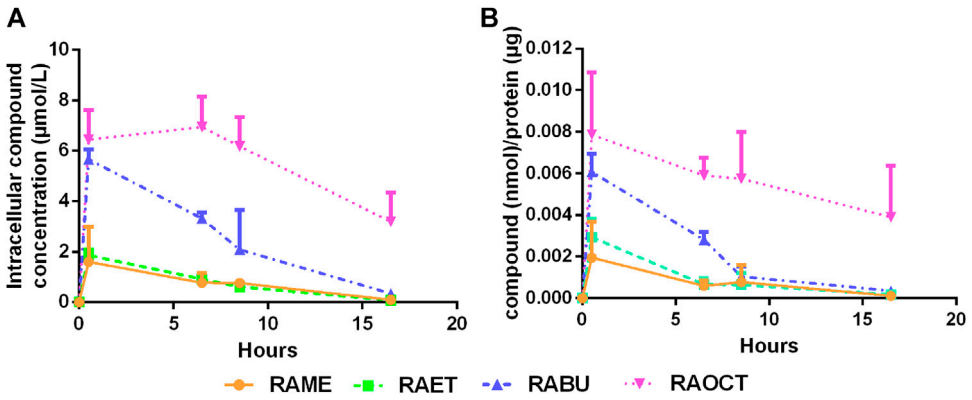

FIGURE 6 | Esters of rosmarinic acid with longer alkyl groups show increased intracellular bioavailability. Cytoplasmic fractions of cells treated with rosmarinic acid (RA) esters (10 µmol/L) for up to 16 h were diluted with MeOH, centrifuged to remove proteins and subjected to LC-MS/MS analysis **(A)** Graph shows means ± SD of detected concentrations of RA esters within cytoplasmic fractions over time out of three independent experiments **(B)** Detected concentrations of respective esters were normalized to protein concentrations. Graph shows mean ± SD of the amount of compound per µg protein out of three independent experiments.

(Setchell and Clerici, 2010). However, in the case of the antiproliferative effect of RA in VSMC, we show that the active principle is RA, rather than any of the so far reported gut-microbial metabolites of RA.

Therefore, we concentrated on improving the absolute bioavailability and the potency of RA to inhibit VSMC proliferation by increasing its lipophilicity via esterification. After oral/intravenous administration of RA alkyl esters to rats, the bioavailability of RA does not entirely progress with the increase of the alkyl ester chain length from methyl to dodecyl. From methyl to butyl side chains, the bioavailability gradually increases. From butyl to dodecyl chains, the bioavailability decreases. Oral administration of RA ethyl- and butyl-esters in rats increased the bioavailability of RA up to around 7-fold, and the respective C_{max} values were in the range of previously reported IC_{50} values of RA against the PDGF-BB-induced VSMC proliferation (Liu et al., 2018). Our *in vivo* pharmacokinetic results showing T_{max} at around 0.2 h (apart from RADOD) indicate that RA, as well as RA esters are being absorbed in the upper parts of the GIT and are eliminated fast. Bioavailability and T_{max} data for RA presented here are well in accordance with previous studies performed in rats (Nakazawa and Ohsawa, 1998; Konishi et al., 2005; Wang et al., 2017). Previous reports on RA absorption suggest the involvement of paracellular diffusion of RA in the upper intestine (Konishi et al., 2005; Konishi and Kobayashi, 2005), in contrast to the caffeic acid that is being absorbed via monocarboxylic transporters (MCT), and showed about 10 times higher absorption efficiency (Konishi et al., 2005). It would, thus, be interesting to examine whether esterification of RA, with ethanol and butanol in particular, increases its diffusion properties or whether some cellular transporters are involved. Unlike methyl to octyl esters of RA, oral administration of dodecyl ester decreased the bioavailability of RA. A possible reason for this might be the poor dissolution of RADOD in the aqueous milieu of the gastrointestinal tract, which is almost always a prerequisite for oral absorption.

In contrast to systemic application, in cultured VSMC, RA esters did not undergo hydrolysis by intracellular esterases and remained in their original form. This result calls for caution when interpreting results of ester prodrugs in cultured cells, as these cells might lack the necessary carboxylesterases. In the context of local drug delivery to prevent aberrant VSMC proliferation and neointima formation, as in the case of antiproliferative drugs used in drug-eluting stents (Alfonso et al., 2014), RABU achieved the highest intracellular concentration (60% of applied concentration) and the highest potency against VSMC proliferation, without exhibiting significant toxicity.

We showed previously that RAME inhibits VSMC proliferation by decreasing the Rb protein phosphorylation, presumably via direct inhibition of CDK2, and subsequently arresting cells in G_0/G_1 phase (Liu et al., 2018). Our current results strongly indicate that the esterification of RA with alkyl chains longer than one carbon atom does not alter the mechanism of action. While RAME and RAET were detected intracellularly at only about 20% of the administered concentration, levels of absorbed RABU and RAOCT were about 3- and 4-fold higher, respectively. This was reflected in the cell-cycle progression results, as RABU and RAOCT arrested VSMC in G_0/G_1 phase at lower applied concentrations than RAME and RAET. The inhibition of Rb protein phosphorylation was also more pronounced after RABU and RAOCT administration.

Intracellular concentrations of RA esters increased shortly after the treatment, as detected at the 30 min time point. RA esters-mediated inhibition of PDGF-induced Rb protein phosphorylation was, however, observed 6 h after RAME, RAET and RABU had intracellularly reached their peak concentrations. In our previous study, RAME could inhibit the CDK2 kinase activity *in vitro* with an IC_{50} of 11.4 µmol/L, while the activity of CDK4 was affected only at much higher concentrations (Liu et al., 2018). The expression and activity of CDK2 becomes relevant at later stages of the G_1 phase and at the transition into S-phase of the cell cycle (Tadesse et al., 2019). CDK3/cyclin C complex was shown to be crucial for the transition of dormant G_0 cells into the G_1 phase, by phosphorylating the Rb protein at $Ser^{807/811}$ (Ren and Rollins, 2004). CDK2 and CDK3 share 87% alignment score in

their ATP binding pocket regions (Sridhar et al., 2006). It would, thus, be quite interesting to examine whether RA esters act already at the PDGF-induced cell cycle entry by inhibiting the activity of CDK3.

In summary, this study is the first to investigate how derivatization of rosmarinic acid affects both its bioavailability and biological activity. Of all tested esters, esterification of RA with a C4-alkyl chain resulted in the highest bioavailable fraction of RA *in vivo* and the highest potency against VSMC proliferation *in vitro*. Considering the enhanced intracellular bioavailability, RABU might exhibit an even more potent effect against neointima formation in a femoral artery cuff model than previously shown for RAME (Liu et al., 2018). However, further studies are needed to investigate the safety of long-term RABU use as well as its effect on the neointima formation *in vivo*.

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 animal study was reviewed and approved by Ethics Committee of Yantai University (IACUC No. 2018-DA-12).

AUTHOR CONTRIBUTIONS

TB and RL designed the study and drafted the manuscript; TB, GR, PH, LD, and GY performed experiments and analyzed data; EH and VD supervised the execution of experiments and their interpretation. All authors revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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

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SYSTEMATIC REVIEW
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A Systematic Review of the European Rapid Alert System for Food and Feed: Tendencies in Illegal Food Supplements for Weight Loss

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Background: Slimming products represent a dynamically growing group of food supplements worldwide. The efficacy of safely usable natural ingredients is usually below consumers' expectations. Certain manufacturers add unauthorized or prohibited ingredients to weight loss supplements in order to increase their efficacy. Hence, many of these products are adulterated and may pose a risk to the consumers' health.

Aims: The aim of our work was to give an overview on natural ingredients used in slimming products, to summarize the frequently used synthetic adulterants and also to assess the trends of adulterated and illegal food supplements in the European Union based on the warnings of the Rapid Alert System for Food and Feed (RASFF) in the time period of 1988–2019.

Methods: Reports between 1988–2019 were extracted from the RASFF portal on January 1, 2020. Each entry was individually reviewed.

Results: 2,559 records of food supplements with quality problems were identified in the RASFF, several of which [319 (12,5%)] were marketed to facilitate weight loss. 202 (63,3%) contained unapproved, synthetic drug ingredients. The major adulterant (113 of 319, 35.4%) was DNP (2,4-dinitrophenol), whereas sibutramine was the second most frequent adulterant agent (69 products, 21,6%) between 1988 and 2019.

Conclusion: The number of approved medicines for the indication of weight loss is relatively low and their efficacy (and also that of the natural ingredients) is limited. Therefore, a significant number of weight loss supplements is adulterated to satisfy patients' expectations. Hence, these products may cause serious adverse effects in sensitive patients.

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Abbreviations: 5-HT2C, 5-hydroxytryptamine; ATP, adenosine triphosphate; BMI, body-mass index; CB1-receptor, cannabinoid receptor type 1; CHMP, Committee for Medicinal Products for Human Use; CLA, conjugated linoleic acid; DNP, 2,4-dinitrophenol; EC, European Commission; EGCG, epigallocatechin-3-gallate; EMA, European Medicines Agency; EU, European Union; FA, fatty acid; GCE, green coffee extract; GLP-1, glucagon like peptide-1; GM, glucomannan; HCA, (-)-hydroxycitric acid; NA, noradrenaline; NR, no reports; POMC, pro-opiomelanocortin; PPA, phenylpropanolamine; RASFF, rapid alert system for food and feed; RCT, randomized controlled trial; SCOUT, sibutramine cardiovascular outcomes trial; T2DM, type 2 diabetes mellitus; WMD, weighted mean difference.

Keywords: food supplements, rapid alert system for food and feed, illegal medicines, obesity, weight loss

INTRODUCTION

Obesity is an emerging health problem worldwide, the number of affected people doubled from 1980 to 2008 (Finucane et al., 2011). Not only the incidence of obesity has been growing, but the world's population's mean BMI has also been increasing by a significant 0.4–0.5 kg/m^2 in each decade (Finucane et al., 2011). Based on the World Health Organization's (WHO) estimates, almost two billion adults had a body-mass index (BMI) ≥ 25.0 and of these adults more than 650 million people were classified as obese (BMI ≥30.0) in 2016 (World Health Organisation, 2018; Montan et al., 2019). Obesity does not only affect the United States, but it is becoming an epidemic also in Europe and even in several developing countries (Barness et al., 2007). According to a recently issued report, approximately 23% of the female and 20% of the male population of Europe were considered obese (World Health Organisation, 2019). If game-changing measures are not going to be applied, by the end of this decade, more than half of the world's adult population will be obese or at least overweight (Kelly et al., 2008). Obesity is associated with many comorbidities, including cardiovascular (e.g. heart disease, hypertension) and cerebrovascular (e.g. stroke) ailments. The incidence of other severe chronic diseases, such as type 2 diabetes mellitus (T2DM) and degenerative musculoskeletal diseases (e.g. arthritis) are higher among overweight and obese people than among people with normal BMI. Moreover, Alzheimer's disease and some malignant tumors affect more often obese people (World Health Organisation, 2018). Lifestyle interventions, involving dietary modifications and increased physical activity are of high importance in avoiding obesity; however, a high proportion of the individuals loses interest shortly after they have started their new lifestyle and return to their original one; therefore, the results are rarely permanent (Curioni and Lourenço, 2005). In the past century, many pharmacons have been approved to support weight loss; however, currently only three APIs and one combination product are available for this indication in the European Union (EU) (Tonstad et al., 2016). Several medicines are no longer available on the market because of safety concerns. Alternative methods are sought-after in the treatment of obesity. The most commonly used products for this purpose are food supplements, which are easily available on the market. However, these products are often counterfeited, containing illegal components. RASFF was set up to aid the national competent authorities in harmonizing their actions and informing one another in the control of food and feed that are posing serious risks (Rapid Alert System for Food and Feed (RASFF), 2020). RASFF is used in the European Union to help obtain the minimally required safety and quality of feed and food (i.e. safety and quality).

The goals of our study were to give an overview on the most widely used natural products, and on the safety profiles of available and withdrawn pharmacons that might be used to facilitate weight loss. We also aimed to assess their presence in food supplements on the European market associated with a warning released by RASFF and to summarize the trends from 1988 to 2019.

PHARMACOTHERAPY OF OBESITY

According to current guidelines, complex measures should be considered in obese patients and pharmacological therapy should only be used along other lifestyle modifications. Actions to address weight management should not only be applied in obese people (BMI ≥30), but also in patients with a BMI ≥27 kg/m^2 if they already have metabolic syndrome or sleep apnea. The efficacy of pharmacotherapy should be assessed and reconsidered within 3 months of initiating the treatment (Yumuk et al., 2015), and it is highly recommended to complete pharmacotherapy with comprehensive lifestyle modifications including calorie deficit and pronounced physical activity.

A great deal of pharmacons previously used in weight management are no longer marketed because of safety issues. These drugs involve amphetamine derivatives, fenfluramine, lorcaserin, phenylpropanolamine, rimonabant, and sibutramine (Bray, 2014; Morris, 2018). The emerged safety concerns highlight that the potential side-effects (CNS-depressing effects, insomnia, toxicity, primary pulmonary hypertension) of pharmacons inducing weight loss should be monitored and assessed more thoroughly (Montan et al., 2019). Currently available medications for this purpose in the European Union are orlistat, liraglutide, naltrexone/bupropion and phentermine resinate (Yumuk et al., 2015; EMA 2020b; The State Institute for Drug Control (SUKL), 2020).

In the meantime, the popularity of food supplements for weight loss management and their market share have been increasing (Ríos-Hoyo and Gutiérrez-Salmeán, 2016). Unfortunately, some products are adulterated with synthetic substances, usually formerly approved weight loss compounds, which had already been withdrawn from the market. Hence, these food supplements can pose serious health risks for consumers.

Medications of Obesity Available in the European Union

Orlistat

Orlistat (1) gained its marketing approval for obesity management by the European Commission (EC) in 1998 (**Supplementary Table S1**). It was the first selective, irreversible inhibitor of gastric and pancreatic lipase enzymes. Its mechanism of action involves the reduction of dietary fat lipolysis and absorption (Al-Suwailem et al., 2006). It is a prescription-only medicine in the European Union. Based on the results of a meta-analysis, orlistat decreased average body weight with 2.35 and 2.94 kg, at doses 60 and 120 mg, respectively (Li et al., 2005). Its use may be associated with clinically relevant mild-to-moderate gastrointestinal adverse effects (e.g. abdominal

pain, diarrhea, fecal spotting, and steatorrhea). Apart from the above-mentioned minor side-effects, serious adverse reactions were also associated with the use of orlistat (e.g. subacute liver failure, cholelithiasis and cholestatic hepatitis). The safety of the chronic use of orlistat is highly questionable because it affects the absorption of other pharmacons and fat-soluble vitamins (Filippatos et al., 2008).

Liraglutide

Liraglutide acts as a glucagon like peptide-1 (GLP-1) receptor agonist and it was first used for the treatment of T2DM (Collins and Costello, 2019). Liraglutide was a promising pharmacon in the therapy of T2DM, because it improved the patients' cardiovascular status and outcome (Marso et al., 2016). After being proved in human studies that GLP-1 analogues promote weight loss, it has become an approved drug for weight management. The mechanism of action of liraglutide involves appetite suppression and delayed gastric emptying (Mehta et al., 2017). Nevertheless, liraglutide was authorized by the European Medicine Agency (EMA) as an adjunct to a comprehensive lifestyle measures to induce weight loss (**Supplementary Table S1**; Christou et al., 2016; EMA, 2020b).

Liraglutide is available as an injection, but another GLP-1 receptor antagonist, semaglutide can be given *per os*. Semaglutide gained its EMA approval recently for the treatment of adults with insufficiently controlled T2DM to improve glycemic control (Gomez-Peralta and Abreu, 2019). Semaglutide is also monitored; therefore, rapid identification of safety concerns and unknown side effects are made possible (EMA, 2020a).

In human studies, the most common side effects associated with the intake of liraglutide included gastrointestinal symptoms (e.g. nausea and vomiting, risk of pancreatitis), and increased pulse rate (Marso et al., 2016). In a clinical trial, from randomization to the 20th week, the mean weight loss in the intention-to-treat (ITT) population, was statistically significantly bigger in the liraglutide treated groups when compared to placebo. The effect was dose-dependent, daily doses of 1.2, 1.8, 2.8, and 3.0 mg liraglutide resulted in a weigh loss of 4.8, 5.5, 6.3, and 7.2 kg, respectively. Taking placebo resulted in a 2.8 kg weigh loss (Astrup et al., 2009).

Naltrexone-Bupropion

After subsequent clinical trials demonstrated its safety, a combination therapy, containing naltrexone (2) and bupropion (3) has been approved in the EU for weight management (**Supplementary Table S1**). Naltrexone acts as an opioid antagonist on the μ-opioid receptor and decreases appetite by inhibiting β-endorphin-mediated autoinhibition of POMC (pro-opiomelanocortin) neurons (Grossman et al., 2003; Cone, 2005; Greenway et al., 2009). The anorectic effects of the antidepressant bupropion is mediated via the stimulation of the activity of POMC cells in the arcuate nucleus of the hypothalamus (Caixàs et al., 2014). Significant weight loss was observed in participants assigned to the combination group after the end of a 56-week-long trial. Patients in the verum group received 32 mg of naltrexone and 360 mg bupropion daily. In groups showing the highest weight loss, the combination was applied for 28–36 weeks, mean change in bodyweight was −6.1 kg (Greenway et al., 2009). Based on the above mentioned evidence, this combination may serve as a possible treatment adjunct to lifestyle modifications by promoting satiety, reducing appetite, enhancing energy expenditure; therefore, it helps patients to achieve weight loss goals (European Medicines Agency, 2015; Sherman et al., 2016). Initially, the first clinical trials focused on the cardiovascular side effects of the combination (Sherman et al., 2016; Tek, 2016). The combination of naltrexone and bupropion is contraindicated in patients with uncontrolled hypertension; however, the possible risks of this combination on cardiometabolic parameters of the patients is not fully understood (Connolly et al., 1997; James et al., 2010). Based on the clinical trials, frequently occurring adverse effects in participants in the verum group were nausea, headache, constipation, dizziness, vomiting, and xerostomia (Vorsanger et al., 2016). Bupropion alone, and in combination with naltrexone increases the blood pressure; therefore, therapies in which bupropion is administered should only be initiated in patients whose blood pressure is well-controlled. Moreover, the patient's blood pressure is to be checked regularly by either the patient itself or by a health care professional throughout the whole course of the treatment.

Phentermine

Phentermine (4) has great potential as a weight loss drug. It also acts on the POMC neurons of the hypothalamus by inhibiting the norepinephrine transporter (Narayanaswami and Dwoskin, 2017). Weigh loss of up to 6 kg have been reported by taking phentermine (15–30 mg/daily) (Lonneman et al., 2013). Phentermine had been used as an approved drug to overcome obesity in Europe since 1956 (**Supplementary Table S1**), and it was a frequently prescribed medicine for decades (Colman, 2005). However, in 2012 the EMA withdraw the marketing authorization of phentermine because of its side-effects. Based on the published evidence, the compound might affect the cardiovascular and the central nervous system when used for a long time. Moreover, the authority was concerned about the possibility of the use of the drug by obese adults for whom taking phentermine may have deteriorating effects (Shin and Gadde, 2013). Nevertheless, phentermine resinate is available in modified-release capsules in the Czech Republic based on the authorization of the national competent authority (The State Institute for Drug Control (SUKL), 2020). Phentermine was combined with topiramate (PHEN/TPM) to achieve weight loss goals and enhance quality of life of obese people. The combination was to be used once daily, but its approval was withdrawn in Europe in 2012, due to concerns regarding its safety (Shin and Gadde, 2013). Although, a 56-week-long trial conducted with a controlled release formulation of phentermine and topiramate (containing 15 mg phentermine and 92 mg topiramate) was proved to have an outstanding efficacy; however, it has to be noted that its use increased dose-dependently the occurrence of psychiatric and cognitive adverse events (Scheen and Van Gaal, 2014).

Withdrawn Amphetamine Derivative-type Medications in the European Union

Amphetamine and its derivates (e.g. phenylpropanolamine, fenfluramine, dexfenfluramine) were used for obesity the first

time in the 1930s (Bray and Greenway, 1999). These compounds are mainly derived from phenylethylamine from which neurotransmitters dopamine, epinephrine and norepinephrine are derived. The weight loss mechanism of action of amphetamine derivatives involves the stimulation of norepinephrine- and dopamine-release in the hypothalamic and limbic regions' satiety centers (Bray, 1993). Amphetamine derivatives exert their anorexigenic effect for a few hours; but tolerance develops relatively fast within only a few weeks. Amphetamine use and abuse often result in cardiac complications (Bazmi et al., 2017). Amphetamine derivatives used as weight loss therapy were removed from the legal market in Europe due to safety concerns and the quick development of tolerance (**Supplementary Table S2**).

Phenylpropanolamine

Phenylpropanolamine (PPA, 5) is available without prescription, and commonly administered as an appetite suppressant for weight loss, and also in cases of cough and common cold (Kernan et al., 2000). PPA is chemically related to the amphetamine-like anorectic agents (Walker et al., 1996). Effects of PPA in weight loss is documented, but the exact mechanism of action is not fully elaborated (Wellman and Sellers, 1986).

Despite the fact that its safety and efficacy profile is controversial, the drug is still available in some European countries. There are safety concerns suggesting a link between the consumption of PPA and stroke (Kernan et al., 2000). In a clinical trial, complementary to a 5,023 kJ (1,200 kcal) diet, patients in the verum group took 75 mg of sustained release PPA for 6 weeks and the achieved weight loss was higher than in the non-treated placebo group (Schteingart, 1992). Patients in the verum group lost 2.59 kg, while the results in the placebo-receiving group was less pronounced (−1.07 kg). 36 patients of the original study were agreed to be enrolled in a further double-blind trial up to 20 weeks, and the difference remained significant (PPA −5.1; placebo −0.4 kg, $p = 0.01$). In spite of the more weight loss in the PPA group, patients did not report a greater anorexigenic effect. The authors of the article concluded that phenylpropanolamine can be used in combination with a diet based on calorie deficit to promote safe weight management.

Fenfluramine, Dexfenfluramine

(+)-Norfenfluramine (6), the active metabolite of prodrugs fenfluramine and dexfenfluramine, induces weight loss and it is a potent agonist on 5-hydroxytryptamine (5-HT_{2C}) receptors (Porter et al., 1999; Fitzgerald et al., 2000). Both drugs, fenfluramine and its (S)-isomer, dexfenfluramine were used in monotherapy, the former one for short-term, and the latter one for long-term weight management, even if its long-term safety was not yet documented (Weintraub et al., 1992). The effects of dexfenfluramine were examined on obese women (n = 52) in a placebo-controlled, double-blind study. Patients in the dexfenfluramine group followed a 1,500 kcal/day diet and took 15 mg dexfenfluramine twice a day (Ditschuneit et al., 1996). After completing the 12-months-long trial, patients in the verum group lost 14.2 ± 2.20 kg, while patients in the placebo group lost only 4.92 ± 2.99 kg. The side-effects of dexfenfluramine are quite alarming though; based on a case-control study, it increased the prevalence of cardiovascular diseases, and its use was associated with pulmonary hypertension (Abenhaim et al., 1996). Therefore, because of safety concerns, both drugs, and the so called fen-phen formulation (combination of fenfluramine and phentermine) were withdrawn from the market in 1997 (Weintraub et al., 1992; Wadden et al., 1998; European Medicines Agency, 2003).

Withdrawn Non-amphetamine Derivative Type Medications in the European Union

2,4-Dinitrophenol

The compound 2,4-dinitrophenol (DNP, **7**) was initially applied in explosive mixtures, but in 1933 it was discovered that DNP causes significant weight loss, and soon it was marketed in slimming products (Tainter et al., 1933). DNP contributes to weigh management by increasing the basal metabolic rate (Cutting et al., 1933). However, serious adverse effects occurred so often that it was withdrawn from the market, and it was labelled as an 'extremely dangerous' drug (**Supplementary Table S3**; Tainter et al., 1934; McFee et al., 2004; Colman, 2007). The side-effects of DNP are associated with its mechanism of action: DNP induces a hyper-metabolic state of the body via uncoupling oxidative phosphorylation, and the excess energy becomes thermal energy in the mitochondria. Hyper-metabolite state is followed therefore with an uncontrolled thermogenesis causing hyperthermia and undesirable elevated body temperature associated with systemic responses (Tainter et al., 1935). After 1938, DNP was no longer prescribed and reports on severe side-effects did not occur, but it is assumed that the use of the compound has not been vanished completely, because case reports of DNP caused deaths still emerged after it has been withdrawn from the legal market (Colman, 2007). Today, DNP is sold illegally as a weight loss aid under a number of different names and its use is encouraged by reports of rapid and safe weight loss (McFee et al., 2004).

Rimonabant

Rimonabant (8), the first antagonist on the cannabinoid receptor type 1 (CB_1-receptor) entered the European market in 2006 (**Supplementary Table S3**) (Rinaldi-Carmona et al., 1995). Initially, it was a promising medication, since several trials proved its effects on weight loss and it also improved several parameters of metabolic syndrome. A meta-analysis of RCTs evaluating the efficacy and safety of rimonabant (20 mg/day) found that the average weight loss in the treated group was 4.7 kg (4.1–5.3 kg), significantly higher, compared to the placebo group (Christensen et al., 2007). However, the use of rimonabant was linked to diverse psychiatric adverse events (e.g. anxiety, depression, and suicidal ideation); therefore, the EMA withdrew the market authorization of rimonabant in the EU in January 2009 (Sam et al., 2011).

Sibutramine

The antidepressant sibutramine (9) inhibits the reuptake of neurotransmitters serotonin (5HT)- and noradrenaline (NA). Apart from its original application later on it was found to

reduce appetite (McNeely and Goa, 1998). In a 12-week-long study, the effects of sibutramine was similar to that of dexfenfluramine. Patients in both groups lost significant amount of weight (4.5 kg, and 3.2 kg, respectively). Sibutramine was used at daily doses ranging from 5 to 30 mg. Based on the results of this study, the optimum daily dose of the drug is 10–15 mg (Lean, 1997). After reports on increased diastolic and systolic blood pressure and pulse rate, concerns were raised regarding the safety of sibutramine (Sharma et al., 2009). Hence, its safety was assessed in the so called Sibutramine Cardiovascular Outcomes Trial (SCOUT) (James, 2005). In this trial the harmlessness of sibutramine was evaluated on patients with a history of cardiovascular disease. As a result, the EMA concluded that the risk-benefit ratio was unfavorable for sibutramine and recommended to suspend all marketing authorizations for sibutramine-containing medicines in Europe (Williams, 2010). Sibutramine was approved in 2001, whereas its market authorization was suspended in 2010 (**Supplementary Table S3**; Ioannides-Demos et al., 2005).

Lorcaserin

Lorcaserin (10), an 5-HT_{2C} receptor agonist, was an approved drug for long-term treatment of obesity and it was intended to be used along with reduced-calorie diet and increased physical activity (Smith et al., 2010). In 2013 (**Supplementary Table S3**), only one year after its marketing approval, the marketing authorization holder officially notified the EMA's Committee for Medicinal Products for Human Use (CHMP) about his wish to withdraw the marketing authorization for lorcaserin, because based on the CHMP' opinion the benefits of lorcaserin—a medicine intended for helping to achieve weight control in obese and overweight patients—did not outweigh its risks (e.g. depression, valvulopathy) (European Medicines Agency, 2013). The weight loss achieved after a one year treatment with lorcaserin ranged from 4.5 to 5.8 kg in the published clinical trials when taking 10 mg lorcaserin once or twice daily (Fidler et al., 2011). Patients taking lorcaserin experienced a significant increased risk of depression (DiNicolantonio et al., 2014). Long-term use might be associated with increased cancer risk (LiverTox, 2012b).

FOOD SUPPLEMENTS FOR WEIGHT LOSS

Considering the limited efficacy and unfavorable side-effect profiles of synthetic drugs, there is a high demand for alternative treatments like herbal products to induce weight loss (Bahmani et al., 2015). One further reason for turning to alternative preparations is the fear from the possible side-effects of synthetic drugs. However, natural origin does not guarantee safety, as it can be demonstrated on the example of ephedrine, a natural alkaloid of species of Ephedraceae having a remarkably high cardiovascular risk (Samenuk et al., 2002). It is a myth that the use of herbal substances are always safe and harmless, and it is important to highlight that herbal compounds can have an interaction with medicines and products of natural origin can cause adverse events as well (Pittler and Ernst, 2001; Dwyer et al., 2005; Poddar et al., 2011; Astell et al., 2013). Moreover, since the regulation and control of food supplements is less strict compared to medicines, the ratio of conterfeit or mislabbelled, potentially dangerous products might be higher.

According to a recent review, most popular natural ingredients marketed for weight management include chitosan, glucomannan, capsaicin, carnitine, and conjugated linoleic acid (CLA) (Wharton et al., 2020). In Europe, other popular herbal ingredients include *Camellia sinensis* (L.) Kuntze (Theaceae), *Garcinia cambogia* (Gaertn.) Desr. (Clusiaceae) and unroasted seed of *Coffea arabica* L. (Rubiaceae) (Barrea et al., 2019; Ríos-Hoyo and Gutiérrez-Salmeán, 2016; The Plant List, 2013). *Hoodia gordonii* (Masson) Sweet ex Decne. (Apocynaceae), *Stevia rebaudiana* (Bertoni) Bertoni (Compositae), *Acaciopsis rigidula* (Benth.) Britton and Rose (Leguminosae family, syn. of *Acacia rigidula*) also occurred frequently as constituent of weight loss products in the warnings of RASFF (The Plant List, 2013). In the following, we present an overview of the most popular ingredients of weight loss products, including some plants that were common constituents of products reported in the RASFF system.

Chitosan

Chitosan, a polysaccharide composed of β-(1→4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units, is formed by the deacetylation of chitin. This compound can be found in the animal kingdom (e.g. the exoskeleton of crustaceans and insects) (Mesa Ospina et al., 2015). It contributes to weight management by lowering the absorption of dietary fat and cholesterol, and it might also promote fat excretion leading to weight loss without dietary modifications (Pokhis et al., 2015). A meta-analysis of 14 RCTs studied the effects of chitosan on body weight, serum lipids and blood pressure (Moraru et al., 2018). The results indicated that by the use of chitosan as a food supplement for up to 52 weeks might promote weight loss (average −1.01 kg). Apart form its slight effects on the body weight, the consumption of chitosan was associated with improvements of serum lipid profile and a significant reduction blood pressure, both systolic and diastolic (−2.68 mmHg, and −2.14 mmHg, respectively).

Based on the published studies, the short-term use of chitosan is safe, but except for those with shellfish allergy (Waibel et al., 2011). Adverse effects include flatulence, constipation, indigestion, nausea, and heartburn (Gallaher et al., 2000). Chitosan might interact with warfarin and partially interferes with the absorption of the fat-soluble vitamins (i.e. vitamins A, D, E, and K); however, its effects on the fecal fat excretion are not fully proven (Huang et al., 2007; Jull et al., 2008).

Glucomannan

The most commonly used type of glucomannan (GM) in weight loss products is extracted from tubers of *Amorphophallus konjac* K. Koch (Araceae) (Xiao et al., 2000, The Plant List, 2013). GM, a hemicellulose-type polysaccharide induces weight loss through several mechanism. It makes the absorption in the small intestine slower; however, reduces the transit time in the small intestine, because it increases the viscosity of the content; furthermore, GM increases energy loss via fecal excretion. GM induces satiety in

several ways: the consumption of GM enhances mastication efforts, and after its consumption it delays gastric emptying; moreover, elevated levels of plasma cholecystokinin induces cephalic– and gastrointestinal-phase satiety signals (Gallaher et al., 2000; Ríos-Hoyo and Gutiérrez-Salmeán, 2016). A meta-analysis found that the daily consumption of GM (1.2–15.1 g/day) for 5 weeks improves the patient' metabolic profile, but only slightly affects the body weight (WMD: −0.79 kg) (Sood et al., 2008). However, contrasting results were reported by Zalewski and Szajewska, 2015, claiming that in otherwise healthy overweight or obese adults, short-term use of GM may promote a slight weight loss, but it does not affect the BMI. The effects of GM on children are not enough to establish a firm conclusion. Mild gastrointestinal adverse effects (bloating, diarrhea) were associated with the use of GM (Keithley and Swanson, 2005).

Capsaicin

Capsaicin (11) and capsaicinoids are agonists of the TRPV1 (transient receptor potential vanilloid subfamily 1) receptor, and they mimic the effects of cold, which decreases the fat mass through the activation and recruitment of brown adipose tissue (Saito, 2015). Capsaicin affects the oxidation of lipid and influences energy expenditure (Whiting al., 2014; Ludy et al., 2012; Zheng et al., 2017). Food rich in capsaicin contributes to weight management by preventing obesity (Zheng et al., 2017). There are also reports that the body weight of healthy women who regularly use chili peppers is slightly reduced when compared to those who do not use chili (Henry and Emery, 1986; Yoshioka et al., 1998). A meta-analysis based on eight studies involving 191 participants concluded that patients who took 2 mg capsaicin before each meal consumed less calorie by an average of 74 kcal; therefore, capsaicin may help maintain weight by reducing total energy intake (Whiting et al., 2014). However, based on other literature data, the level of its effects on thermogenesis and fat oxidation is moderate and its long-term efficacy is questionable (Lejeune et al., 2003). A middle-aged man with normal BMI would lose approximately 0.5 kg over 6.5 years if he pursued a calorie deficit diet of 10 kcal and consumed hedonically acceptable doses of capsaicin (Ludy et al., 2012), whereas a weight loss of 2.6 kg over 8.5 years would be reachable if he was in a 50 kcal negative energy balance (Galgani and Ravussin, 2010; Hall, 2010). Capsaicin at acceptable doses is safe, although it might cause mild-to-moderate gastrointestinal side-effects, sweating, flushing, and rhinorrhea (Avesaat et al., 2016). In addition, capsaicin compounds can interfere with antihypertensive agents (Patanè et al., 2010).

Carnitine

Carnitine (12) transports long-chain fatty acids (FAs) into the mitochondria for transformation, and energy is produced from these FAs via β-oxidation, and it also aids eliminate toxic compounds from the cell (NIH, 2017). L-carnitine is the isomer of carnitine that is used to enhance weight loss (Elmslie et al., 2006). Nine RCTs involving 911 patients were summed up and analyzed in a systematic review and meta-analysis (Pooyandjoo et al., 2016). Participants receiving carnitine (in doses varying from 1.8 g/day to 4 g/day) lost significantly more weight (−1.33 kg) and their BMI decreased significantly more (−0.47 kg/m^2) than patients receiving the control treatment. The results revealed that the weight loss effects of carnitine diminished over time. L-carnitine is very well tolerated; at doses of up to 15 g daily and there were only a few, mild side effects like infrequent diarrhea, gastralgia and nausea (Goa and Brogden, 1987).

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) and its isomers activate different nuclear receptors and, thus, they differentially regulate the expression of those genes that are related to lipid metabolism (Chin et al., 1994). The natural sources of are beef meat and dairy products, but it can also be found in dietary supplements (Schmid et al., 2006). A meta-analysis of human studies indicated that the effect of CLA on weight loss was superior to that of the placebo: median doses of 3.2 g was effective and reduced fat mass (Whigham et al., 2007). However, in certain studies, the association between CLA and weight loss was not observed (Wharton et al., 2020). Nevertheless, there is a clear need for further, larger human trials assessing the efficacy and safety of CLA dietary supplements (Whigham et al., 2004). In animal studies CLA interfered with glucose metabolism (e.g. increased insulin resistance in mice) and lead to a change in liver function inducing lipodystrophy; therefore, these effects should be evaluated in human studies as well to rule out any safety concerns (Clément et al., 2002).

Green Coffee

Unroasted seed of *Coffea arabica* L. are good sources of chlorogenic acids (13) that are not present in roasted coffee because of their thermolability (Farah et al., 2008). The possible use of green coffee in weight management is related to its chlorogenic acid content (Shimoda et al., 2006). Chlorogenic acid-rich green coffee extracts reduce blood lipid and glucose levels, blood pressure, and reduce the risk of certain cardiovascular diseases (Sanlier et al., 2019). A meta-analysis of three RCTs involving a total of 142 participants revealed that by the consumption of green coffee extract (GCE) a significant reduction in body weight was achieved (Onakpoya et al., 2011b). The authors of the above-mentioned meta-analysis could not establish an effective dose for the extract. The grade of evidence of this analysis is moderate, and if more rigorous trials with longer duration are published, the efficacy and safety of GCE in weight management might become appraisable.

Green Tea

The non-fermented leaves of *Camellia sinensis* (L.) Kuntze is green tea. Its main active compounds are catechin polyphenols, such as epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate (EGCG, 14). From these constituents, EGCG is the one in which green tea is the most abundant and this compound is the most important with respect to its pharmacological effects (Musial et al., 2020). Caffeine is also a major pharmacologically active ingredient of green tea (Jeukendrup and Randell, 2011). It was found that a

combination of green tea and caffeine contributed to weight management in people who usually consume low amounts of caffeine. The effect was attributed to enhanced thermogenesis and fat oxidation (Dulloo et al., 1999). This might be explained by the caffeine content of tea, since high doses of caffeine elevates thermogenesis and fat oxidation and lowers leptin levels; therefore, it might have body weight reducing effects (Westerterp-plantenga et al., 2005). Moreover, catechins, especially EGCG, inhibits catechol-*O*-methyltransferase (COMT) and therefore enhances fat oxidation (Westerterp-plantenga et al., 2005). A meta-analysis of human studies carried out with green tea proved that EGCG-containing extracts have significant effect on weight loss and its maintenance (WMD: −1.31 kg; duration at least 12 weeks). When analyzing the data from studies in which high regular caffeine intake was recorded, the effect of caffeine intake on body weight was not significant. However, the studied population differed in these two groups, i.e. low caffeine intake was studied on Asian participants and moderate-to-high caffeine doses were studied in Caucasian people; therefore, the conflicting results might derive form the heterogeneity of the included studies (Hursel et al., 2009). Green tea extracts are common constituents of slimming products; however, there are concerns about the hepatotoxicity of extracts with high (>100 mg/day) EGCG content (Oketch-Rabah et al., 2020).

Garcinia cambogia

The main acid compound of *Garcinia cambogia* (Gaertn.) Desr. is (-)-Hydroxycitric acid (HCA, 15). This compound have proven adenosine triphosphate (ATP) citrate lyase inhibitory effects (Watson et al., 1969). The inhibition of the above mentioned enzyme restricts the availability of acetyl coenzyme A (acetyl-CoA) units that are necessary for fatty acid synthesis and lipogenesis during a so called lipogenic diet when patients consume high amounts of carbohydrates (Kornacker and Lowenstein, 1965; Bressler and Brendel, 1966; Linn and Srere, 1979). The compound restrains the synthesis of FAs, lipogenesis, appetite, and therefore aids weight loss (Sullivan et al., 1972). Despite its promising mechanisms, clinical studies have shown controversial findings (Jena et al., 2002). Nine RCTs were analyzed in a meta-analysis which revealed a small but significant weight loss promoting effect of HCA when compared to placebo (Onakpoya et al., 2011a). The duration of the included studies varied from 2 to 12 weeks, and the participants took 1–2.8 g of HCA daily. More recently, cases of acute liver injury have been emerged in association with a product claimed to contain *Garcinia cambogia*. It is alarming that not only mild side effects (transient and moderate enzyme elevations) were reported but symptomatic acute hepatitis and acute liver failure were also described (LiverTox, 2012a). The frequency of hepatic adverse reactions is not known but it seems to be uncommon (<1:10,000). HCA might influence glucose homeostasis by modifying insulin sensitivity, and increasing gluconeogenesis and the formation of ketone bodies (McCarty and Majeed, 1994; Jena et al., 2002).

Hoodia gordonii

The consumption of *Hoodia gordonii* (Masson) Sweet ex Decne. has its tradition among a native South African people (van Heerden, 2008). Bushmen used to eat this succulent plant for its appetite reducing effects. In Europe, *H. gordonii* can only be marketed after appropriate safety assessment, since it was not used as a food or food ingredient before 15 May 1997 (European Commission, 2020). An oxypregnane glycoside, P57 (16) is assumed to be responsible for the appetite reducing effects of *H. gordonii*. It was found that after intracerebroventricular administration, P57 increased ATP production in the hypothalamus (MacLean and Luo, 2004). No published, peer-reviewed meta-analysis of RCTs examining the efficacy of *Hoodia* were found in the literature (PubMed/Medline, the Cochrane Library, ClinicalKey and Google Scholar). In a placebo-controlled study involving overweight women, the weight loss efficacy of *H. gordonii* was compared to placebo. Participants were classified by body fat percentages, and 25 of them took *H. gordonii* and 24 received placebo (Blom et al., 2011). To ensure identical circumstances for each participant during the 15-day-long study, they stayed in the clinic 4 days prior to the study for a run-in period and during the 15-days treatment period. Participants were asked to consume a yogurt drink 1 h prior to each breakfast and dinner. The yogurt contained 1,110 mg *H. gordonii* or placebo. There were no serious adverse events but nausea, vomiting, and disturbances of skin sensation occurred in the verum group. Blood pressure, pulse rate, bilirubin and alkaline phosphatase levels increased significantly in the verum group. Recently alarming side effects (elevated blood pressure and heart rate) that are in line with the previously describe study have been reported (Roza et al., 2013).

Stevia rebaudiana

The plant *Stevia rebaudiana* (Bertoni) Bertoni is native to South America, and Native Americans used it for centuries to sweeten their food and also for medicinal purposes, as herbal tea to alleviate several ailments, such as heartburn (Lemus-Mondaca et al., 2012). Glucosides obtained from *S. rebaudiana* are approximately 300 times sweeter than sucrose. Nowadays, when obesity has become a worldwide problem, low- and no-calorie sweeteners, such as *S. rebaudiana* offers an alternative that might help reduce sugar intake, and decrease the incidence of diseases derived from high refined sugar consumption (Samuel et al., 2018).

The whole plant and also the dried leaves of *S. rebaudiana* are novel foods in the EU based on the Regulation (EC) No 258/97 (European Commission, 1997). Extracts prepared from the leaves of *S. rebaudiana* are authorized as novel food, based on the Regulation (EC) No 1333/2008 on food additives or Regulation (EC) No 1334/2008 on flavorings, respectively (European Commission, 2008). The regulation covers only herbal tea containing or prepared with leaves of *S. rebaudiana* and preparations that are to be used for sweetening or flavoring purposes, every other use of *Stevia* is still unauthorized in the European Union.

S. rebaudiana appears to be safe. Human and animal studies have shown that steviol glycosides do not possess nor carcinogenic, nor mutagenic, nor teratogenic activates, and they do not have acute or subacute toxicity (Momtazi-Borojeni et al., 2017).

Acacia rigidula

Extracts of *Acaciopsis rigidula* (Benth.) Britton and Rose leaves are used in weight loss products with little or no published clinical data about their potential biological effects, and has no documented history of use as food or traditional herbal treatment (Pawar et al., 2014). A comprehensive literature search in several databases (PubMed/Medline, the Cochrane Library, ClinicalKey and Google Scholar) yielded no results regarding its safety and efficacy. The consumption of *A. rigidula* might be dangerous because it contains appreciable amounts of toxic azotoids (Clement et al., 1998). *A. rigidula* is still not a novel food in the European Union, hence it cannot be market as food supplement, only taxons *Acacia arabica* (Lam.) Willd., *Acacia nilotica*(L.) Delile, *Acacia senegal* (L.) Willd. *and Acacia verek* Guill. and Perr. are authorized as a novel food ingredient (European Commission, 2020).

MATERIALS AND METHODS

Retrospective data were extracted from the RASFF portal. Data from individual warnings were recorded (date, product, product category, notification type, countries concerned, subject, action taken, distribution status and risk decision). Records were grouped into four main categories:

(1) "A" for unauthorized ingredients;
(2) "B" for unsafe ingredients;
(3) "C" if there was a problem with the level of the ingredient (too high or too low);
(4) "D" other problems (eg mislabeling, taste disturbance).

RASFF signals are classified as alert, information notification or border rejection as part of its RASFF Portal. Subcategories were created based on the intended use of the reported product. The risks and adverse effects were also assessed. Data from 1988 to 2019 were extracted from the reported supplements database on January 1, 2020. Each entry was individually reviewed. After the data set was categorized, descriptive analyses were performed using Microsoft Excel 2010 (Microsoft Excel, RRID:SCR_016137) for Windows (Microsoft Inc.).

RESULTS

The raw data set from the RASFF database included 2,559 records of food supplements with quality problems and several of these products were marketed to facilitate weight loss [319 (12.5%)]. 202 (63.3%) of these slimming products contained unapproved, synthetic weight loss pharmacons. Other frequently used adulterants were erectile dysfunction drugs and performance-enhancers which are not included in this article.

The overall reports extracted from RASFF show that the first notifications were created in 2003, and the number of the reported signals kept growing until 2019 (especially in case of DNP). The majority of the adulterated anti-obesity products contained DNP (113 of 319, 35.4%). Sibutramine was the second most frequent adulterant (69 products, 21.6%) in the weight loss food supplement category and it was reported in almost every year, in contrast with DNP, which was reported only in four different years, in 2003, 2017, 2018, 2019. Phenolphthalein, a laxative with genotoxic and carcinogenic potential was the less common synthetic adulterant, with 20 reports. Unauthorized plant ingredients such as *Hoodia gordonii* (Masson) Sweet ex Decne., *Stevia rebaudiana* (Bertoni) Bertoni, and *Acaciopsis rigidula* (Benth.) Britton and Rose (in RASFF portal recorded as syn.: *Acacia rigidula*) were reported less frequently (**Figure 1**).

Based on our statistical overview, adulterated DNP products have been reported mainly in the United Kingdom. Whereas sibutramine has been reported with the highest number in Germany followed by Cyprus and Slovenia, it was reported less frequently in other countries (**Supplementary Figure S1**).

Based on the reports, DNP as adulterant first appeared in 2003, in Finland, and there were no reports on DNP until 2017, and then in 2017, it appeared again, and the number of DNP-containing products started to increase dramatically. The notifications originated from the United Kingdom and Cyprus.

Sibutramine was first detected in food supplements in the 2005, and since then it is a common adulterant in the EU. The number of reports on sibutramine-containing products peaked in 2012 (21 reports) and concerned many countries in the EU. The first 2 phenolphthalein reports originated from Hungary and Cyprus. The maximum number of phenolphthalein was seven reports in 2013 from Germany (**Supplementary Table S4**).

The ratio of the emergence of these three, commonly used synthetic compounds can be seen in **Supplementary Figure S2**. Based on the reports, it can be concluded that sibutramine emerged more frequently, but it occurred in fewer products. DNP was mainly present in 2018 (41 times) and in 2019 (70 times), while the popularity of sibutramine seemingly peaked in the early 2010s, in 2011 and 2012 14 and 21 reports were registered, respectively. Phenolphthalein emerged with the highest reports in 2012 with six reports and with seven reports in 2013.

Based on the RASFF signals *Hoodia gordonii* [66 of 117 (56.4%)], Stevia rebaudiana [23 (19.66%)] and *Acacia rigidula* [28 (23.93%)] were reported as unauthorized herbal products. The countries affected by adulterated products were Poland, Lithuania, France, Malta, Spain, Belgium, Austria, Switzerland, Ireland, Sweden, and Finland as shown in **Supplementary Figure S3**.

Hoodia gordonii was reported for the first time, in 2005 in the Netherlands (2 reports). The greatest number of products containing *Hoodia goordonii* was reported in 2017 (30 reports). In 2016, there were no reports on *Hoodia gordonii*. The first appearance of a product containing unauthorized *Stevia rebaudiana* was reported in Denmark in 2006. The highest number of reports on products containing unathorized *Stevia rebaudiana* was four in 2010. Except for 2005, 2015, 2018, 2019, it was present in every year from 1988–2019. *Acacia rigidula* was first reported in 2016, firstly in the Netherlands, but later also in Belgium, Austria, France, Malta, Spain and other European countries, overall 28 records were found in RASFF (**Supplementary Table S5**).

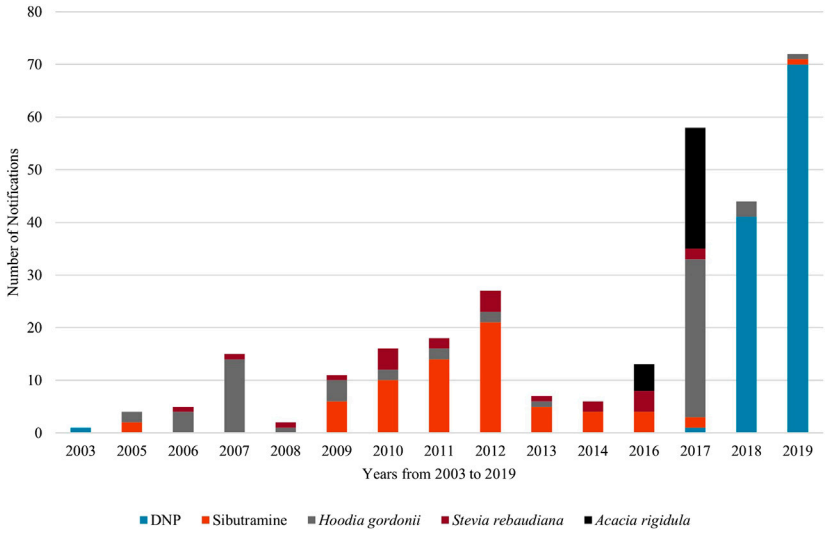

FIGURE 1 | Notifications on weight loss food supplements in the RASFF (2003–2019).

The ratio of these three reported, natural products with safety concerns are represented in **Supplementary Figure S4**. *Hoodia gordonii* was present more often, in smaller quantities, with the highest occurrence of 30 reports in 2017. *Stevia rebaudiana* has been reported in almost every year, but the highest number of reports was only four. The appearance of *Acacia rigidula* only started at 2016 and it emerged again in 2017 with 23 records.

DISCUSSION

The aim of our work was to summarize the trends concerning adulterated food supplements associated with a warning released by the RASFF between 1988 and 2019, focusing on products with intended use as slimming agent. RASFF is a platform for reporting food safety issues within the European Union. When a RASFF member suspects that a food or feed poses a serious risk to the people's health, the member state should notify the European Commission (EC) via RASFF without any delay. In cases of withdrawing or recalling products from their market or in cases when rapid measures are needed, the members are obliged to notify the EC to help protect peoples' health.

The increasing number of signals on illegal food supplements in RASFF reveals that the presence of undeclared ingredients poses an important public health concern. Illegal supplements marketed for weight loss were most commonly adulterated with DNP or sibutramine between 1988 and 2019. The use of former one may result in quick weight loss, but often causes serious adverse events (Colman, 2007). Several deaths attributable to DNP have been published (Cann and Verhulst, 1960). DNP was detected as an adulterant for the first time in 2003, but the number of products containing this compound has been increasing from 2017 through 2019, reported mainly in the United Kingdom. Sibutramine was reported in several countries; however, the number of products containing sibutramine was lower. SCOUT confirmed that sibutramine (at daily doses ranging from 10 to 15 mg) increases the risk for nonfatal myocardial infarction and nonfatal stroke in patients with preexisting cardiovascular disease, and have an increased potential to develop high blood pressure or pulse rate (Sharma et al., 2009).

It is alarming that the majority of the reported signals were in connection with unsafe synthetic substances. There have been increasing number of reports on DNP, and since this substance can cause serious side effects it is necessary to monitor the use of DNP more closely in the future.

Out of the most popular food supplements with natural origin the extracted materials of *Hoodia gordonii* (Masson) Sweet ex Decne., *Stevia rebaudiana* (Bertoni) Bertoni, and *Acaciopsis rigidula* (Benth.) Britton and Rose were unauthorized products registered in RASFF from 1988–2019. *Stevia rebaudiana* seems to be the least dangerous component based on the reports of our review on RASFF. It was reported in small quantity, and for now became authorized as a novel food according to EC Regulation EC No. 258/97 (European Commission, 1997). In spite of that it has been traditionally used for hundreds of years, more scientific and clinical studies are needed to verify its safety, because it was represented almost in every year in RASFF from 1988–2019, and it is very popular among the consumers.

The other two plants (*H. gordonii*, *A. rigidula*) are still not authorized as a novel food and their safety is not scientifically proven (Roza et al., 2013; Clement et al., 1998).

Despite the fact that *H. gordonii* is often used as an adulterant, and advertised for its weight loss promoting effects, there is still little known about its chemical constituents and their mechanism of action. Recent research suggests that the use *H. gordonii* may cause increased blood pressure and pulse rate (Roza et al., 2013). Taken into consideration that *H. gordonii* emerges regularly from 1988, it would be important to monitor food supplements containing *Hoodia*.

A. rigidula is a shrub native to the Southeastern United States, and it contains several biogenic amines. The plant has been marked in products promoting weigh loss; however, its effects are not yet supported by either its traditional usage, since it has never been used in the traditional medicine, or by research results. *Acacia rigidula* occurred in the RASFF portal recently, the presence of *A. rigidula* should be monitored closely in weight loss dietary supplements.

CONCLUSION

As several medications used to manage body weight are no longer available on the market, because of their serious adverse effects; there is a clear need for effective products to support weight loss because currently there are only a few therapeutic options to address this issue. However, the efficacy of natural ingredients usually does not meet the customers' expectations.

Some products (typically sold as food supplements) are adulterated with synthetic compounds to increase their efficacy. Adulterated food supplements may cause serious adverse effects, and their interactions with other medicines are also unpredictable. Therefore, it is alarming that the number of signals on adulterated products in RASFF is increasing. As the food supplement industry continues to grow worldwide, it is important to mark these signals as a public health issue, and to elaborate various measures to decrease the number of these signals by improving the safety, quality and testing of food supplements.

AUTHOR CONTRIBUTIONS

DK. collected and analyzed the data and drafted the manuscript. BT and OR. analyzed the data and checked the manuscript for validity. DC. conceptualized the research and did the final check of the manucsript.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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The Potential use of Honey as a Remedy for Allergic Diseases: A Mini Review

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Honey has been conventionally consumed as food. However, its therapeutic properties have also gained much attention due to its application as a traditional medicine. Therapeutic properties of honey such as anti-microbial, anti-inflammatory, anti-cancer and wound healing have been widely reported. A number of interesting studies have reported the potential use of honey in the management of allergic diseases. Allergic diseases including anaphylaxis, asthma and atopic dermatitis (AD) are threatening around 20% of the world population. Although allergic reactions are somehow controllable with different drugs such as antihistamines, corticosteroids and mast cell stabilizers, modern dietary changes linked with allergic diseases have prompted studies to assess the preventive and therapeutic merits of dietary nutrients including honey. Many scientific evidences have shown that honey is able to relieve the pathological status and regulate the recruitment of inflammatory cells in cellular and animal models of allergic diseases. Clinically, a few studies demonstrated alleviation of allergic symptoms in patients after application or consumption of honey. Therefore, the objective of this mini review is to discuss the effectiveness of honey as a treatment or preventive approach for various allergic diseases. This mini review will provide insights into the potential use of honey in the management of allergic diseases in clinical settings.

Keywords: allergic rhinoconjunctivitis, atopic dermatitis, allergic rhinitis, allergic asthma, allergy, honey, allergic fungal rhinosinusitis, mini review

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INTRODUCTION

Allergic diseases, one of the most commonly occurring diseases worldwide, are a group of hypersensitivity disorder mediated by immunological mechanisms which can cause tissue damage and life-threatening reactions (Jutel and Akdis, 2011; Kaplan et al., 2012). Their prevalence continues to increase at an alarming rate across gender, age and racial groups, thereby increasing the global health burden substantially over the last 20 years in developed and developing countries (Pawankar et al., 2011). According to World Allergy Organization, allergies now affect up to 30%–40% of the population worldwide with children and young adults bearing the greatest burden of these diseases (Pawankar et al., 2011). Some of the common examples of allergic diseases include allergic rhinitis, asthma, conjunctivitis, atopic eczema, and life-threatening anaphylaxis. Several factors are considered to be responsible for the dramatic rise in allergic cases which include increase in pollution, climate change, reduction in biodiversity, urbanization

of societies, and change in lifestyle and dietary habits (Pawankar et al., 2011). The current treatment available for allergic diseases include antihistamines (carbinoxamine; hydroxyzine), corticosteroids (beclomethasone; ciclesonide), biologics (omalizumab; dupilumab), and allergen immunotherapy. Each besets by several side effects such as lipodystrophy, glucocorticoid-induced osteoporosis, purpura, and rosacea (Schacke et al., 2002; Coondoo et al., 2014). The drawbacks of existing treatments for allergies has driven an interest in complementary and alternative medicine (CAM) as an alternative treatment. An example of CAM that has been receiving attention in modern medicine is honey.

Honey is a natural food substance produced from nectar and plant sweet deposits that is collected, processed and stored by bees. It is high in both nutritional and therapeutic values, and has been traditionally used to treat burns, wounds, cough, asthma, and several other gastrointestinal and cardiovascular problems (Eteraf-Oskouei and Najafi, 2013). Honey can be classified according to its source of nectar, i.e., floral or non-floral honey. Its color, thickness, aroma, taste and composition vary greatly depending on its source, the bee species, weather, geographical location, harvesting season and its processing and storage conditions (Subramanian et al., 2007; Silvano et al., 2014). Honey is a complex substance predominantly comprised of sugar, mainly fructose and glucose, and small quantities of other sugars such as maltose and sucrose (Khan et al., 2007). The protein content in honey ranges from trace amounts of amino acids such as proline, alanine, glycine (Hermosin et al., 2003) to enzymes such as catalase, amylase, invertase (Jeffrey and Echazarreta, 1996), which varies depending on the bee species that produce the honey. It has a very low content of vitamins and minerals, constituting only 0.02% of its weight (Alqarni et al., 2014). The average pH of honey is 3.9 (ranging from 3.4 to 6.1); this acidic pH is greatly attributed to the 0.57% organic acids, mainly, gluconic acid and citric acid present in honey (Gündoğdu et al., 2019). In terms of phytochemical composition, honeys like Tualang, Manuka, and Gelam have been shown to contain a high number of flavonoids and polyphenols such as quercetin, kaempferol, chrysin, and apigenin. The quantitative analysis for some of the phytochemicals have also been reported by other studies shown in **Table 1**. These phytochemical compounds have been reported to be responsible for the medicinal properties of honey such as anti-inflammatory, anti-oxidant, anti-microbial, anti-allergic, anti-diabetic, anti-cancer, anti-parasitic activity, anti-ulcer, wound healing, and cardiovascular disease prevention (Cornara et al., 2017; Samarghandian et al., 2017). Among these reported beneficial effects, anti-inflammatory, anti-oxidant, anti-cancer, and anti-microbial activities from various honey are well documented (Cornara et al., 2017). For example, apigenin, chrysin, and quercetin isolated from honey have been proven in one reported study to inhibit the growth of various bacterial species (Das et al., 2015), whereas isolated kaempferol from honey contains anti-cancer and anti-inflammatory activity (Hämäläinen et al., 2007; Ghaffari et al., 2012). However, very limited research had been conducted to study the anti-allergic properties of honey. Thus, this mini review aims to summarize the existing findings on honey and its potential as an anti-allergic agent. This review critically analyses the findings from both preclinical and clinical studies, and discusses the limitations and future prospects of honey in the management of allergy.

CLINICAL STUDIES ON THE EFFECTS OF HONEY IN VARIOUS ALLERGIC DISEASES

Atopic Dermatitis

A study conducted by Alangari et al. (2017) investigated the effectiveness of manuka honey on Atopic Dermatitis (AD) lesions by comparing the pathological status of honey-treated site to non-treated site. Three Item Severity score (TIS) was used to determine the degree of erythema, edema/papulation, and excoriation. Fourteen AD candidates from United Kingdom with bilateral similarly affected areas were requested to apply a layer of honey on the lesion site at night and wash it off the next morning for seven consecutive days. The authors reported that the honey-treated lesion had a significant improvement in the mean TIS score after 7 days of honey treatment. Interestingly, a 1 year follow up revealed that three of the participants reported an overall improvement in their eczema conditions without using honey after the study period, indicating that the beneficial effect of honey may retain in the skin even after stopping its topical application.

Another study reported by Al-Waili (2003) was a patient-blind clinical study where the authors compared the effects of natural honey obtained from Al-Theed City, UAE premixed with olive oil (honey mixture) in two groups of AD patients. The first group of patients consisted of 10 AD patients who did not receive any form of drug treatment before and during the study period. On the other hand, the second group of patients consisted of 11 AD patients who received corticosteroid treatment before and during the study period. All patients in the first group were subjected to treatment of honey mixture only whereas all patients in the second group were subjected to treatment of both honey mixture and corticosteroid ointment in different v/v ratios: mixture A (1:1), mixture B (2:1) and mixture C (3:1). From this study, eight out of 10 AD patients in the first group showed a significant improvement in AD symptoms after 1-week topical application of honey mixture (**Table 2**). Meanwhile, the patients who were in the second group showed a successful reduction in their dependence on corticosteroid at a range of doses by at least 50%. Although this clinical study demonstrated that topical application of honey mixture was able to relieve the symptoms of AD as well as decreasing the dependency of AD patients on corticosteroid, whether the olive oil itself exerted any anti-allergic properties was not demonstrated nor discussed. This is relevant since more recent studies have reported anti-allergic effects of olive oil (Isoda et al., 2012; Wani et al., 2015). Thus, the outcome of the study by Al-Waili (2003) could significantly be improved by an additional control group where the patients receive olive oil alone. This would be able to address any possible anti-allergic activities exerted by olive oil in the honey mixture. Not only that, the absence of a control group with corticosteroid only as the treatment in the second group may make the data interpretation to be difficult. Moreover, the number of participants in each

TABLE 1 | The common reported phenolic and flavonoid compounds found in Tualang, Gelam, and Manuka honey as well as each of their respective quantitative data. design and study outcome of reported.

Author	Type of honey	Common reported phenolic and flavonoid compounds (μg/100 g honey)			
Sarfarz and Nor Hayati (2013); Khalil et al. (2011)	Tualang honey	Catechin		12.9–35.6	
		Caffeic acid		2.5–3.2	
		Benzoic acid		0.2–1.0	
		Naringenin		0.6	
		Trans-cinnamic acid		0.01–0.5	
		Gallic acid		0.4–0.4	
		Kaempferol		0.02–0.2	
		Syringic acid		0.02–0.1	
		p-Coumaric acid		0.04	
		Luteolin		ND	
		Hyacinthin		ND	
Putri Shuhaili et al. (2016); Hussein et al. (2011)	Gelam honey	Quercetin		1,588.9–1,594.3	
		Chrysin		1,498.6–1,504.6	
		Hesperetin		1,475.2–1,477.8	
		Gallic acid		859.4–876.8	
		Ellagic acid		558.8–575.7	
		Chlorogenic acid		502.8–528.1	
		Caffeic acid		428.8–442.0	
		Ferulic acid		356.9–381.4	
		p-Coumaric acid		301.5–308.3	
Alvarez-Suarez et al. (2014); Sarfarz and Nor Hayati (2013); Yao et al. (2003)	Manuka honey	Quercetin	180–550	4-Methoxybenzoic acid	ND
		Isorhamnetin	320–470	Abscisic acid	ND
		Chrysin	370–400	Methyl syringate	ND
		Luteolin	130–430	Phenyllactic acid	ND
		Pinocembrin	150–230	2-Methoxybenzoic acid	ND
		Kaempferol	130–260	(4-Methoxyphenyl)-acetic acid	ND
		Protocatechuic acid	45.7–53.7	Pinostrobin chalcone	ND
		Syringic acid	37–43	Desoxyanisoin	ND
		Genistic acid	24.7–36.1	Methyl syringate	ND
		Gallic acid	24.2–34.6	3,5-Dimethoxybenzoic acid	ND
		Benzoic acid	6.5–29.9	3-Phenyllactic acid	ND
		Protocatechualdehyde	6.3–20.1	Salicylic acid	ND
		Caffeic acid	17.2–18.6	Apigenin	ND
		Chlorogenic	16.1–16.9	Tectochrysin	ND
		p-Coumaric acid	ND	8-Methoxykaempferol	ND
		Myricetin	ND	Isoferulic acid	ND
		Pinobanksin	ND	Galangin	ND

ND, no data.

TABLE 2 | Shows summary of the disease model, experimental design and study outcome of reported clinical studies on the anti-allergic potential of various types of honey.

Author	Type of allergic disease	Type of honey	Number of patients	Age and gender	Grouping and treatment method	Honey treatment frequency	Scoring scale on disease symptoms	Symptoms evaluated	Study outcome (improvement of disease symptoms)	Remarks
Alangari et al. (2017)	Atopic Dermatitis	Manuka honey	14	Gender: 8 Female; 6 Male Mean age: 23–43 years	All participants: topical application of honey over the skin lesions at night and cover it with gauze. Remove the covering and wash the site in the morning	Once/day for 1 week	0 to 9 points (Three Item Severity Score)	Erythema Edema/papulation Excoriation	Yes	NA
Al-Waili et al. (2003)	Atopic Dermatitis	Natural unprocessed honey	21	Gender: 4 Female; 17 Male Age range: 5–16 years	Group 1 (10 participants with no topical treatment during recruitment) Right side body skin lesions: topical application of Vaseline Left side body skin lesions: topical application of honey mixture Group 2 (11 participants with ongoing topical corticosteriods treatment during recruitment) Right side body skin lesions: topical application of Vaseline and betamethasone esters 0.1% Left side body skin lesions: topical application of honey mixture with corticosteroids ointment	Three times/ day for 2 weeks	0 to 4 points	Erythema Scaling Lichenification Excoriation Indurations/papulation Oozing/crusting Pruritis	Yes	Most patients also successfully reduced their dose of corticosteroid in honey mixture
Asha'ari et al. (2013)	Allergic rhinitis	Tualang honey	40	Gender: 26 Female; 14 Male Age range: 20–50 years	Group 1 (20 participants): daily 10 mg of loratadine for 4 weeks Oral ingestion of honey-flavored corn syrup (placebo) for another 4 weeks Group 2 (20 participations): daily 10 mg of loratadine for 4 weeks Oral ingestion of Tualang honey at 1 g/ kg body weight for another 4 weeks	Once/day for 28 days	0 to 4 points (Allergic Rhinitis and Its Impact on Asthma Classification)	Nasal blockage Rhinorrhea Hyposmia Nasal, eye and palatal itchiness Sneezing	Yes	NA
Thamboo et al. (2011)	Allergic Fungal Rhinosinusitis	Manuka honey	34	Gender: No data Age range: >19 years	All participants: Intranasal application of 2 ml 1:1 honey/saline mixture using a mucosal atomization device in selected nostril	Once/day for 30 days	0 to 9 points (Philpott-Javer Endoscopic Scoring System)	Mucosal oedema and polyps Presence of mucin	No	Only nine out of 34 patients showed improment
Rajan et al. (2002)	Allergic rhinoconjunctivitis	Local honey Clover honey	36	Gender: 24 Female; 12 Male Age range: 20–72 years	Group 1 (12 participants): Oral ingestion of locally collected honey Group 2 (12 participants): Oral ingestion of Clover honey Group 3 (12 participants): Oral ingestion of honey-flavored corn syrup (placebo)	Once/day for 30 weeks	0 to 3 points	Nasal symptoms: Runny nose Sneezing Itchy nose Post-nasal drip Stuffy/blocked nose Ocular symptoms: Sore eyes Swollen eyes Watery eyes Itchy eyes	No	NA

NA, Not applicable.

subgroup was small (three to four patients per subgroup) if we were to consider the different dilutions utilized in the second group and therefore, the results obtained may not be generalizable. Finally, the insolubility of honey in olive oil might affect the outcome of this study.

Allergic Rhinitis

A randomized placebo-controlled trial was done by Asha'Ari et al. (2013) to study the inhibitory effects of Tualang honey against Allergic Rhinitis (AR). Forty patients were first treated with 10 mg of second-generation antihistamine (loratidine) once daily for the first 4 weeks, followed by oral honey treatment (1 g per kg body weight per day) or placebo for another 2 months. At week 0, four and eight of the study, the patients were assessed according to the Allergic Rhinitis and Its Impact on Asthma (ARIA) classification focusing on seven symptoms (**Table 2**). The authors concluded that ingestion of honey significantly improves the mean of total symptoms score between honey treated group and placebo group, suggesting a progressive amelioration of AR symptoms following consumption of Tualang honey. In fact, the cardinal symptoms of AR were significantly improved in these patients even after discontinuation of antihistamines. This was not observed in the placebo group whose improvement in AR symptoms declined after week 4, possibly due to the cessation of the antihistamine treatment. Thus, this study demonstrates that the ingestion of honey along with usual standard medication is beneficial in relieving the AR symptoms without any reported adverse effect.

Allergic Rhinoconjunctivitis

A clinical study by Rajan et al. (2002) demonstrated the effect of honey consumption on rhinoconjunctivitis by comparing the effects of locally collected honey from Bristol, England and clover honey (from Lancaster, England). Thirty-six Allergic Rhinoconjunctivitis (ARC) patients were randomly assigned into three groups; the first and second group of participants were instructed to consume a locally collected honey and clover honey respectively, while the third group was given placebo with honey-flavoured corn syrup. All the patients were requested to consume one tablespoonful of honey or corn syrup once per day for 30 weeks. Ten main symptoms were tracked and scored in this study which consisted of both nasal and ocular symptoms (**Table 2**). At the end of the study, no significant improvement in terms of symptoms score was found in the two honey treatment groups in comparison to the placebo group. This is thought to be due to inadequate dosage of honey to relieve the symptoms. The authors also highlighted that the dosage used caused 1/3 of the volunteers to withdraw from the study due to the unpleasant taste of the honey to consume on a daily basis.

Allergic Fungal Rhinosinusitis

In a clinical trial performed by Thamboo et al. (2011), 34 Allergic Fungal Rhinosinusitis (AFRS) patients who had undergone bilateral functional endoscopic sinus surgery (FESS) and exhibited the main symptoms of AFRS were recruited. The patients were requested to spray 2 ml of manuka honey-saline solution (1:1 ratio) in their selected nostril once a day for 30 days, and those who were on prescribed medication were instructed to take their medication before spraying the honey solution. All patients acted as their own control as they continued with their current medical management in both nostrils, but only one nasal cavity was selected to undergo honey treatment. The evaluation was performed using Philpott-Javer Endoscopic Scoring System, which mainly grades the symptoms based on the presence of mucin, mucosal oedema, and polyps. The study demonstrated no significant improvement as only nine out of 34 patients receiving honey treatment showed positive response upon completion of the study.

Clinical Prospect of Honey in the Treatment of Allergic Diseases

Although limited, the clinical evidence described above suggests that honey has great potential in the management of AD and AR. The general symptoms of AD such as erythema, excoriation and oedema can be improved significantly by topical application of honey, without major adverse effect. (Al-Waili, 2003; Alangari et al., 2017). However, the exact dosage of honey was not well-defined in these studies because the honey was either applied as a thin layer on the lesion site, or the honey mixture was prepared with other ingredients such as olive oil. Although the consumption of honey daily for 4 weeks was also reported to improve symptoms of AR. (Asha'Ari et al., 2013), it is still difficult to compare the effects of honey that used different treatment methods (oral ingestion, intranasal, and topical application) across different studies, regardless of the allergic diseases. Therefore, all these factors should be taken into consideration in any future studies as it may contribute to discrepancy when comparing the effectiveness of honey against various allergic diseases.

Although remarkable improvements were observed in certain allergic cases such as AD and AR, other studies reported no significant inhibitory effects of honey in some allergic diseases such as AFRS and ARC (**Table 2**), suggesting that honey might only be effective against certain types of allergic diseases. Even though honey had been reported to have no significant effect on AFRS, it is interesting to note that those AFRS patients who responded positively with honey treatment had been shown to have high level of specific IgE (Thamboo et al., 2011). Such observation can be explained by another study where honey has been shown to contain specific IgE lowering effect (Duddukuri et al., 1997), resulting in its ability to exert an inhibitory effect against AFRS through suppression of IgE levels in the patients. Nevertheless, further research will need to be conducted on these AFRS patients to identify the possible factors that contribute to their positive responses. Any future studies on the effects of honey on AFRS may consider to divide the patients into two groups (Group 1: with honey treatment; Group 2: without honey treatment) in comparison to the current experimental design of Thamboo et al. (2011), where the patients acted as their own control and only one nasal cavity was selected to undergo honey treatment. Based on the literature review, several studies provided limited information on the type of honey used. For instance, the clinical study on ARC and AD by Rajan et al. (2002) and a clinical study on AD by Al-Waili (2003) investigated

TABLE 3 | Pre-clinical studies on the anti-allergic potential of various types of honey.

Author	Type of allergic disease	Type of honey	Cell/Animal model	Number of animals	Age, gender, and weight	Treatment method	Control grouping	Honey treatment Grouping	Experimental outcome	Study conclusion (anti-allergic effects of honey)
Kamaruzaman et al. (2014)	Allergic asthma	Tualang honey	Rabbits (*Oryctolagus cuniculus*)	40 (5 in each group)	Mean age: ND Gender: 3 Female; 37 Male Mean weight: 2.40 ± 0.56 kg	Pre-treatment	Group 1: Normal control Group 2: i.p. injection with OVA (day 1 and 14) Group 3: i.p. injection with OVA (day 1 and 14), followed by aerosolised OVA (day 28–30) Group 4: i.p. injection with PBS (day 1 and 14), followed by aerosolised PBS (day 28–30)	Induction of allergic asthma was done with i.p. injection of OVA at day 1 and 14 Group 5: Aerosolised 25% (v/v) honey (day 23–27) Group 6: Aerosolised 50% (v/v) honey (day 23–27) Group 7: Aerosolised 25% (v/v) honey (day 23–27), followed by aerosolised OVA (day 28–30) Group 8: Aerosolised 50% (v/v) honey (day 23–27), followed by aerosolised OVA (day 28–30)	Inflammatory cell response (Wright-Giemsa stain): ↓ neutrophils, eosinophils, and macrophages infiltration in bronchoalveolar lavage fluid (BALF) Goblet cells (AB-PAS stain): ↓ cell hyperplasia and mucus accumulation in bronchioles	Yes
Shamshuddin and Mohd Zohdi (2018)	Allergic asthma	Gelam honey	Mice (BALB/c)	42 (6 in each group)	Mean age: 8–12 weeks old Gender: 42 Female Mean weight: ND	Pre-treatment	Group 1: Normal control Group 2: i.p. injection with OVA (days 0, 7, and 14), followed by intranasal instillations with OVA (day 14, 25, 26, and 27) Group 3: i.p. injection with OVA (days 0, 7, and 14), followed by intranasal instillations with OVA (day 14, 25, 26, and 27) and oral feeding of PBS Group 4: i.p. injection with OVA (days 0, 7, and 14), followed by intranasal instillations with OVA (day 14, 25, 26, and 27) and oral feeding of 3 mg/kg dexamethasone	Induction of allergic asthma was done with with i.p. injection of OVA at day 0, 7, and 14 Group 5–7: Intranasal instillations with OVA (day 14, 25, 26, and 27) and oral feeding with 10, 40 or 80% (v/v) of honey (day 14, 25, 26, and 27)	Histopathological analysis (hematoxylin and eosin (H&E) stain): ↓ eosinophil, neutrophils and lymphocytes infiltration in lung tissue Mucin expression (PAS stain): ↓ mucin expression in airway epithelium Mast cell count (toluidine stain): ↓ number of mast cells per bronchiole Total inflammatry cell count (wright-giemsa stain): ↓ number of inflammatory cells in BALF Beta hexosaminidase release assay: ↓ beta hexosaminidase release in BALF	Yes

(Continued on following page)

TABLE 3 | (*Continued*) Pre-clinical studies on the anti-allergic potential of various types of honey.

Author	Type of allergic disease	Type of honey	Cell/Animal model	Number of animals	Age, gender, and weight	Treatment method	Control grouping	Honey treatment Grouping	Experimental outcome	Study conclusion (anti-allergic effects of honey)
El-Aidy et al. (2015)	Allergic asthma	Apiary honey	Mice (Albino CD1)	36 (6 in each group)	Mean age: 6 weeks old Gender: 42 Male Mean weight: 18–20 g	Co-treatment	Group 1: Normal control Group 2: i.p. injection with conalbumin (days 0 and 7), followed by intratracheal administration with conalbumin (day 14, 20, and 30) Group 3: i.p. injection with conalbumin (days 0 and 7), followed by intratracheal administration with conalbumin (day 14, 20, and 30) and i.p. injection with 0.5 mg/kg dexamethasone (day 15–33)	Group 4: i.p. injection with conalbumin (days 0 and 7), followed by intratracheal administration with conalbumin (day 14, 20, and 30) and i.p. injection with 650 mg/kg honey (day 15–33)	Histopathological analysis (H&E stain): No significant ↓ in eosinophil, monocytes, neutrophils, and lymphocytes infiltration in lung tissues	No
Duddukuri et al. (1997)	Passive cutaneous anaphylaxis	Rock bee honey	Mice (BALB/c, C57BL/6, and SWR/J)	ND	Mean age: 8 weeks old Gender: Female Mean weight: ND	Co-treatment	Group 1: Normal control Group 2–4: i.p. injection with OVA/cowpea/finger millet (days 0, 21, and 35)	Group 5–7: i.p. injection with OVA/cowpea/finger millet mixed with 100 **μ**l rock bee honey (days 0, 21, and 35)	↓ IgE antibody level in the peripheral blood of all 3 species of mice	Yes
Alangari et al. (2017)	Mast cell degranulation	Manuka honey	LAD-2 (human mast cell line)	NA	NA	Pre-treatment	Group 1: Normal control cells Group 2 and 3: Cells induced with calcium ionophoreA23187	Group 3–5: Cells pre-treated with 0.5, 1 or 2% honey and later induced with calcium ionophore-A23187	Histamine EIA kit: dose-dependent ↓ in histamine release	Yes

ND, no data; NA, Not applicable.

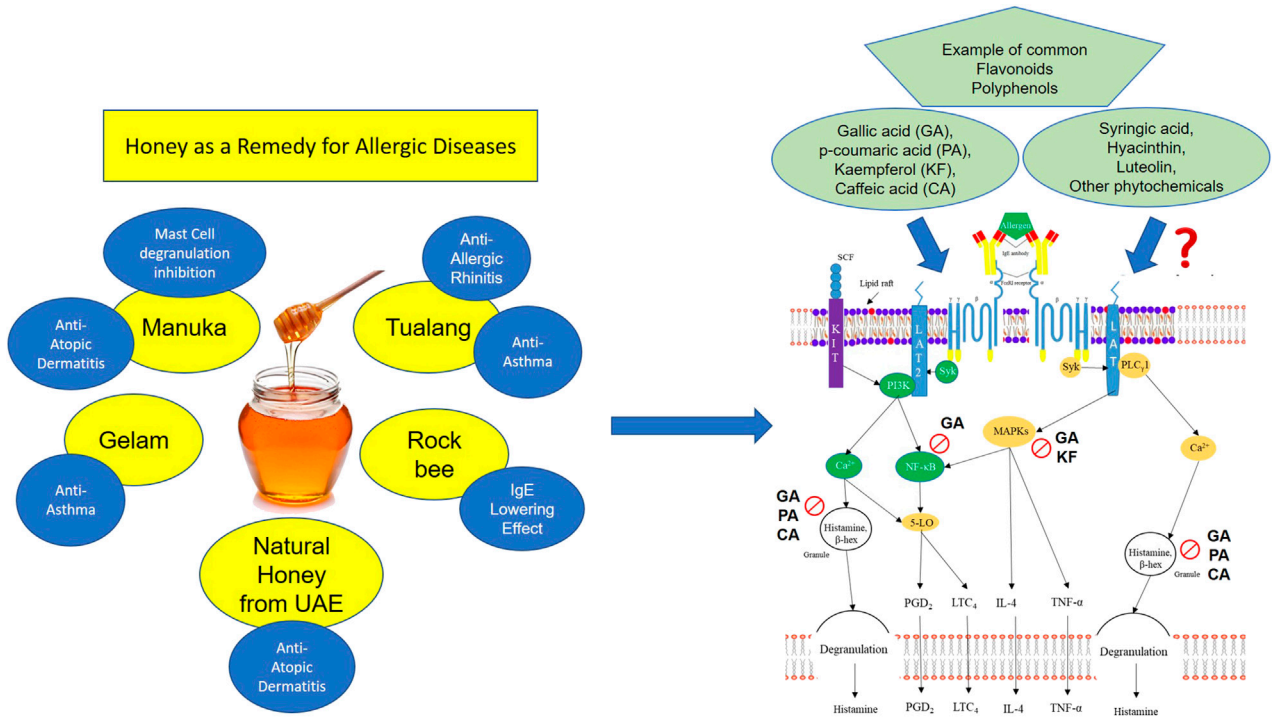

FIGURE 1 | illustrates the association of phytochemical compounds present in honey (such as gallic acid, p-coumaric acid, kaempferol, caffeic acid, and etc.) which exhibit anti-allergic properties and their mechanism of action. Many of these commonly reported phytochemical compounds have been reported to exert their anti-allergic effects by inhibiting various mast cell degranulation signaling pathways. For example, gallic acid (GA) has been reported to inhibit NF-kB, MAPKs, and the release of granules containing histamine and β-hexosaminidase, which eventually leads to attenuation of allergic symptoms

the effect of a local honey but additional details such as the species of bee or the flora sources which the bees collect the nectar to produce the honey were not declared. This additional information may provide a better explanation for the outcomes of any related studies as there is evidence that these factors can influence the therapeutic effects of honey (Liu et al., 2013).

PRECLINICAL STUDIES ON THE EFFECTS OF HONEY

In vitro Model of Mast Cell Degranulation

A study demonstrated that manuka honey is able to inhibit allergic disease by modulating mast cell response (Alangari et al., 2017). In the study, the LAD-2 human mast cell line induced by calcium ionophore was used as an *in vitro* model of allergic reaction to measure the inhibition of histamine release, a key indicator of mast cell degranulation. They reported that pretreatment of honey (0.5, 1, and 2%) was able to inhibit the release of histamine in a concentration-dependent manner.

Specific IgE Lowering Effect

A study conducted by Duddukuri et al. (1997) showed that intraperitoneal (i.p.) administration of 100 µl Rock bee honey (*Apis dorsata*) inhibited antibody responses in BALB/c, C57BL/6, and SWR/J mice induced by 10 µg OVA. Specifically, its anti-passive cutaneous anaphylaxis was indicated by the suppression of antigen-specific IgE levels (IgE titer <4). The fact that this effect was observed in different mice strains with different haplotypes highlights that the specific IgE lowering effect of Rock bee honey is less likely to be influenced by genetic variation. In addition, the authors also demonstrated that the Rock bee honey was able to inhibit the antigen-specific IgE levels in BALB/c mice induced by 100 µg of cowpea or finger millet, suggesting that honey treatment can even dampened the humoral antibody response following exposure to different types of allergen. Apart from Rock bee honey, the study also showed that Apiary and Dabur honey obtained from West Bengal, India were effective at downregulating the antigen-specific IgE production in OVA induced-BALB/c mice. This demonstrates the potential lowering effect of antigen-specific IgE in other Indian commercial honey. However, in contrast to rock bee honey, the authors did not specify any details on the Apiary and Dabur honeys used in their study, which are important to make a better justification on the positive outcome of the findings.

Allergic Asthma

The beneficial effects of honey in allergic asthma have been shown in both mice and rabbit models where previous studies have supported that honey is a promising candidate to treat allergic asthma. The anti-asthmatic effect of raw Gelam honey (*Apis mellifera*) which originated from Malaysia has been reported previously (Shamshuddin and Mohd Zohdi, 2018). This study showed that oral administration of Gelam honey

(40% and 80% (v/v)) exhibits a significant dose-dependent reduction in the airway epithelium thickening and infiltration of inflammatory cells (lymphocytes, neutrophils, and eosinophils) at peribronchiolar region and in the BALF of OVA-induced BALB/c mice. In addition, Gelam honey (10, 40, and 80% (v/v)) was also effective at attenuating the mast cells infiltration in the bronchial region and their beta-hexosaminidase production in the BALF, suggesting that this honey exerts anti-asthmatic effect by modulating the activities of mast cells, apart from other inflammatory cells. However, Gelam honey treatment only caused a marked reduction in airway mucin expression at 80% (v/v), an observation which the authors claimed is comparable to dexamethasone (3 mg/kg).

Another study investigated the effectiveness of aerosolized Tualang honey (25 and 50% (v/v)) as both rescue and preventative agents in OVA-induced rabbits (Kamaruzaman et al., 2014). Regardless of the dosage and treatment method (pretreatment or cotreatment), aerosolized Tualang honey was able to significantly inhibit goblet cell hyperplasia, mucus overproduction, and infiltration of inflammatory cells (eosinophils, mononuclear, neutrophils, and macrophage) in the peribronchial region and BALF in OVA-induced rabbits. These findings highlight the potential of Tualang honey in preventing allergic asthma as well as alleviating the symptoms. However, aerosolized Tualang honey only showed a significant decrease in the airway thickening of both epithelial and mucosal regions, but not in the submucosal region, regardless of the dosage and treatment methods. As submucosal thickening depends mostly on the innermost smooth muscle layer thickening (Kamaruzaman et al., 2014), it is speculated that aerosolized Tualang honey is unable to inhibit the thickening of smooth muscle layer within the airway.

On the other hand, a study conducted by El-Aidy et al. (2015) demonstrated the effect of Apiary honey, obtained from Dakahlia, Egypt, in a conalbumin-induced murine model of allergic asthma. The study reported that intraperitoneal administration of 650 mg/kg honey, along with intratracheal administration of conalbumin as the inducer, did not have any significant inhibitory effects in CD1 mice. In particular, there was no significant decrease in the number of infiltrated inflammatory cells (eosinophil, monocytes, neutrophils, and lymphocytes) within lung tissues when compared to the sensitized group.

CONTROVERSIAL OUTCOMES FROM PRECLINICAL STUDIES ON THE USE OF HONEY AS AN ANTI-ALLERGIC AGENT

As described above, although limited, the preclinical studies mainly demonstrate that honey can significantly inhibit mast cell degranulation, anti-allergen IgE levels, as well as improve all the histopathological parameters of allergic asthma (**Table 3**). However, contradictory to all these positive results, the study conducted by El-Aidy et al. (2015) suggests that honey does not exert any anti-allergic effects. This discrepancy may be due to the variations between studies. For example, El-Aidy et al. (2015) used conalbumin as the inducer which was administered with the honey, whereas Shamshuddin and Mohd Zohdi (2018) induced the mice with ovalbumin. According to a previous study, conalbumin has a more faster response time and a stronger induction level compared to ovalbumin (Ho, 1979). These factors could influence the immune response and may partly explain the differential responses reported by these two studies. The findings reported by El-Aidy et al. (2015) may also be influenced by the route of administration. In the study, honey was administered intraperitoneally. A recent research has highlighted that the administration of honey by the aerosolized method allows it to readily deposit and be absorbed more easily in the airways (Abbas et al. 2019). Apart from that, similar with the clinical studies on ARC and AD, additional details on the honey used in the present study such as the bee species or the flora sources used by the bees to produce their honeys were not specified. This additional information may provide a better explanation for the negative outcomes of the study. More importantly, the bioactive compounds in honey have yet to be identified in previous studies. This may affect its anti-allergic activity as the chemical composition within the honey itself could give rise to different therapeutic responses (Rao et al., 2016).

Honey is known to contain various types of sugars and macronutrient; however, research mainly focuses on the biochemical compounds such as polyphenols which include flavonoids and phenolic acids which largely determine the bioactivity of honey. (Alvarez-Suarez et al., 2014; Putri Shuhaili et al., 2016; Ranneh et al., 2018). Although the general sugar composition of honey might be similar, the polyphenolic content may vary depending on various factors such as floral sources and local climate. Therefore, the identification of the bioactive compounds in honey as well as the inhibitory signaling pathways involved is necessary to provide a more complete insight on its mechanism of action against allergy. For example, clinical and preclinical evidence suggests that Manuka and Tualang honey may be good candidates in treating allergic diseases. Chemical composition analyses have shown that both Manuka and Tualang honey contain a vast number of phytochemicals which have been associated with their bioactivity including antioxidant, wound healing and anti-cancer properties (Alvarez-Suarez et al., 2014). Interestingly, both Manuka and Tualang honey share similar chemical composition including gallic acid, p-coumaric acid, kaempferol, syringic acid, and caffeic acid (Sarfarz and Nor Hayati, 2013; Alvarez-Suarez et al., 2014; Ranneh et al., 2018) and these bioactive compounds have been reported to demonstrate anti-allergic effects in other studies (**Figure 1**). For instance, Kim et al. (2006) reported that gallic acid inhibits the release of histamine, intracellular calcium and pro-inflammatory cytokines (TNF-α and IL-6) in rat peritoneal mast cells (RPMC) possibly via the regulation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPKs) activity. Besides, Lee et al. (2010) also reported that kaempferol exhibits anti-allergic properties through the suppression of β-hexosaminidase and cytokines (TNF-α and IL-4) release, as well as IL-4-induced activation of p38 MAPK in an *in vitro* model using RBL-2H3 cells (Lee et al., 2010). Another study conducted by Zhu et al. (2015) showed that p-coumaric acid and caffeic acid were able to inhibit the release of β-hexosaminidase through IgE-mediated RBL-2H3

cell degranulation. Nonetheless, there are many other phytochemical compounds identified in honey that are yet to be scientifically proven for its anti-allergic properties such as hyacinthin and luteolin. Furthermore, the possible synergistic effect of these phytochemicals in the attenuation of an allergic reaction can also be explored.

CONCLUSION

In summary, this mini review summarizes the evidence on the effectiveness of honey in various allergic diseases in order to demonstrate the potential of honey as CAM. Although there is limited evidence, some studies showed remarkable improvements against certain types of allergic illnesses and support that honey is an effective anti-allergic agent. However, several research gaps remain to be filled, especially the identification of bioactive phytochemical compounds that are responsible for the anti-allergic effects of specific honey. Finally, more clinical studies are required to confirm the activity of honey in the pathogenesis of various allergic diseases and its mechanism of action to further justify its role as a future potential anti-allergic agent.

AUTHOR CONTRIBUTIONS

PA and FI prepared the manuscript. JT and CT conceived the idea, reviewed the drafts and provided important information for the completion. HH and DI reviewed the draft and provided important information for the completion. All authors approved the final version of this manuscript for submission.

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Hypericum triquetrifolium and *H. neurocalycinum* as Sources of Antioxidants and Multi-Target Bioactive Compounds: A Comprehensive Characterization Combining *In Vitro* Bioassays and Integrated NMR and LC-MS Characterization by Using a Multivariate Approach

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Hypericum triquetrifolium and *H. neurocalycinum* were evaluated for their phytochemical content and *in vitro* bioactivity. NMR analyses were performed on the methanol extract of the aerial parts of *H. triquetrifolium* to establish the main classes of phytoconstituents. Then, LC-DAD-MS^n analyses were performed in order to compare the composition of aerial parts and roots extracts of both *Hypericum* species, obtained using either methanol or water as solvents. Results, processed using multivariate data analysis, showed a significantly higher phenolic content of methanol extracts compared to water extracts, while minor qualitative differences were observed between the two. Distinctive flavonoid and PAC patterns were observed for *H. triquetrifolium* and *H. neurocalycinum*, and specific compounds were exclusively detected in one or the other species. Specifically, the phloroglucinols 7-epiclusianone, hyperfirin and hyperforin were present only in *H. neurocalycinum,* while hyperforin was detected only in *H. triquetrifolium.* Extracts were assayed using different *in vitro* tests to evaluate their antioxidant properties and their inhibitory activity against several enzymes, showing significant antioxidant and metal chelating activities. Furthermore, inhibitory properties against acetylcholinesterase, butyrylcholinesterase and tyrosinase were observed. Multivariate approaches were used to correlate biological data with the phytochemical composition of the different extracts. The results, showing positive correlations between specific chemical constituents and the measured bioactivities, represent preliminary data that could guide future studies

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aimed at isolating bioactive constituents from *H. neurocalycinum* and *H. triquetrifolium* for further pharmacological evaluations.

Keywords: LC-MS, NMR, AChE, multivariate analysis, BChE, antioxidant-phytochemical studies, tyrosinase

INTRODUCTION

Hypericum genus (Hypericaceae) encompasses 465 species distributed worldwide and of which nearly 100 taxa (out of which 45 are endemic) are grouped under 19 sections in Turkey (Öztürk et al., 2009; Eroglu Ozkan et al., 2018). Folk populations across the globe have been using *Hypericum* species in traditional medicine for centuries, and even nowadays the therapeutic potential of the same species is harnessed in complementary and alternative medicine. *Hypericum perforatum* L., commonly known as St. John's Wort, is the most studied species of the *Hypericum* genus. Comprehensive reviews have been published highlighting its applications in nutraceutical, phytopharmaceutical and cosmetic products. Furthermore, numerous biological activities of *H. perforatum* L. have been studied, including antibacterial, antiviral, antidepressant and pain-relieving (Agostinis et al., 1995; Biffignandi and Bilia, 2000; Saddiqe et al., 2010; Coppock and Dziwenka, 2016; Galeotti, 2017). The increase in research awareness towards *Hypericum* species might have been nurtured by the interesting pharmacological activity of *H. perforatum* L., which has led to advanced clinical research. Although a meaningful number of scientific studies have focused on the phytochemical composition and bioactivity of numerous *Hypericum* species, some of them still require more scientific assessment and validation due to limited information.

Petroleum ether and methanol extracts of the flowering aerial parts of *H. neurocalycinum* Boiss. & Heldr., endemic to Turkey, have been previously reported as potential antimicrobial agents against methicillin resistant *S. aureus* and *S. epidermidis,* while demonstrating low toxicity against HeLa and NRK-52E cell lines (Ozkan et al., 2013; Özkan et al., 2019). The methanol extract of *H. triquetrifoium* Turra aerial parts, a species known as curled-leaved St. John's Wort and distributed in the Mediterranean basin (Volkov, 2018), has been reported to exert anti-inflammatory activity in carrageenan-induced paw edema rats and antinociceptive activity in mouse model challenged with formalin (Apaydın et al., 1999; Ozturk et al., 2002). I3-II8-biapigenin isolated from *H. triquetrifolium* aerial parts exhibited cytotoxic activity against amelanotic melanoma cell line C32 and large cell lung carcinoma cell line COR-L23, with IC_{50} of 5.73 and 37.42 mg/ml, respectively (Conforti et al., 2007).

The present study aimed at investigating and comparing the phytochemical composition and the antioxidant and enzyme inhibitory potential of two *Hypericum* species of the Turkish flora, namely *H. neurocalycinum* and *H. triquetrifolium*. NMR technique was used to preliminary investigate the class of metabolites in *H. triquetrifolium* extracts. NMR data allowed to set up an appropriate integrated liquid chromatography coupled to diode array detector and multi-step tandem mass spectrometry (LC-DAD-MS^n) and LC coupled to quadrupole-time of flight MS (QTOF) method for the comprehensive phytochemical characterization of methanol and water extracts prepared from both aerial parts and roots of the two *Hypericum* species. Finally, the results from phytochemical screening and *in vitro* bioassays were analyzed using multivariate techniques, in order to find correlations between specific chemical constituents and the monitored bioactivities.

MATERIALS AND METHODS

Plant Material and Preparation of Extracts

The plant material for extraction was collected in the area of Turkey during summer 2019 (*H. neurocalycinum*: Hadim village, Dedemli Valley, 3,140 m, Konya; *H. triquetrifolium*: Anamur village, the ancient city of Anemurium, 5 m, Mersin). Taxonomic identification was performed by the botanist Dr. Evren Yıldıztugay (Selcuk University, Department of Biotechnology, Konya, Turkey) and one voucher specimen for each species (voucher ID numbers: EY-3110 for *H. neurocalycinum* and EY-3072 for *H. triquetrifolium*) was deposited at the herbarium of Selcuk University. The aerial parts (flowers, leaves and stem as mix) and roots were carefully separated. Then, plant materials were dried in a shaded and well-ventilated environment. After drying (about 10 days), plant materials were powdered using a laboratory mill. Powdered plant materials were stored in a dark and cool place and they were kept away from sunlight.

In the study, maceration was preferred to obtain methanol extract. Maceration could be useful to extract thermolabile compounds and this method could be easily performed in further applications. Briefly, powdered plant samples (5 g) were stirred with 100 ml of methanol for 24 h at room temperature. Afterwards, the mixture was filtered and the solvent was evaporated by using rotary-evaporator. Infusion was selected for water extracts. Briefly, the material (5 g) was kept in boiled water (100 ml) for 15 min, then the extract was filtered and lyophilized. Obtained dry extracts were stored at 4°C (Etienne et al., 2021; Sinan et al., 2021).

Determination of Total Phenolic and Total Flavonoid Contents

Spectrophotometric methods were used to determine total phenolic and flavonoid contents, as already reported in earlier papers. Standard equivalents (gallic acid equivalent: GAE, for phenolics; rutin equivalent: RE, for flavonoids) were used to explain the contents in the plant extracts (Slinkard and Singleton, 1977; Zengin et al., 2016).

Phytochemical Investigations

For the preliminary NMR analyses, a sample of *H. triquetifolium* methanol extract was dissolved in methanol/water (50%) mixture (22.5 mg/ml) and the solution was loaded on a Bondelute C-18 solid phase extraction (SPE) cartridge (3 ml). Cartridge was washed with water (2 column volumes), then compounds were eluted using methanol/water (2 volumes) and methanol (2 volumes).

NMR Spectroscopy

NMR spectra were recorded at 600 MHz on Bruker Avance NEO spectrometer equipped with a Cryo probe Prodigy TCI 5 mmm. All experiments were performed at 298 K. COSY, TOCSY, edited-HSQC, HMBC spectra were obtained using gradient selected pulse sequences. The spectral widths were 7,000 and 25,000 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 1,024 for ^{1}H-dimension with a recycle delay of 1.5 s. TOCSY experiments were acquired with 16 transients, 512 increments in second dimension and a 70 ms of spin lock period. Heteronuclear spectra were acquired with 64–96 transients, and 140–200 time increments in ^{13}C-dimension. HSQC experiments used a one-bond carbon-proton coupling constant of 145 Hz, HMBC experiments used a long-range carbon-proton coupling constant of 8 Hz. 2D spectra were processed (software Topspin 4.0.6, Bruker BioSpin) using zero filling to 1,024 in F1 dimension, squared sine-bell apodization in both dimensions, prior to Fourier transformations.

LC-DAD-MS^{n} (Ion Trap) and UPLC-QTOF Analyses

LC-DAD-MS^{n} analyses were obtained using an Agilent LC system (Series 1260) equipped with DAD, autosampler and column oven. After the chromatographic column, a "T" connection splitted the flow equally to DAD and MS. As mass spectrometer, a Varian MS 500 Ion trap equipped with Electrospray Ion Source (ESI) was used, working in negative ion mode and acquiring the data in the *m/z* range 100–2,000. Fragmentation of most intense ion species was obtained using the turbo data depending scanning (tdds®) function of the instrument. Parameters were as follows: spray shield, 600 V; nebulizer pressure, 25 psi; drying gas pressure, 15 psi; capillary voltage, 80 V; RF loading, 80%; needle voltage, 4,500 V. An Agilent XDB C-18 column (3.0 × 150 mm, 3.5 µm) was used as stationary phase. Solvents were: 1% formic acid in water A), acetonitrile B) and methanol C). Gradient was as follows: 0 min, 98% A and 2% B; isocratic up to 5 min; 25 min, 80% A, 10% B, and 10% C; 40 min, 60% A, 30% B, 10% C; 45 min, 20% A, 70% B, and 10% C; isocratic up to 60 min. The flow rate was 400 ml/min.

As reference compounds for quantitative analyses, chlorogenic acid, gallic acid, epicatechin, quercetin-3-glucoside, quercetin, hyperoside, rutin, hypericin and hyperforin were used, and calibration curves were built. Chlorogenic acid solutions were used for quantification of hydoxycinnamic derivatives at 330 nm, and the calibration curve was $y = 165.6x - 382.1$ ($R = 0.99991$). For the quantification of small phenolics, catechin and procyanidin derivatives, gallic acid and epicatechin solutions were used and analyzed at 280 nm. Calibration curves were $y = 122.2x + 16.0$ ($R = 1$) and $y = 27.8x + 111.6$ ($R = 0.9908$), respectively. Quercetin, quercetin-3-glucoside, hyperoside and rutin solutions were used for the quantification of flavonoid and flavonoid glycosides, and they were analyzed at 280 nm. Calibration curves were $y = 80.9x - 74.4$ ($R = 0.9999$), $y = 39.3x + 227.1$ ($R = 0.9889$), $y = 89.9x + 417.9$ ($R = 0.9964$) and $y = 39.2x + 19.6$ ($R = 0.9996$), respectively. Naphthodianthrone derivatives were quantified with hypericin solutions at 590 nm, and the calibration curve was $y = 266x + 8{,}199$ (R = 0.9988). Quantification of phloroglucinols was obtained using MS. Hyperforin solutions were used, and the calibration curve was $y = 5.58e^{+5}x + 1.14e^{+7}$ ($R = 0.9999$). Solutions were prepared in the range of 100–0.1 µg/ml.

Identification of compounds was obtained comparing the MS fragmentation spectra with the literature, and MS and retention times (R.T.) with those of available standard compounds.

Accurate *m/z* values were obtained using a Waters Acquity UPLC system coupled to a Waters Xevo G2 QTOF MS detector, operating in ESI (-) mode. For chromatographic separation, an Agilent Eclipse plus C18 column (2.1 × 50 mm, 1.8 µm) was used as stationary phase, and a gradient mixture of methanol 1) and 0.1% formic acid in water 2) as mobile phase. The gradient was: 0 min, 2% A; 0.75 min, 2% A; 11 min, 100% A; 13.5 min, 100% A; 14 min, 2% A and isocratic up to 15 min. Flow rate was 0.4 ml/min. MS parameters were as follows: sampling cone voltage, 40 V; source offset, 80 V; capillary voltage, 3,500 V; nebulizer gas (N_2) flow rate, 800 L/h; desolvation temperature, 450°C. The mass accuracy and reproducibility were maintained by infusing lockmass (leucine-enkephalin $[M-H]^-$ = 554.2620 m/*z*) thorough Lockspray at a flow rate of 20 µl/min. Centroided data were collected in the *m/z* range 50–1,200, and the *m/z* values were automatically corrected during acquisition using lockmass.

Determination of Antioxidant and Enzyme Inhibitory Effects

Different protocols were performed to explain the antioxidant properties of *Hypericum* extracts. The protocols included reducing power (CUPric Reducing Antioxidant Capacity assay: CUPRAC and Ferric Antioxidant Power assay: FRAP), metal chelating, phosphomolybenum and free radical scavenging (2,2-diphenyl-1-picryl-hydrazyl-hydrate assay: DPPH; and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay: ABTS). Experimental details were given in our previous paper (Grochowski et al., 2017). Inhibitory effects of *Hypericum* extracts were tested against different enzymes (tyrosinase, α-amylase, α-glucosidase and cholinesterase). Both antioxidant and enzyme inhibition assays were explained by standard equivalents (trolox and EDTA for antioxidant; galantamine for cholinesterase; kojic acid for tyrosinase; acarbose for amylase and glucosidase) (Grochowski et al., 2019; Stojković et al., 2020).

Data Analysis

One-way ANOVA followed by Turkey post-hoc test was performed to assess the difference between the averages of the samples. The analysis was performed using XLSTAT software v. 2018. The *p*-value for each parameter was evaluated, and a

$p < 0.05$ was considered as statistically significant. After the univariate analysis, a supervised PLS-DA analysis was carried out through the *R* package mixOmics to discriminate the two studied species. The variable importance on projection (VIP) score of each bioactivity was calculated to reveal the most discriminant, and Student's t-test was performed to compare the species considering those discriminant bioactivities.

Correlation Analysis

Correlation analysis between phenolic compounds identified in *Hypericum* methanol extracts and biological activities was performed using the Spearman rank correlation test. Data pre-processing included the removal of variables with more than 80% missing values, the imputation of the remaining missing values using the *K*-nearest neighbors (KNN) algorithm, and finally data normalization by means of *log* transformation and Pareto scaling. Analysis was performed using the Metaboanalyst v. 4.0 platform (Chong et al., 2019).

RESULTS AND DISCUSSION

Total Bioactive Components

Supplementary Table S1 summarizes the total phenolic and flavonoid contents of methanol and water extracts of the aerial parts and roots of *H. triquetrifolium* and *H. neurocalycinum*. In most of the cases, phenolic and flavonoid contents of the methanol extracts were significantly ($p < 0.05$) higher compared to water extracts. This finding has been already reported in several studies (Boeing et al., 2014; Osmić et al., 2018). In addition, it was also noted that phenolic and flavonoid contents of the aerial parts of both *Hypericum* species were significantly higher than the roots (**Supplementary Table S1**). Thus, on the basis of these preliminary data, phytochemical investigations were initially performed on *H. triquetifolium* aerial parts.

NMR Analysis

An initial screening on *H. triquetifolium* was performed using different NMR approaches. ^{1}H NMR was acquired on the methanol extracts of the aerial parts of *H. triquetifolium* (ATM) after partial fractionation obtained by C-18 SPE. Extract was suspended in water and loaded in the column. After washing with water, a first methanol/water (50–50%) fraction and a 100% methanol fraction were eluted. NMR analyses of the two fractions were used to support the elucidations of the main constituent present in the extract, and to compare the data obtained in LC-DAD-MS^{n}.

NMR Analysis of Methanol/Water (50–50%) ATM Fraction

The aromatic part presents a large number of signals that support the presence of hydroxycinnamic acid, *p*-coumaric acid, quercetin and a protocatechuic acid derivative. Main assignments were deduced combining the data obtained from 2D-NMR experiments as well as comparing literature data and reference compounds. **Figure 1** represents an enlarged portion of the edited-HSQC spectrum with the main assignments highlighted, while **Supplementary Figure S1** reports the chemical structures of the identified compounds. Further signals are observed in the spectrum range δ 5.00–3.00 and most of the constituents can be ascribed to sugars as α- and β-glucose, fructose and sucrose. Signals in the spectral region δ 5.5–4.5 can be ascribed to the anomeric proton of glycosidic substituents (**Figure 2**). Main assignments are reported in **Supplementary Table S2**.

NMR Analysis of 100% Methanol ATM Fraction

Methanol fraction eluted from SPE resulted less rich in compounds compared to the methanol/water one. Intense signals in the aliphatic part can be ascribed to fatty acids, as expected. Signals supporting the presence of phloroglucinols were detected, and main assignments are reported in **Supplementary Table S3**. Considering the reported phloroglucinols for different *Hypericum* species (Porzel et al., 2014), signals were assigned to hyperforin-type derivatives. In particular, signals supporting the presence of three prenyl moieties were observed, mostly due to the three sp^2 olefinic CH that showed COSY coupling with a signal in the aliphatic part of the spectrum (δ 1.65), deriving from a quaternary methyl group. Signals suggesting the presence of a keto-isobutyl moiety were also observed. Diagnostic HMBC correlations were observed from singlet at δ_H 1.05 (δ_C 16.5) assigned to methyl group 31, with keto functions at δ_C 210.0, 208 as well as with quaternary carbon at δ_C 55.0 (C-1). Further diagnostic HMBC were observed from methyl group 14 with C-6 (δ 55.0), C-5 (δ 83.7), C-7 (δ 45.0) and C-15 (δ 37.0). Comparison with literature (Porzel et al., 2014) suggested the presence of hyperpolyphyllirin as one of the most abundant derivatives.

LC-DAD-MS^{n} Phytochemical Analysis of the Different *Hypericum* Extracts

Once established the different classes of compounds by NMR, an integrated LC-DAD-MS^{n} (ion trap) and LC-QTOF (high resolution) approach was used to compare the phytochemical composition of roots and aerial parts extracts of the two *Hypericum* species obtained with water and methanol as solvents. Compound identification was performed by combining the data from DAD, ion trap (MS^{n}) and high resolution MS and comparing the results from NMR. Identified compounds could be classified in four different classes of phytoconstituents.

Phloroglucinols

Hypericum species are known for containing different isoprenyl phloroglucinols, in many cases related to hyperforin (Tawaha et al., 2010; Pang et al., 2020). In our analysis, hyperforin was used as reference compound in order to assess main fragmentation pathways and to compare the MS behaviour of other detected phloroglucinols (**Table 1**).

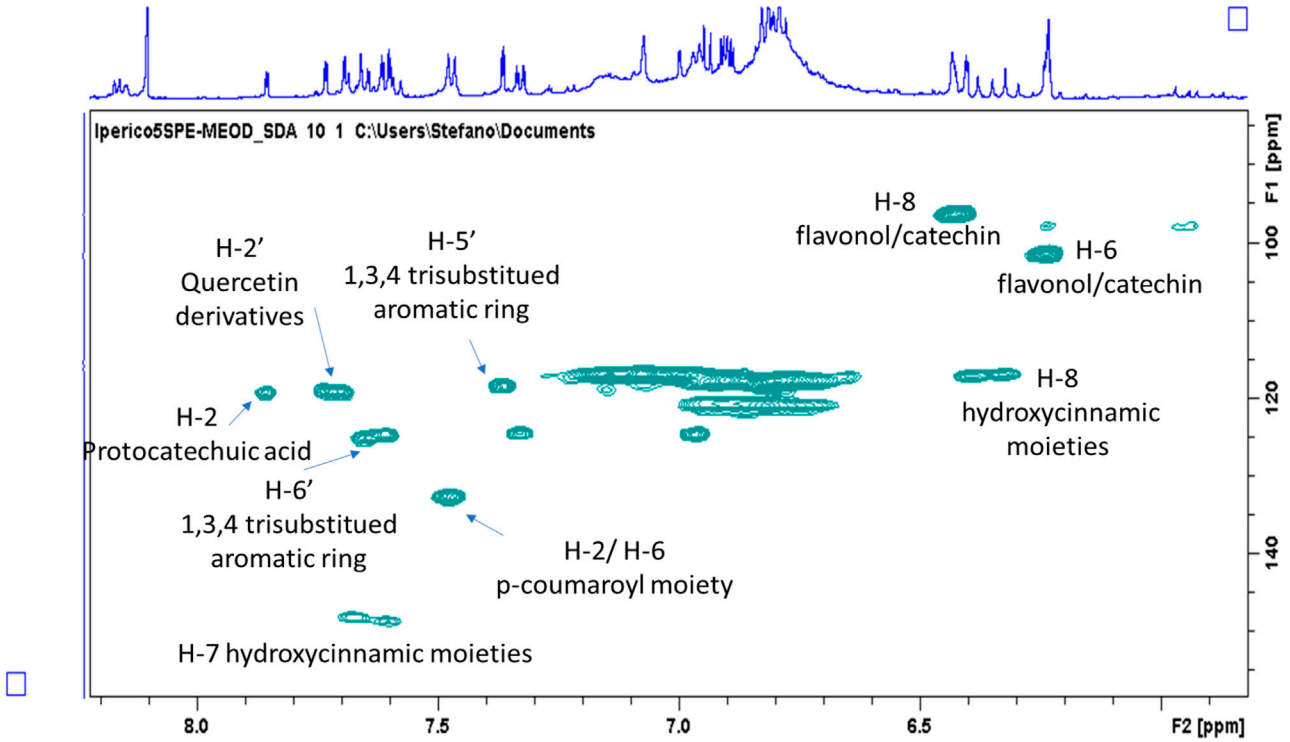

FIGURE 1 | Enlargement of the HSQC-DEPT of the methanol/water (50–50%) ATM fraction with highlighted resonances assigned to principal classes of phenolic constituents.

Considering hyperforin (R.T. 59.4 min), the most abundant fragment in MS^2 is ascribable to the loss of a prenyl (3-methylbut-2-en-1-yl) unit (− 69 a.m.u.), leading to the fragment ion at *m/z* 467. Further ions formed by the MS^2 fragmentation of the *m/z* 467 ion were observed at *m/z* 315 and 313. The fragment at *m/z* 315 could be explained with the neutral loss of two prenyl units and one 4-methylpent-3-en-1-yl one (− 83 a.m.u.). The fragment at *m/z* 313 can be ascribed to the loss of one prenyl unit, the isopropenyl moiety (−71 a.m.u.) and 4-methylpent-3-en-1-yl one (−83 a.m.u.). Other significant fragments were observed: one at *m/z* 451.8, corresponding to the loss of 4-methylpent-3-en-1-yl; one at *m/z* 398, corresponding to the loss of two 3-methylbut-2-en-1-yl units (−138 a.m.u.); one at *m/z* 384, corresponding to the loss of both prenyl and isoprenyl moieties.

MS^3 data from *m/z* 398 showed fragments at *m/z* 354 and 352, corresponding to the loss of CO_2 (−44 a.m.u.) and HCOOH (−46 a.m.u.) from the keto-enolic moiety of hyperforin. A lower molecular weight fragment at *m/z* 259 was observed after the fragmentation of the ion at *m/z* 398, due to the loss of two 3-methylbut-2-en-1-yl units (−138 a.m.u.). Ions at *m/z* 315 and 313 were formed by the fragmentation of the species at *m/z* 384, as observed for the parent ion at *m/z* 467, together with small fragments at *m/z* 271 (−113 a.m.u.), ascribable to the loss of CO_2 and 3-methylbut-2-en-1-yl (−69 a.m.u.).

Hyperfirin was assigned to the compound eluted at R.T. 58.7 min, presenting *m/z* 467 and similar fragmentation pattern as hyperforin, as suggested by previous publications (Porzel et al., 2014).

Compounds at *m/z* 481 were observed at R.T. 57.7 min and 59.3 min. Both the peaks showed MS^2 fragments related to loss of CO_2 (*m/z* 437) and a prenyl moiety (*m/z* 411), together with other signals at *m/z* 276 (corresponding to the loss of 205 a.m.u.) and 233 (−249 a.m.u.), being this latter common to other phloroglucinol derivatives (Porzel et al., 2014). The difference with hyperforin was related to the loss of CO_2 in MS^2, while in the fragmentation of hyperforin this loss was observed only in MS^3 after the loss of a prenyl unit, namely from the ion at *m/z* 467. We could suggest that these derivatives are similar to hyperforin, but with a missing prenyl unit. On the basis of the HSQC-DEPT-NMR spectrum of the methanol ATM fraction obtained by SPE (**Figure 3**) and previously published MS data (Porzel et al., 2014), the structure of the derivatives could be assigned to hyperpolyphyllirine. Due to the presence of the two chromatographic peaks, we could suggest that one is ascribable to hyperpolyphyllrine and one to an isomer.

Based on literature data and on the MS fragmentations reported in **Table 1**, tentative identification of 1′3′pren45′me4′oxoPIB, geranyl phlorisobutyrophenone, garsubellin E, 7-epiclusianone and adhyperforin were annotated. To estimate the amount of phloroglucinol derivatives in the different extracts, hyperforin was used as reference compound. As reported in **Table 1**, hyperforin was detected in the root and aerial parts extracts of *H.*

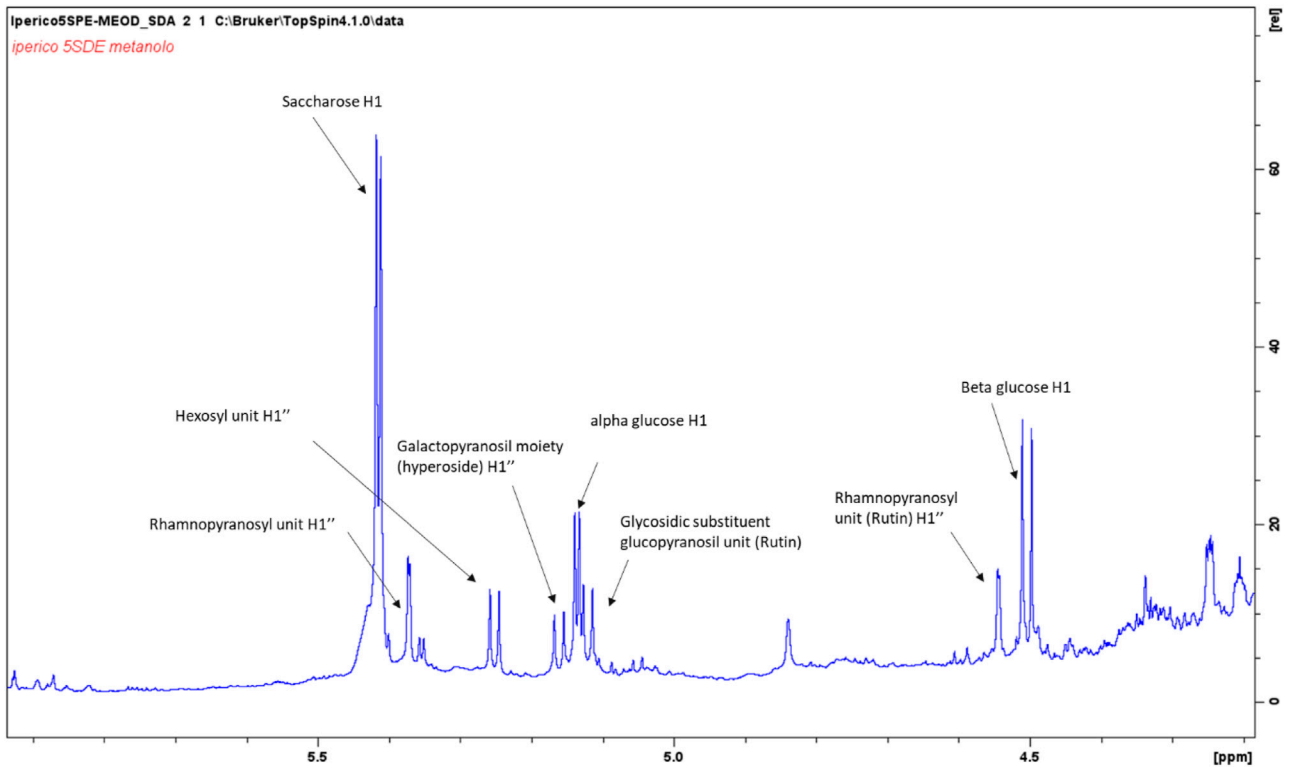

FIGURE 2 | Enlargement of the ^{1}H NMR spectrum of the methanol/water (50–50%) ATM fraction with highlighted signals assigned to principal anomeric sugar protons.

neurocalycinum. Other derivatives detected only in this species included 7-epiclusianone, hyperfirin and adhyperforin, mostly in roots. On the other hand, hyperpolyphyllirine and its isomer were observed only in *H. triquetifolium,* mostly in the aerial parts.

Hydroxycinnamic Derivatives

LC-DAD-MS^{n} and LC-QTOF analyses of both *H. triquetifolium* and *H. neurocalycinum* aerial and root extracts revealed numerous peaks with UV spectra and *m/z* values corresponding to hydroxycinnamic derivatives. These were eluting in the first part of the chromatogram, i.e. between 13–28 min. Peaks at R.T. 13.2, 22.9 and 23.5 min presenting *m/z* 353 were ascribable to 1-caffeoylquinic acid, 3-caffeoylquinic acid and 5-caffeoylquinic acid, respectively, due to their fragmentation patterns (Clifford et al., 2003). Several peaks with $[M-H]^{-}$ at *m/z* 337 were assigned to *p*-cumaroylquinic acid derivatives. LC-MS structural information are reported in **Table 2**. Overall, our findings are consistent with previously published data on *H. triquetifolium* and *H. neurocalycinum*, reporting caffeoyl- and p-coumaroylquinic acid conjugates among the most abundant phytoconstituents (Ozkan et al., 2013; Karakashov et al., 2015).

Flavonoid Glycosides and Catechin, Procyanidin and Napthodiantrone Derivatives

H. triquetifolium and *H. neurocalycinum* extracts revealed the presence of epicatechin and procyanidin derivatives (PACs) up to pentamers. Protocatechuic acid glucoside was present in all the samples. Although, to the best of our knowledge, previous studies reporting procyanidins in *H. triquetifolium* and *H. neurocalycinum* have not been published, catechin and several oligomeric procyanidins (such as A2, B2, and B3) have been identified and isolated from *H. perforatum* (Ploss et al., 2001; Hellenbrand et al., 2015) and *H. hircinum* subsp. *Majus* (Tocci et al., 2018).

Among the flavonoid constituents of *H. triquetifolium* and *H. neurocalycinum* extracts, quercetin derivatives, rutin, quercetin-3-O-glucoside and hyperoside were detected in all the samples. Hypericin was also revealed in all the analysed extracts (**Table 2**).

Qualitative and Quantitative Differences in the Composition of the Tested Extracts

Quantitative results are reported in **Tables 1** and **2**, summarising the amount of each identified metabolite. To observe differences between the two *Hypericum* species, the data matrix was initially elaborated using PCA. Results, reported the scatter plot in **Figure 4**, show a net clusterization: *H. neurocalycinum* samples are located in the upper part of the plot (green dots), while those of *H. triquetifolium* are grouped in the lower part (blue dots). The same plot shows also the clusterization of *Hypericum* samples based on the plant part extracted, i.e., roots and aerial parts populate respectively – x and + x parts of the plot. Differences between the two species could be ascribed to specific compounds, i.e. myricetin hexoside,

TABLE 1 | LC-DAD-MS^n and LC-QTOF data used for the identification of phloroglucinols in *Hypericum triquetrifolium* and *Hypericum neurocalycinum* methanol and water extracts of both aerial parts and roots.

R.T. (min)	[M-H]$^-$	MS2 *	Theoretical *m/z*	Exp. HR *m/z* **	Δppm	Molecular Formula ([M-H]$^-$)	HT-AP-MeOH	HT-AP-Water	HN-AP-MeOH	HN-AP-Water	HT-R-MeOH	HT-R-Water	HN-R-MeOH	HN-R-Water	Identification [reference]
50.4	359		359.2222	359.2225	−0.88	C22H31O4	ND	ND	ND	ND	ND	0.02 ± 0.01	ND	0.15 ± 0.01	1′3′pren45′me4′oxoPIB (Crispin et al., 2013)
54.4	331	287 (217,151) 262,207	331.1909	331.1909	0.00	C20H27O4	1.63 ± 0.03	0.04 ± 0.01	0.23 ± 0.01	ND	ND	ND	1.23 ± 0.01	0.12 ± 0.01	Geranyl phlorisobutyrophenone (Porzel et al., 2014)
54.7	497	427,357,276 233 207	497.3267	497.3276	−1.92	C31H45O5	1.12 ± 0.04	0.04 ± 0.01	ND	ND	ND	ND	1.07 ± 0.04	ND	Garsubellin E (Fukuyama et al., 1998)
56.4	501	432 (363,327,305 271)	501.3005	501.2993	2.54	C33H41O4	ND	ND	0.47 ± 0.01	0.57 ± 0.01	ND	ND	9.94 ± 0.06	1.78 ± 0.04	7-Epiclusianone (Porzel et al., 2014)
57.7	481	437 (369,245) 411,369 301 277 233	481.3318	481.3325	−1.58	C31H45O4	6.74 ± 0.06	3.36 ± 0.01	ND	ND	0.87 ± 0.02	0.33 ± 0.01	ND	ND	Hyperpolyphyllirin (Porzel et al., 2014)
58.7	467	423,398 (329,277,219) 329,287	467.3161	467.3159	0.45	C30H43O4	ND	ND	3.20 ± 0.01	3.24 ± 0.03	ND	ND	6.07 ± 0.05	6.10 ± 0.05	Hyperfirin (Porzel et al., 2014)
59.3	481	437 (369,245) 411,369 301 277 233	481.3318	481.3323	−1.14	C31H45O4	1.64 ± 0.04	0.69 ± 0.01	ND	ND	0.12 ± 0.01	ND	ND	ND	Hyperpolyphyllirin isomer (Porzel et al., 2014)
59.4	535	467,451,398 384 327 271 234	535.3787	535.3775	2.40	C35H51O4	ND	ND	0.01 ± 0.01	0.01 ± 0.01	ND	ND	0.24 ± 0.01	0.04 ± 0.01	Hyperforin (Alali et al., 2009; Porzel et al., 2014)
59.8	549	413 (369,343,327 271)	549.3944	549.3931	2.50	C36H53O4	ND	ND	0.08 ± 0.01	0.08 ± 0.01	ND	ND	ND	1.34 ± 0.01	Adhyperforin [16]

HT-AP-MeOH: H. triquetrifolium *aerial parts, methanol extract; HT-AP-Water:* H. triquetrifolium *aerial parts, water extract; HT-R-MeOH:* H. triquetrifolium *root, methanol extract; HT-R-Water:* H. triquetrifolium *root, water extract; HN-AP-MeOH:* H. neurocalycinum *aerial parts, methanol extract; HN-AP-Water:* H. neurocalycinum *aerial parts, water extract; HN-R-MeOH:* H. neurocalycinum *root, methanol extract; HN-R-Water:* H. neurocalycinum *root, water extract; ND: not detected. *: fragments in bold indicate the source of the MS3 fragments, reported in brackets; **: experimental values obtained from LC-QTOF analysis.Quantitative data of all the extracts are also reported.*

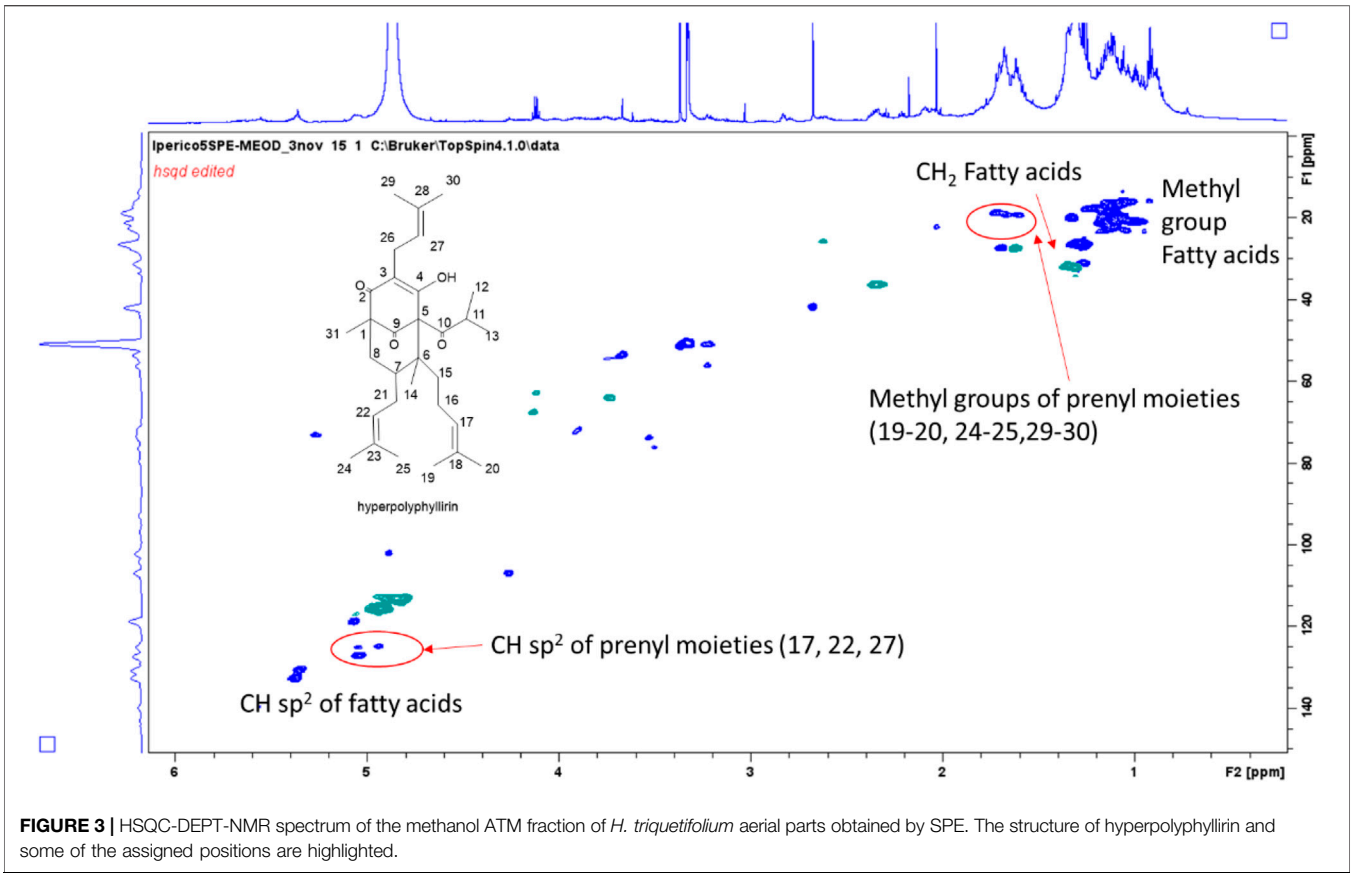

FIGURE 3 | HSQC-DEPT-NMR spectrum of the methanol ATM fraction of *H. triquetifolium* aerial parts obtained by SPE. The structure of hyperpolyphyllirin and some of the assigned positions are highlighted.

quercetin-7-O-pentoside, quercetin-3-O-pentoside, cinchonain-Ib, 7-epiclusianone, hyperfirin, and hyperforin for *H. neurocalycinum*, and PAC trimer, PAC dimer and apigenin-7-O-glyucoside for *H. triquetifolium* (**SupplementaryFigure S2**).

The PCA indicated that limited variations occurred to the samples extracted with either methanol or water. To empathise the differences in composition related to the two extraction solvents, a supervised PLS-DA was performed, and the obtained plot is reported in **Figure 5**. The model was validated using the permutation test (1,000 random permutations), and the goodness of fit as well as the predictability of the model were expressed by R2X (cum) = 0.757 and R2Y (cum) = 0.997, and Q2 (cum) = 0.68, respectively. Overall, the parameters indicated the robustness of the model. Considering the VIP coefficients (>1) and the significance level calculated comparing methanol *vs.* water samples, we summarised the findings supporting the compounds related to different extraction solvents for the two considered plant parts (aerial parts and roots) in **Supplementary Table S4**. Furthermore, data are grouped in the Table showing the sum of mg/g of total hyroxycinnamic, small phenolics and PACs, total flavonoids, and total phloroglucinols. Considering the roots of *H. triquetifolium,* methanol extract reached more than 50% (w/w) of small phenolics and PACs, while water extract reached only 27% (w/w). A similar behaviour was observed for *H. neurocalycinum* roots, where small phenolics and PACs were extracted reaching 14% in methanol and 2.2% in water. Aerial parts of *Hypericum* species were rich in hydroxycinnamic acids, accounting for 100–185 mg/g, while 6–25 mg/g were measured from the roots. Flavonoids were, as expected, in large amount mainly in the aerial parts. Going further in detail on the different constituents that have been quantified, we observed some variation. Phloroglucinols presented a different behaviour: geranyl phlorisobutyrophenon, garsubellin E, and hyperpolyphyllirin, for example, were better extracted in methanol, while hyperfirin and 7-epiclusianone were better extracted in water. This finding might be related to the different solubility of each derivative in the two solvents as well as to the different composition of the tissues that were extracted, i.e., aerial parts and roots. Considering the phenolic constituents, a good extraction could be obtained with both the solvents. Some of the most lipophilic flavonoids as quercetin aglycone were better extracted with organic solvent, while for most of the hydroxycinnamic derivatives water extraction appeared to be more effective than methanol.

Antioxidant Properties of H. triquetrifolium and H. neurocalycinum Extracts

Multiple assays were performed to assess the antioxidant capacity of the extracts and the results are given in **Table 3**. The total antioxidant capacity of the extracts was measured by recording

TABLE 2 | LC-DAD-MS^n and LC-QTOF data used for the identification of phenolic constituents in *Hypericum triquetrifolium* and *Hypericum neurocalycinum* methanol and water extracts of both aerial parts and roots. Quantitative data of all the extracts are also reported.

R.T. (min)	[M-H]$^-$	MS2 fragmentation *	Theoretical *m/z*	Experimental HR *m/z* **	Δppm	Molecular Formula ([M-H]$^-$)	Identification	HT-AP-MeOH	HT-AP-Water	HN-AP-MeOH	HN-AP-Water	HT-R-MeOH	HT-R-Water	HN-R-MeOH	HN-R-Water
Hydroxycinnamic acids															
13.2	353	191,179,135	353.0873	353.0869	1.08	C16H17O9	1-Caffeoylquinic acid	36.12 ± 0.08	61.29 ± 0.21	17.81 ± 0.08	15.09 ± 0.05	2.16 ± 0.08	1.89 ± 0.06	3.37 ± 0.08	5.71 ± 0.03
16.5	341		341.0872	341.0872	0.00	C15H17O9	Caffeoyl hexose	ND	ND	ND	4.78 ± 0.08	0.07 ± 0.05	ND	1.42 ± 0.05	1.53 ± 0.05
17.3	337		337.0923	337.0922	0.31	C16H17O8	3-*p*-Cumaroylquinic acid	11.00 ± 0.18	22.78 ± 0.08	7.37 ± 0.09	ND	0.08 ± 0.04	0.38 ± 0.04	ND	ND
17.9	337	163,119 93	337.0923	337.0920	0.94	C16H17O8	trans-5-*p*-Cumaroylquinic acid	20.00 ± 0.08	18.36 ± 0.01	0.29 ± 0.04	1.48 ± 0.05	2.27 ± 0.09	1.54 ± 0.03	0.31 ± 0.05	0.46 ± 0.05
19.1	337		337.0923	337.0922	0.31	C16H17O8	4-*p*-Cumaroylquinic acid	1.44 ± 0.08	1.09 ± 0.01	1.92 ± 0.01	1.01 ± 0.08	ND	ND	ND	ND
20.1	337		337.0923	337.0921	0.63	C16H17O8	1-*p*-Cumaroylquinic acid	1.15 ± 0.08	34.18 ± 0.07	5.10 ± 0.04	5.20 ± 0.05	ND	ND	0.15 ± 0.01	0.55 ± 0.05
21.6	337		337.0923	337.0920	0.94	C16H17O8	cis-5-*p*-Cumaroylquinic acid	25.63 ± 0.07	27.63 ± 0.01	66.52 ± 0.77	70.37 ± 0.91	1.00 ± 0.08	2.20 ± 0.02	12.39 ± 0.10	11.90 ± 0.08
22.9	353	191	353.0873	353.0867	1.68	C16H17O9	3-Caffeoylquinic acid	2.38 ± 0.04	6.11 ± 0.08	0.19 ± 0.08	ND	0.22 ± 0.01	0.32 ± 0.05	0.41 ± 0.05	ND
23.5	353	191	353.0873	353.0869	1.08	C16H17O9	5-Caffeoylquinic acid	4.63 ± 0.08	4.59 ± 0.04	1.17 ± 0.06	13.00 ± 0.12	0.35 ± 0.02	1.11 ± 0.01	0.35 ± 0.01	5.37 ± 0.03
26.8	367		367.1029	367.1027	0.60	C17H19O9	Feruloylquinic acid	2.80 ± 0.01	3.43 ± 0.06	0.34 ± 0.06	0.40 ± 0.08	ND	ND	0.00	0.04 ± 0.08
28.9	337		—	337.0923	—	—	Feruoyl derivative	1.03 ± 0.01	6.32 ± 0.12	0.41 ± 0.04	0.80 ± 0.06	0.30 ± 0.06	ND	0.22 ± 0.01	0.28 ± 0.02
Small phenolics and PACs															
10.4	315		315.0716	315.0716	0.00	C13H15O9	Protocatechuic acid glucoside	4.79 ± 0.05	2.41 ± 0.03	10.32 ± 0.06	15.32 ± 0.06	1.12 ± 0.02	0.59 ± 0.08	9.94 ± 0.04	5.10 ± 0.04
25.5	577	451,425,407	577.1346	577.1349	−0.55	C30H25O12	PAC B dimer	38.12 ± 0.08	37.09 ± 0.05	25.77 ± 0.06	34.03 ± 0.17	103.16 ± 0.08	59.65 ± 0.11	14.65 ± 0.14	1.80 ± 0.02
26.8	865	739,576,289	865.1989	865.1993	−0.49	C45H37O18	PAC trimer	2.37 ± 0.05	29.51 ± 0.06	2.55 ± 0.01	0.72 ± 0.05	20.89 ± 0.07	6.51 ± 0.05	1.68 ± 0.05	ND
27.8	289		289.0712	289.0711	0.37	C15H13O6	Epicatechin[§]	51.19 ± 0.05	14.64 ± 0.05	9.39 ± 0.08	51.70 ± 0.16	113.38 ± 0.15	48.59 ± 0.09	30.68 ± 0.14	2.08 ± 0.05
28.9	577	451,425,407	577.1346	577.1349	−0.55	C30H25O12	PAC dimer	5.16 ± 0.04	22.13 ± 0.04	0.68 ± 0.07	3.44 ± 0.04	6.94 ± 0.05	4.39 ± 0.05	0.11 ± 0.02	ND
30.8	1153	576,289	1153.2614	1153.2628	−1.29	C60H49O24	PAC tetramer	2.50 ± 0.00	4.58 ± 0.02	9.10 ± 0.05	2.52 ± 0.05	84.68 ± 0.15	32.10 ± 0.12	0.32 ± 0.00	ND
31.4	1153	576,289	1153.2614	1153.2626	−1.10	C60H49O24	PAC tetramer	13.77 ± 0.05	1.16 ± 0.08	16.43 ± 0.05	4.95 ± 0.01	ND	3.29 ± 0.05	0.36 ± 0.09	ND
31.7	1153	576,289	1153.2614	1153.2628	−1.29	C60H49O24	PAC tetramer	26.50 ± 0.05	14.18 ± 0.05	6.34 ± 0.08	26.21 ± 0.14	84.68 ± 0.14	8.99 ± 0.10	8.69 ± 0.10	ND
32.0	865	739,576,289	865.1980	865.1993	−1.59	C45H37O18	PAC trimer	17.39 ± 0.05	54.47 ± 0.23	55.51 ± 0.21	25.85 ± 0.17	74.97 ± 0.18	20.02 ± 0.10	16.78 ± 0.10	13.03 ± 0.05
34.3	1153	576,289	1153.2614	1153.2625	−1.01	C60H49O24	PAC tetramer	6.84 ± 0.05	7.12 ± 0.08	15.93 ± 0.05	15.93 ± 0.05	8.08 ± 0.07	5.41 ± 0.03	3.79 ± 0.03	ND
34.5	865	739,576,289	865.1980	865.1990	−1.23	C45H37O18	PAC trimer	10.76 ± 0.01	1.74 ± 0.01	5.74 ± 0.00	1.06 ± 0.09	8.99 ± 0.02	23.81 ± 0.05	16.67 ± 0.05	ND

(Continued on following page)

TABLE 2 | (*Continued*) LC-DAD-MS^n and LC-QTOF data used for the identification of phenolic constituents in *Hypericum triquetrifolium* and *Hypericum neurocalycinum* methanol and water extracts of both aerial parts and roots. Quantitative data of all the extracts are also reported.

R.T. (min)	[M-H]$^-$	MS2 fragmentation *	Theoretical *m/z*	Experimental HR *m/z* **	Δppm	Molecular Formula ([M-H]$^-$)	Identification	HT-AP-MeOH	HT-AP-Water	HN-AP-MeOH	HN-AP-Water	HT-R-MeOH	HT-R-Water	HN-R-MeOH	HN-R-Water
35	1441		1441.3248	1441.3247	0.07	C75H61O30	PAC pentamer	3.52 ± 0.06	5.01 ± 0.03	5.89 ± 0.09	7.42 ± 0.01	13.58 ± 0.01	13.54 ± 0.03	9.48 ± 0.05	ND
35.9	720***	644,577,407	—	—	—	—	PAC pentamer	ND	ND	ND	ND	6.28 ± 0.04	13.64 ± 0.15	9.55 ± 0.05	ND
36.9	1153	576,289	1153.2614	1153.2624	−1.01	C60H49O24	PAC tetramer	ND	ND	ND	ND	1.75 ± 0.05	2.34 ± 0.01	1.64 ± 0.01	ND
37	865	739,576,289	865.1980	865.1991	−1.23	C45H37O18	PAC trimer	ND	ND	ND	ND	2.06 ± 0.02	1.28 ± 0.05	0.90 ± 0.05	ND
38	865	739,576,289	865.1980	865.1991	−1.23	C45H37O18	PAC trimer	ND	ND	ND	ND	26.58 ± 0.16	12.14 ± 0.16	8.50 ± 0.18	ND
41	865	739,576,289	865.1980	865.1990	−1.23	C45H37O18	PAC trimer	7.08 ± 0.19	0.93 ± 0.11	10.25 ± 0.08	0.97 ± 0.15	5.17 ± 0.18	3.27 ± 0.15	ND	ND
41.9	1730		—	—	—	—	PAC derivative	4.17 ± 0.15	0.24 ± 0.05	3.18 ± 0.05	0.96 ± 0.07	1.80 ± 0.09	0.47 ± 0.02	6.94 ± 0.05	ND
Flavonoids and napthodiantrone derivatives															
34.3	479	316,287,271 243 179 151	479.0826	479.0823	0.67	C21H19O13	Myricetin hexoside	ND	ND	1.39 ± 0.01	1.75 ± 0.01	ND	ND	0.74 ± 0.02	1.86 ± 0.05
35.3	609		609.1456	609.1452	0.70	C27H29O16	Rutin§	70.24 ± 0.09	79.30 ± 0.23	ND	18.65 ± 0.02	ND	ND	0.23 ± 0.05	0.22 ± 0.04
35.3	463	301	463.0877	463.087	1.60	C21H19O12	Quercetin-3-galactoside (hyperoside)§	54.35 ± 0.12	13.15 ± 0.09	123.49 ± 0.33	46.27 ± 0.15	4.27 ± 0.04	4.06 ± 0.00	29.50 ± 0.16	6.60 ± 0.05
35.6	463	301	463.0877	463.0874	0.69	C21H19O12	Quercetin-3-glucoside§	15.87 ± 0.01	13.56 ± 0.05	26.34 ± 0.05	15.08 ± 0.03	5.46 ± 0.09	5.01 ± 0.06	8.50 ± 0.09	2.97 ± 0.09
36.8	433	300,271,255 179 151	433.0771	433.0769	0.49	C20H17O11	Quercetin 7-O-pentoside	ND	ND	43.10 ± 0.09	20.27 ± 0.10	ND	ND	12.32 ± 0.10	1.62 ± 0.09
37.1	433	301,271,179 151	433.0771	433.0766	1.22	C20H17O11	Quercetin 3-O-pentoside	ND	ND	38.65 ± 0.17	19.32 ± 0.12	ND	ND	12.92 ± 0.09	2.10 ± 0.01
37.6	447	301	447.0927	447.0928	-0.24	C21H19O11	Quercetin-3-rhamnoside	56.59 ± 0.15	56.72 ± 0.09	ND	0.34 ± 0.02	8.20 ± 0.05	3.57 ± 0.05	1.14 ± 0.01	0.66 ± 0.01
41	461		—	—	—	—	Flavonoid derivative	10.65 ± 0.00	11.61 ± 0.04	ND	ND	ND	ND	2.92 ± 0.06	2.21 ± 0.01
41.1	451	341,323,297 217 177	451.1029	451.1025	0.94	C24H19O9	Cinchonain-Ib	ND	ND	0.30 ± 0.09	1.48 ± 0.00	ND	ND	1.29 ± 0.00	1.00 ± 0.01
41.3	301		301.0348	301.0343	1.76	C15H9O7	Quercetin§	4.75 ± 0.10	1.95 ± 0.03	5.24 ± 0.06	0.80 ± 0.01	ND	ND	0.55 ± 0.03	0.06 ± 0.01
44.3	537	443,385	537.0822	537.0824	−0.39	C30H17O10	Biapigenin	6.72 ± 0.00	ND	ND	ND	ND	ND	ND	ND
44.5	537		537.0822	537.0826	−0.79	C30H17O10	Amentoflavone	0.73 ± 0.03	0.72 ± 0.03	3.43 ± 0.08	ND	ND	ND	ND	ND
59	503		503.0767	503.0766	−0.00	C30H15O8	Hypericin	1.56 ± 0.01	1.58 ± 0.03	1.49 ± 0.06	1.57 ± 0.07	1.66 ± 0.03	1.60 ± 0.01	1.18 ± 0.01	1.47 ± 0.00

HT-AP-MeOH: H. triquetrifolium *aerial parts, methanol extract; HT-AP-Water:* H. triquetrifolium *aerial parts, water extract; HT-R-MeOH:* H. triquetrifolium *root, methanol extract; HT-R-Water:* H. triquetrifolium *root, water extract; HN-AP-MeOH:* H. neurocalycinum *aerial parts, methanol extract; HN-AP-Water:* H. neurocalycinum *aerial parts, water extract; HN-R-MeOH:* H. neurocalycinum *root, methanol extract; HN-R-Water:* H. neurocalycinum *root, water extract; ND: not detected. *: fragments in bold indicate the source of the MS3 fragments, reported in brackets; **: values obtained from LC-QTOF analysis; ***: ion detected as [M-2H]$^{2-}$; §: identification was confirmed by co-injection with reference standard.*

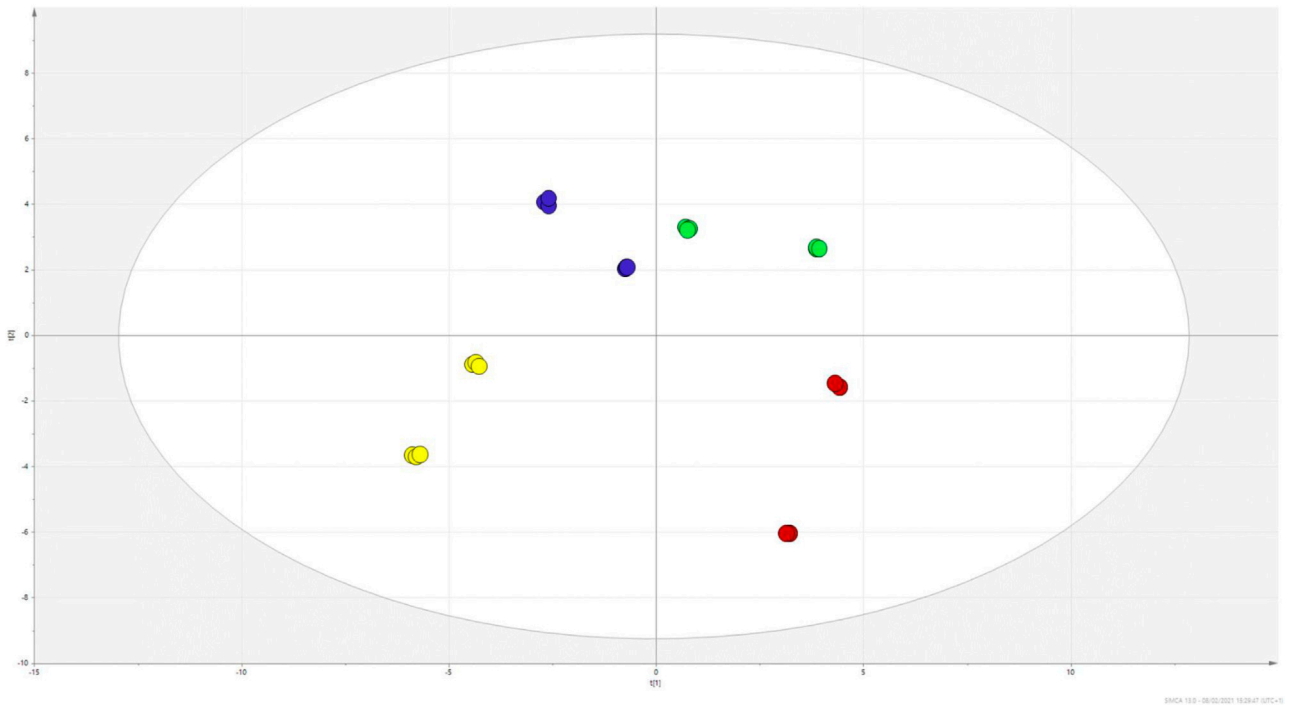

FIGURE 4 | PCA scatter plot obtained from the quantitative data of *H. triquetifolium* aerial and roots extracts (red and yellow dots, respectively) and *H. neurocalicinum* aerial and roots extracts (green and blue dots, respectively).

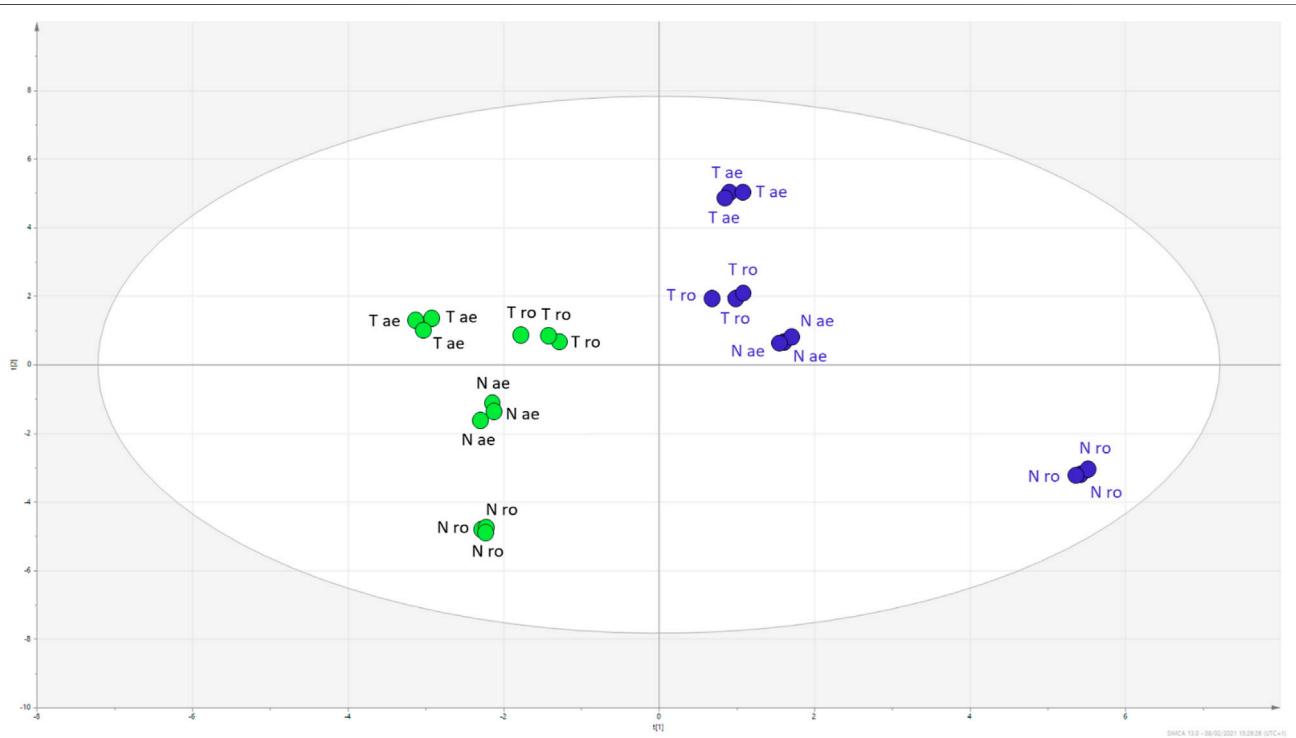

FIGURE 5 | PLS-DA scatter plot of *H. triquetifolium* (T) and *H. neurocalicinum* (N) aerial (ae) and roots (ro) extracts obtained with either methanol (green dots) or water (blue dots).

TABLE 3 | *In vitro* antioxidant properties of *Hypericum triquetrifolium* and *Hypericum neurocalycinum* extracts.

Hypericum species	Parts/ solvents	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Phosphomolybdenum (mmol TE/g)	Metal chelating ability (mg EDTAE/g)
H. neurocalycinum	Aerial parts-MeOH	251.90 ± 5.35^{d}	473.77 ± 3.04^{d}	591.69 ± 7.16^{c}	305.34 ± 0.75^{d}	2.59 ± 0.11^{d}	21.87 ± 0.64^{d}
	Aerial parts-Water	288.01 ± 3.40^{c}	400.89 ± 5.40^{e}	484.12 ± 1.39^{d}	285.40 ± 1.11^{f}	2.32 ± 0.05^{d}	26.04 ± 0.17^{b}
	Roots-MeOH	121.10 ± 1.58^{e}	335.69 ± 14.35^{f}	318.81 ± 3.82^{e}	157.60 ± 1.95^{g}	1.86 ± 0.06^{e}	22.13 ± 0.26^{cd}
	Roots-water	121.83 ± 1.64^{e}	176.28 ± 0.96^{g}	223.42 ± 1.29^{f}	118.51 ± 1.44^{h}	1.35 ± 0.05^{f}	30.00 ± 0.19^{a}
H. triquetrifolium	Aerial parts-MeOH	325.76 ± 13.44^{b}	517.19 ± 5.43^{c}	602.27 ± 14.38^{bc}	297.75 ± 1.39^{e}	3.28 ± 0.18^{bc}	12.61 ± 0.71^{f}
	Aerial parts-Water	400.42 ± 10.03^{a}	628.81 ± 22.46^{a}	694.90 ± 4.98^{a}	434.76 ± 0.34^{a}	3.61 ± 0.11^{a}	17.20 ± 0.29^{e}
	Roots-MeOH	343.17 ± 11.60^{b}	617.53 ± 27.45^{a}	610.04 ± 4.58^{b}	327.29 ± 5.47^{c}	3.36 ± 0.17^{ab}	23.24 ± 0.54^{c}
	Roots-water	407.35 ± 8.76^{a}	556.53 ± 2.00^{b}	608.08 ± 1.59^{bc}	337.30 ± 1.44^{b}	3.02 ± 0.11^{c}	26.88 ± 0.07^{b}

**Values expressed are means ± S.D. of three parallel measurements. TE, Trolox equivalent; EDTAE: EDTA equivalent. Different superscripts [(a-h)] in the same column indicate significant differences in the extracts (p < 0.05 from one-way ANOVA followed by Post Hoc Tukey test is considered significant; the superscript "a" indicates the highest activity).*

TABLE 4 | *In vitro* enzyme inhibitory effects of *Hypericum triquetrifolium* and *Hypericum neurocalycinum* extracts.

Hypericum species	Parts/solvents	AChE inhibition (mg GALAE/g)	BChE inhibition (mg GALAE/g)	Tyrosinase inhibition (mg KAE/g)	Amylase inhibition (mmol ACAE/g)	Glucosidase inhibition (mmol ACAE/g)
H. neurocalycinum	Aerial parts-MeOH	2.13 ± 0.29^{ab}	3.05 ± 0.07^{b}	67.45 ± 0.46^{b}	0.90 ± 0.03^{a}	0.97 ± 0.02^{a}
	Aerial parts-Water	0.73 ± 0.09^{d}	0.92 ± 0.05^{c}	13.96 ± 2.02^{e}	0.21 ± 0.01^{d}	na
	Roots-MeOH	1.60 ± 0.16^{c}	3.70 ± 0.38^{ab}	65.64 ± 0.63^{b}	0.80 ± 0.03^{b}	0.91 ± 0.03^{b}
	Roots-water	0.63 ± 0.08^{d}	1.10 ± 0.25^{c}	na	0.17 ± 0.01^{d}	na
H. triquetrifolium	Aerial parts-MeOH	2.05 ± 0.04^{b}	3.59 ± 0.50^{b}	66.74 ± 0.20^{b}	0.80 ± 0.01^{b}	0.96 ± 0.02^{ab}
	Aerial parts-Water	1.55 ± 0.07^{c}	0.53 ± 0.03^{c}	40.35 ± 0.27^{c}	0.91 ± 0.01^{a}	na
	Roots-MeOH	2.48 ± 0.02^{a}	4.38 ± 0.29^{a}	69.93 ± 0.14^{a}	0.81 ± 0.04^{b}	0.96 ± 0.03^{ab}
	Roots-water	1.41 ± 0.02^{c}	0.62 ± 0.02^{c}	33.89 ± 0.47^{d}	0.34 ± 0.01^{c}	na

** Values expressed are means ± S.D. of three parallel measurements. GALAE: Galatamine equivalent; KAE: Kojic acid equivalent; ACAE: acarbose equivalent; na: not active. Different superscripts [(a-h)] in the same column indicate significant differences in the extracts (p < 0.05 from one-way ANOVA followed by Post Hoc Tukey test is considered significant; the superscript "a" indicates the highest activity).*

the absorbance of green phosphate/Mo(V) complex formed in acidic condition (Boroja et al., 2018). It can be noted from **Table 3** that the methanol and water extracts of *H. triquetrifolium* aerial parts (3.28–3.61 mmol TE/g for methanol and water extracts, respectively) and roots (3.36–3.02 mmol TE/g for methanol and water extracts, respectively) exerted a higher antioxidant activity compared to *H. neurocalycinum* extracts (1.35–2.59 mmol TE/g). Metal chelation is a crucial antioxidant defence mechanism. In fact, metal chelators can sequester metal ions by forming cyclic coordination complexes (Howard and Wilson, 2003). The participation of metal ions such as iron species in Fenton reaction generate oxidizing species which contribute to oxidative stress and cause oxidative damages to biomolecules (Zhao, 2019). In the present study, *H. neurocalycinum* extracts demonstrated the highest metal chelation properties (**Table 3**). Among the *H. neurocalycinum* extracts, the water extract of *H. neurocalycinum* roots exhibited the highest metal chelating activity (30.00 mg EDTAE/g), while the methanol extract of *H. triquetrifolium* aerial parts exhibited the lowest activity.

The reducing potential of the studied *Hypericum* species was evaluated using the standard FRAP and CUPRAC methods. Interestingly, the water extract of *H. triquetrifolium* aerial parts showed highest reducing potential (694.90 and 434.76 mg TE/g). Earlier studies have reported the correlation between phenolic content and reducing properties (Upadhyay et al., 2014; Boroja et al., 2018). Likewise, this observation has been recorded for radical scavenging studies. In the present study, it was observed that the water extract of *H. triquetrifolium* aerial parts showed highest activity against ABTS radical, while the water extract of *H. triquetrifolium* root was more active against the DPPH radical. For the assessment of antioxidant activity, it has been advocated that the ABTS assay is more sensitive due to its faster reaction kinetics and its higher response to antioxidants (Lee et al., 2015). Previous studies have reported the antioxidant activity of the methanol extract of the aerial parts of *H. neurocalycinum* and *H. triquetrifolium*, but a paucity of scientific information regarding the antioxidant activity of the water extracts of these two *Hypericum* species was noted (Conforti et al., 2002; Ozkan et al., 2013; Eroglu Ozkan et al., 2018).

Evaluation of Enzyme Inhibitory Activity

Numerous investigations have attempted to harness the enzyme inhibitory potential of natural compounds for the development of

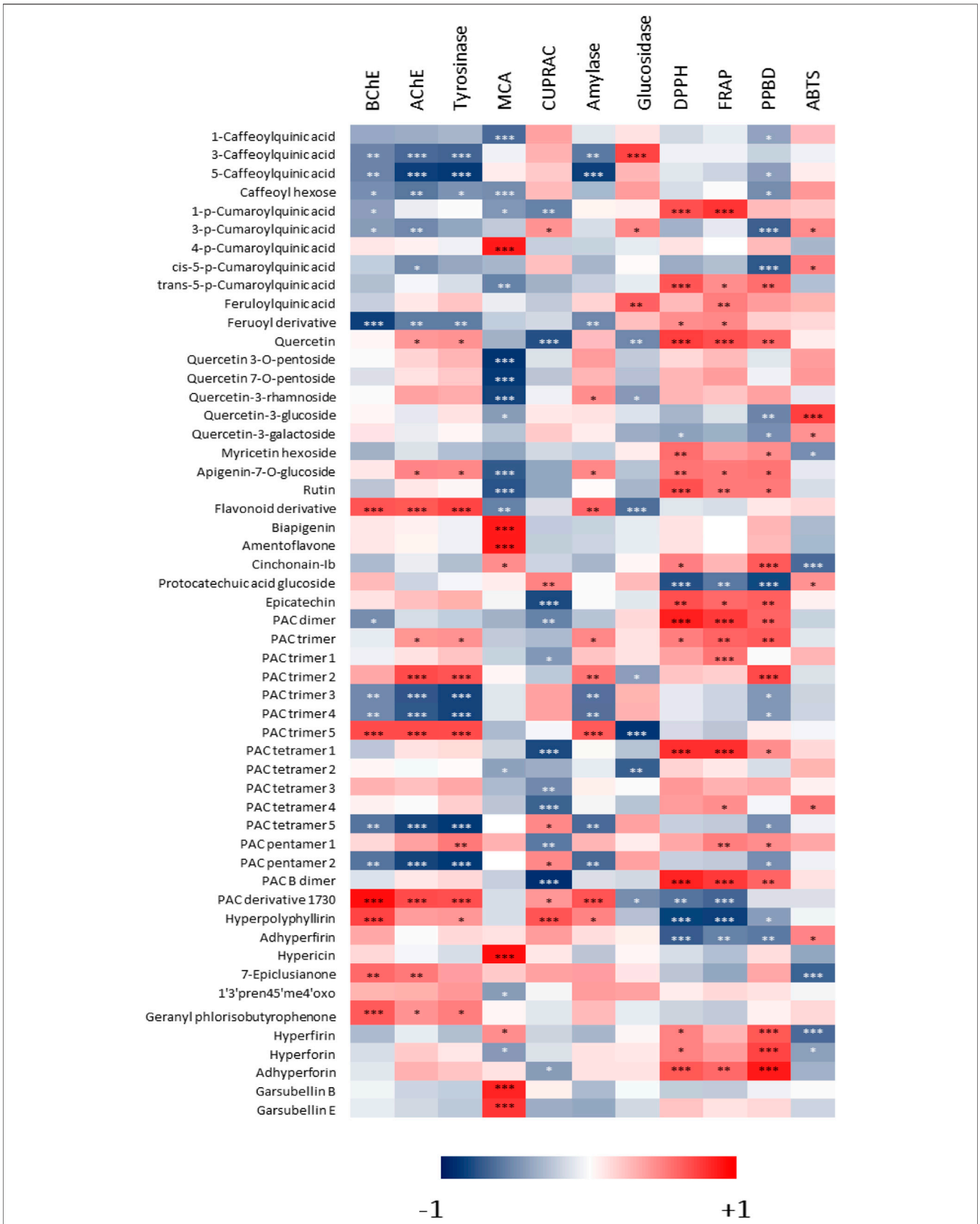

FIGURE 6 | Heatmap showing the correlations among phenolic compounds identified in *Hypericum* methanol extracts and measured biological activities. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001.

novel drug candidates. Endeavors to discover and develop lead compounds are focused on the optimization of candidates that can target specific enzymes, since enzymes characterize high level of disease association, i.e., target validation and druggability, and target tractability (Copeland et al., 2007). Here, the possible inhibitory action of methanol and water extracts of *H. neurocalycinum* and *H. triquetrifolium* aerial parts and roots was determined. As shown in **Table 4**, extracts of the studied *Hypericum* species exhibited poor inhibition on α-amylase and α-glucosidase, of which the latter has been claimed to be an interesting target for the management of diabetes type II due to less adverse effects compared to traditional treatments. It is worth mentioning that only methanol extracts exhibited some activity, while water extracts were not active against α-glucosidase. The aerial parts of another species, namely *H. perforatum* subsp. *perforatum,* have shown to exhibit inhibitory activity against both α-amylase and α-glucosidase (Kladar et al., 2017). The authors reported that the inhibitory effects were 51.10% (for α-amylase at 400 μg/ml) and 41.33% (for α-glucosidase at 400 μg/ml). Taken together, the members of the *Hypericum* genus could be considered as valuable sources of α-amylase and α-glucosidase inhibitors.

As shown in **Table 4**, the extracts of the studied *Hypericum* species exhibited inhibitory potential on AChE and BChE. Previously, it has been reported that the methanol extract of *H. neurocalycinum* (5 mg/ml) inhibited 72.24% of AChE activity (Eroglu Ozkan et al., 2018). Likewise, in the present study, the methanol extract of *H. neurocalycinum* aerial parts (2.13 mg GALAE/g) showed higher inhibitory activity against AChE compared to the other extracts studied (0.63–1.60 mg GALAE/g). However, the methanol extract of *H. triquetrifolium* roots (2.48 mg GALAE/g) showed even higher activity. Although the inhibition of AChE is considered to be a highly viable strategy for the symptomatic management of Alzheimer's disease, the role of BChE in late Alzheimer's disease has been recognized. In the present study, the methanol extract of *H. triquetrifolium* roots (4.38 mg GALAE/g) was found to exhibit high inhibition against BChE (Mehta et al., 2012). In a previously published study, *H. humifusum* exhibited higher inhibition on AChE (4.57 mg GALAE/g), α-amylase (2.55 mmol ACE/g), and α-glucosidase (8 mmol ACE/g) (Béjaoui et al., 2017). When compared to our findings, *H. humifusum* exhibited similar AChE inhibitory effects of the tested *Hypericum* species, but its amylase and glucosidase inhibitory effects were higher than those of both *Hypercium* species.

Tyrosinase is a rate limiting enzyme responsible for the biosynthesis of melanin, which is considered as a key therapeutic strategy for the management of skin hyperpigmentation conditions (Zolghadri et al., 2019). In the present study, the methanol extracts of *H. neurocalycinum* and *H. triquetrifolium* aerial parts and roots exhibited high inhibition against tyrosinase. The methanol extract of *H. triquetrifolium* roots displayed the best tyrosinase inhibitory ability (69.93 mg KAE/g extract). On the contrary, the water extract of *H. neurocalycinum* was not active on tyrosinase.

Data Mining

A PLS-DA model was developed to build an accurate species classification model based on biological data. PLS-DA is a useful supervised multivariate tool dealing with complex data: it minimizes background effects and provides an effective descriptive and predictive modelling of the data itself. It has been used in numerous scientific areas such as genomics, pharmaceutical science, lipidomics, proteomics, and many others (Zontov et al., 2020). **Figure S3A** shows the samples plot for function 1 *vs*. function 2: as illustrated, the samples of *H. neurocalycinum* are separated from *H. triquetrifolium* along the first function of the model. The k-fold cross-validation of the model is reported in **Supplemental Figure S3B**. It can be observed from the classification error rate (BER) and the maximum distance, the best performance of the model seemed to be achieved for function = 1. Additionally, the AUC value is 1, indicating that there is 100% chance that the first function of the model will be able to discriminate both studied species (**Supplemental Figure S3B**). Thus, the PLS-DA model can successfully distinguish between *H. neurocalycinum* and *H. triquetrifolium*.

The identification of differential bioactivities between both the species was carried out using the VIP values. Thus, taking into account the VIP index greater than 1, phosphomolybdenum, DPPH, ABTS, FRAP, and CUPRAC could be considered as the potential assays for distinguish the two species (**Supplemental Figure S3C**). This result entailed that the antioxidant properties played a crucial role for *H. neurocalycinum* and *H. triquetrifolium* discrimination, probably due to the presence/absence and/or great/lower quantity of compounds responsible for the observed antioxidant properties. Afterwards, through comprehensive comparison using Student's *t*-test, superior ABTS and DPPH radical scavenging, CUPRAC and FRAP reducing ability and total antioxidant capacity were recorded for *H. triquetrifolium* samples (**Figure S3D**).

Correlation Between Phytochemical Characterization and Bioactivities

Correlation analysis was performed to determine if the compounds identified in *Hypericum* extracts could be specifically related to the measured biological activities of the different samples. The results, reported in the heatmap in **Figure 6**, show that the presence of specific constituents is positively correlated to the bioactivities exerted by the extracts, while other constituents are not significantly contributing to the same activities, thus yielding in a negative contribution. In a general way, this type of analysis could help to obtain a preliminary indication about the constituents that contribute the most to the observed bioactivities.

Considering BChE, AChE and tyrosinase, the heatmap in **Figure 6** indicates that some specific constituents were related to the observed effects. For example, PAC trimer five and PAC derivative with *m/z* 1730 were positively correlated with these activities, while other PAC trimers appeared to be negatively correlated. This suggests that for these enzymatic inhibitory effects, PACs can be at least in part responsible, nevertheless changes in structure can lead to significant decrease or increase of the activity. Specific evaluation of purified compounds should be performed to assess the contribution of different derivatives. Furthermore, some hydroxycinnamic acid

derivatives were negatively correlated with the inhibition of these enzymes. Data indicate that hypopolyphyllirine and geranyl phloroisobutyrophenone were positively correlated with BChE, suggesting a certain specificity of effect. Further data should be obtained testing the single compounds to better understand the role of phloroglucinols.

With respect to the chelation of metals, two different assays were performed, namely metal chelating activity and CUPRAC. In the first assay, the most significant positive correlations ($p < 0.001$) were observed for 4-coumaroylquinic acid, biapigenin, amentoflavone, hypericin, garsubellin B, and garsubellin E. On the other hand, several flavonoid glycosides were negatively correlated with this activity. For CUPRAC, a strong ($p < 0.001$) positive correlation was observed only with hyperpolyphyllirin. 5-caffeoylquinic acid was negatively correlated with amylase inhibition, while PAC trimer 5 and PAC derivative with *m/z* 1,730 were positively correlated with the effect on this enzyme. The effect was different on glucosidase, in fact 3-caffeoylquinic acid was positively correlated, while PAC trimer 5 was negatively correlated with its inhibition. 3-Caffeoylquinic acid and feruloylquinic acid were positively correlated with α-glucosidase activity, while compounds correlated with α-amylase were some PAC derivatives. The data indicate that mainly procyanidins and simple quinic acid esters with hydroxycinnamic derivatives may exert inhibitory activity on the two enzymes related to sugar metabolism.

CONCLUSION

H. neurocalycinum and *H. triquetrifolium* from Turkey showed different phytochemical compositions, and using multivariate data analysis markers of each species were identified. Specifically, myricetin hexoside, quercetin-7-O-pentoside, quercetin-3-O-pentoside, cinchonain-Ib, 7-epiclusianone, hyperfirin, and hyperforin were assessed as markers for *H. neurocalycinum*, while PAC trimer, PAC dimer and apigenin-7-O-glyucoside were those for *H. triquetifolium*. Comparing methanol *vs.* water extractions, the former was in general more effective for the extraction of phloroglucinols, phenolic acids, PACs and flavonoids. Considering the biological assays, both *Hypericum* species showed significant antioxidant and metal chelating activities, as well as a significant inhibitory effect on tyrosinase. Moderate inhibitory activities were observed against AChE and BChE, while a weak effect on the inhibition of α-amylase and α-glucosidase was observed. In general, methanol extracts were more active than aqueous ones, and this could be explained by the higher phenolic content of the former. Finally, correlation analysis allowed to observe preliminary correlations between specific compounds among those identified and the assayed bioactivities. These results indicate that the integration of comprehensive phytochemical screening, bioactivity assays and multivariate analysis can afford a suitable and rapid approach for the identification of compounds with specific biological activities in complex natural extracts, and it could guide further isolation studies on the most promising compounds.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Conceptualization, SD, IF, GA, GP, SS, and GZ; methodology, IF, SD, SS, GP, ES, and GZ software, KS and GP; validation, SD, SS, and GZ; formal analysis, SD; investigation, IF, GA, FE, SD, MN-A, and MM; GZ resources, EY; data curation, GZ; writing—original draft preparation, IF, SD, MN-A, and MM writing—review and editing, SG, MM, and GZ visualization, KS and GP; supervision, SD, MM, and GZ project administration, GZ; funding acquisition, SD All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.660735/full#supplementary-material.

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Deciphering the Formulation Secret Underlying Chinese Huo-Clearing Herbal Drink

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Herbal teas or herbal drinks are traditional beverages that are prevalent in many cultures around the world. In Traditional Chinese Medicine, an herbal drink infused with different types of medicinal plants is believed to reduce the 'Shang Huo', or excessive body heat, a status of sub-optimal health. Although it is widely accepted and has a very large market, the underlying science for herbal drinks remains elusive. By studying a group of herbs for drinks, including 'Gan' (*Glycyrrhiza uralensis* Fisch. Ex DC.), 'Ju' (*Dendranthema morifolium* (Ramat.) Tzvelev), 'Bu' (*Microcos paniculata* L.), 'Jin' (*Lonicera japonica* Thunb.), 'Xia' (*Prunella vulgaris* L.), and 'Ji' (*Plumeria rubra* L.), the long-term jargon is connected with the inflammation of modern immunology through a few pro-inflammatory markers. *In vitro* studies have indicated that cellular inflammation is lowered by Ju and Jin either individually or synergistically with Gan. Among all herbs, only Gan detoxicated cellular toxicity of Bu in a dose dependent manner. The synergistic formulation of Ju and Gan, or Jin and Gan, in a reduction of Shang Huo, was tested *in vivo*. Both combinations exhibited a lower percentage of neutrophils, monocytes, and $CD4^+/CD8^+$ ratio in the blood, as well as inflammatory cytokines. Furthermore, body weight in the combinatory groups was more stable than treatments using single herbs. The combination of old traditional oriental methods with Western science logistics, has resulted in the formulation of different herbs into one concoction for the use of detoxification and synergism.

Keywords: ICAM-1, traditional Chinese medicine, Shang Huo, inflammation, herbal tea, herbal drink

INTRODUCTION

A great variety of herbal drinks are appreciated across the world. Besides the well-studied green tea which is used for the mitigation of oxidative stress (Serafini et al., 2011), many other kinds of herbs are also used as beverages. For example, barley (*Hordeum vulgare* L.) in East Asia (Tatsu et al., 2020), rooibos tea [*Aspalathus linearis* (Burm.f.) Dahlg] in South Africa (van Heerden and Hardie, 2020), lemongrass [*Cymbopogon citratus* (DC.) Stapf] in India and Pakistan (Kieling and Prudencio, 2019), thyme (*Thymus vulgaris* L.) in Germany (Kulisić et al., 2007), badan [*Bergenia crassifolia* (L.) Fritsch] and Ivan-tea (*Epilobium angustifolium* L.) in Russia (Shikov et al., 2014; Shikov et al., 2017), and

yerba mate (*Ilex paraguariensis* A.St.-Hil.) in South America (Bracesco et al., 2011). Similarly, herbal drinks in Traditional Chinese Medicine (TCM) are used to treat slight ailments. Different from other types of TCM, which are more potent in treating substantial diseases, herbal drinks in TCM are milder, consumed in smaller doses, but is stronger, clearly differentiated from common teas. In TCM theory it is believed that an herbal drink can remove the over-heating, or "Shang-huo" of the body. Over-heating of the body is one of the five culprits incurring disease, based on the oldest TCM transcript "Huang-di Nei-jin," which is over 2000 years old. The concept has been passed down to TCM practitioners ever since. The symptoms of TCM Shang-huo are commonly represented by a sore throat, mouth sores, swollen gums, red eyes, irritability, insomnia, and dry stool (Liu et al., 2016; Xie et al., 2017). Along with the increase in urbanization and industrialization, the rapid pace of living and a high-pressure society have placed more and more people in extended periods of anxiety, tension, insomnia, and melancholy. These signs are characteristic of a state of decreased resistance to stress, which could be ameliorated by adaptogens (Panossian et al., 2021). These symptoms of suboptimal health are also commonly associated with the above Shang-huo indicators of TCM, which are actually similar to the phenomena of topical inflammation in immunology (Pan et al., 2020). In accordance with the observation, many studies have indicated that herbs used in herbal drinks present a certain anti-inflammatory activity (Wang et al., 2008; Huang et al., 2012).

On the other hand, such herbal drinks are usually formulated by a few types of herbal extracts. There is a general formulation principle for all TCM based on another important transcript "Shen-nong Ben-cao Jing" (SBJ), which is contemporary of the "Huang-di Nei-jin." The SBJ states that an ideal formula should contain a group of harmonious herbs, each playing unique roles, mimicking a well-organized society with a monarch, a minister, an assistant, and a guide (Serafini et al., 2011). However, the principle of SBJ is hard to follow in practice because of distinct rules for its formulation, even when treating a disease with clear symptoms, let alone diseases like Shang-huo with obscure manifestations. Many TCM formulas have therefore been passed onto descendants in secret.

In the current study, Shang-huo, which is linked to inflammation through an inflammatory endothelial cell model was studied. A collection of six herbal materials were investigated in this study: *Glycyrrhiza uralensis* Fisch. Ex DC. (Leguminosae), *Dendranthema morifolium* (Ramat.) Tzvelev (Asteraceae), *Microcos paniculata* L. (Malvaceae), *Lonicera japonica* Thunb. (Caprifoliaceae), *Prunella vulgaris* L. (Lamiaceae), and *Plumeria rubra* L. (Apocynaceae). Each herb's ability to reduce Shang-Huo is indicated and then quantified by inflammatory protein markers. With the quantification, each herb's contribution to reducing the Shang-Huo was compared in parallel. A combination of different herbs in one pot also showed synergistic efficacy. Some herbs were not beneficial to reducing Shang-huo, but it did alleviate cytotoxicity. The findings in cells were also verified *in vivo*. Mice studies also showed that a combination of different herbs is favorable for maintaining immune biochemical profiles and keeping a steady body weight while ingesting a single type of herb led to weight loss.

MATERIALS AND METHODS

Herbal Extraction

The herbal materials investigated in this study consisted of six herbs: "Gan" (the root of *Glycyrrhiza uralensis* Fisch. Ex DC.), "Ju" (The flower of *Dendranthema morifolium* (Ramat.) Tzvelev), "Bu" (The leaf of *Microcos paniculata* L.), "Jin" (The flower of *Lonicera japonica* Thunb.), "Xia" (The fruit body of *Prunella vulgaris* L.), and "Ji" (The flower of *Plumeria rubra* L.) (**Figure 1**). All herbs used in this study were of Chinese origin and inspected to confirm their identity by a certified TCM pharmacist (Prof. Mo Wang). The quality of the materials was verified by high-performance liquid chromatography (HPLC; **Supplementary Figure S1**; **Supplementary Table S1**). Each herbal material was soaked in 1.5 L of water for 30 min at room temperature (RT) and then boiled for 2 h, resulting in a volume of 1 L. After filtration, concentration, and lyophilization, the powder was stored at −20°C until use. For Gan and Ju, 20 g of each material was used, and the resultant powder was 6.5 and 3.3 g, respectively. For Ju, Jin, Ji, and Xia, the initial material was 10 g from each, and the resultant powder for further experimentation was 4.57, 3.39, 2.68, and 2.01 g, respectively.

Mammalian Cell Culture

Human microvascular endothelial cells (HMEC-1) were gifted from Dr. Moonsoo M Jin (Weill Cornell Medicine). HMEC-1 cells were cultured in MCDB 131 medium (Sigma) supplemented with 10% FBS (Natocor—Industria Biológica, Argentina), 1 mg/ml hydrocortisone (Sangon Biotech, Shaihai, China), and 10 ng/ml recombinant human epidermal growth factor (Sangon Biotech, Shaihai, China). For induction of inflammation, HMEC-1 cells were treated with 1 mg/ml of LPS 3 h after the treatment with herbal extracts. The mammalian cells were maintained at 37°C in a 5% CO_2 humidity incubator.

MTT Assay

To determine cell viability, the colorimetric MTT assay was used. HMEC-1 cells (1×10^4 cells/well) were cultured in MCDB131 media in a 96-well plate at 37°C and exposed to herbal extract with different concentrations for 24 h. Cells treated with medium concentration were only used as a negative control. After removing the supernatant of each well and followed by PBS washing, cells were added with 20 μL of MTT solution (5 mg/ml in PBS) and 80 μL culture medium. The cells were then incubated for another 4 h until the formazan crystals were dissolved in dimethyl sulfoxide (100 μL). The absorbance intensity was measured by a microplate reader (Bio-RAD 680, United States) at 570 nm with a reference wavelength of 630 nm. All experiments were performed in triplicate, and the relative cell viability (%) was calculated as a percentage relative to the untreated control.

FIGURE 1 | Photos of medicinal materials of 6 herbs used in this study. These are flowers of Ju, Jin and Ji, roots of Gan, Leaves of Bu and ripe flowers and fruits of Xia. All materials were used as dry samples.

CCK-8 Assay

To determine cell viability under a different treatment, the colorimetric CCK-8 metabolic activity assay was used. HMEC-1 cells (1×10^4 cells/well) were cultured in MCDB131 media in a 96-well plate at 37°C, and then exposed to extracts with different concentrations for 24 h. Cells treated with a medium concentration only served as a negative control. After removing the supernatant of each well, PBS washing was done, before adding cells with 10 μL of CCK-8 solution (5 mg/ml in PBS) and 90 μL of culture medium. The cells were then incubated for another 4 h, and the absorbance intensity was measured by a microplate reader (Bio-RAD 680, United States) at 450 nm. All experiments were performed in triplicate, and the relative cell viability (%) was calculated as a percentage relative to the untreated control.

Immunofluorescence Flow Cytometry

Immunofluorescence flow cytometry for ICAM-1 analysis on the HMEC-1 surface was conducted according to the method described previously (Kang et al., 2011). Flow cytometry for IL-8 and MCP-1 analysis followed the instructions of the Multi-Analyte Flow Assay Kit (LEGENDplex™, Biolegend, United States). Mice peripheral blood was treated with lysis buffer (1.5 M NH_4Cl, 10 nM $NaHCO_3$, 1 mM EDTA·2Na) and washed by PBS. The cells were then permeabilized and stained with fluorescent-conjugated monoclonal antibodies to CD3, CD4, CD8, CD14, CD11b, and Ly-6G/Ly-6C (BD Biosciences, United States). After washing, the cells were analyzed on a FACSCalibur (Guava easyCyte, Millipore).

RNA Extraction and Semiquantitative RT-PCR

RNA extraction was conducted according to a previous method (Kang et al., 2011). Then 500 μg of total RNA was reverse-transcribed at 42°C for 20 min, followed by 85°C for 5 s using a reverse transcription kit (TRUEscript 1st Strand cDNA, Aidlab Biotechnologies, China) in a thermal cycler (Thermal Cycler A100, LongGene Scientific Instruments, China). The cDNA product was used for real-time gene amplification analysis. Master mix 2X qPCR kit (2x Sybr Green qPCR Mix, Aidlab Biotechnologies) was used to amplify the specified genes for quantitative PCR. Intron spanning primers were designed from the National Institute of Health qPrimerDepot using accession codes NM_000201 (ICAM-1), NM_001078 (VCAM-1), NM_002982 (MCP-1), NM_000634 (IL-8), and NM_002046 (GAPDH).

Animal Study

The animal experiments were conducted according to the guidelines for the care of animals of Huazhong Agricultural University (Wuhan, China), and were approved by the Institutional Animal Care and Ethics Committee (HZAUMO-2018-057). C57BL/6 mice (female, 6 weeks old) was purchased from the Hubei Provincial Center for disease Control and Prevention and maintained in the animal facility at Huazhong Agriculture University (Wuhan, China). Standard guidelines for laboratory animal care were followed. The study protocol was approved by the Huazhong Agricultural University Animal Care

Committee. The mice were randomly divided into different groups as indicated, each with six to eight mice. Lyophilized herbal extracts of different amounts were resuspended in saline buffer and orally administered to mice (200 μL) twice daily for 4 days. On day 4, 3 h after the administration of the herbal extracts, the mice were intraperitoneally injected with 10 mg/kg lipopolysaccharide (LPS; *Escherichia coli*. 026B6, Sigma, United States) in 300 μL saline buffer. The control was administered the same volume of saline buffer. The body weight of the mice was recorded every 24 h for 10 days.

For lymphocytes analysis, the peripheral blood was obtained by orbital sinus bleeding. The blood samples were centrifuged at 3,000 rpm for 5 min in an anticoagulant tube. The supernatant was then discarded, and red blood cell lysis buffer (Biolegend) was added to disrupt red blood cells. After sitting on a bench for 10 min, the sample was centrifuged, and the pellet was washed again by saline buffer. The pelleted lymphocytes were examined by a cytometer (Guava easyCyte, Millipore).

Statistical Analysis

Data was expressed as mean ± standard deviation (SD) of at least four identical samples. The statistical analysis of the data was carried out using GraphPad Prism 7. The unpaired student's t-test was used to determine statistical significance in comparison to the matched controls (**Figures 2**, **3**). One-way ANOVA was used to compare mean responses among different treatments and the control (**Figures 4**, **5**), followed by a Tukey's post-hoc test to determine statistical significance.

RESULTS

Quantification of Herbal Shang-Huo Reducing Capability in an Endothelial Cell Model

To explore the underlying rationale of herbal drinks, we selected a group of popular herbs, including licorice (*Glycyrrhiza uralensis* Fisch.) or "Gancao" in Chinese ("Gan" for short, hereafter used), chrysanthemum, "Juhua" or "Ju" (*Chrysanthemum morifolium* Ramat.), honeysuckle, "Jinyinhua" or "Jin" (*Lonicera japonica* Thunb.), frangipani, "Jidanhua" or "Ji" (*Plumeria rubra* L.), shiral, "Buzhaye" or "Bu" (*Microcos paniculata* L.), and self-heal, "Xiakucao" or "Xia" (*Prunella vulgaris* L.), to test their effectiveness against Shang-huo (**Figure 1**). The acronym was chosen according to Chinese phonetic pronunciation. These six herbs were individually extracted by hot water infusion. Referring to The Chinese *Pharmacopoeia* (version 2015), one to two chemicals from each herb were quantified by HPLC for quality control and standardization (**Supplementary Figure S1**; **Supplementary Table S1**).

On top of our previous cellular model, lipopolysaccharides (LPS)-induced human microvascular endothelial cells (HMEC-1), an inflammatory cell mode was applied as the cellular model (Kang et al., 2011; Zhang et al., 2018) to test the compatibility and effectiveness of Shang Huo to anti-inflammation. To select an appropriate concentration for the study, the cytotoxicity was assayed for each of these herbal extracts in HMEC-1 cells. It showed that Gan and Ji had no cytotoxicity to the cells, and even promoted the cell viability at all tested concentrations. However, the rest of the herbs all exhibited cytotoxicity at higher concentrations (**Figures 2A,B**), with Bu being the only one showing concentration-dependent toxicity at all concentrations (**Figures 2A,B**). Then HMEC-1 cells were treated with these herbs in a concentration range without cytotoxicity. The protein expression level of a few pro-inflammatory factors, including intercellular adhesion molecule 1 (ICAM-1) on the endothelia surface and chemokines monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8, were investigated. As expected, all three cytokines were greatly induced by LPS. In the presence of LPS, Ju, Bu, Jin, and Xia all imposed a concentration-dependent ICAM-1 reduction, while Gan and Ji did not lower the ICAM-1 level significantly (**Figure 2C**). Another cell surface inflammatory marker, VCAM-1, was barely detected in all treatments (data not shown). The level of a group of cytokines including TNF-α, IL-1β, MCP-1 and IFN-γ, IL-8 and MCP-1 was examined. Because HMEC-1 is not an immune cell, we could only detect IL-8 and MCP-1 expression, which showed a similar trend with ICAM-1 (**Figures 2D,E**). Overall, Ju and Jin reduced the level of these pro-inflammatory factors, while Gan and Bu had the least effects. Correspondingly, the mRNA level of these genes was also reduced when the cells were treated by these herbal extracts (**Figures 2F–I**).

The Synergistic Effects of Herbal Drink Formulation

We then focused on interpreting the rationality of mixing different herbs into a one-pot drink, since the anti-inflammatory strength of each herb is different. Because Bu exhibited a concentration-dependent toxicity, we tested the cellular viability with a combination of Bu and the other five herbal extracts, respectively. It showed that only Gan exhibited a concentration-dependent rescue of the cellular viability (**Figures 4A–E**). Gan is called the "prime minister" of all TCM because it is a core component in most TCM formulas. It is believed to possess the unique ability of harmonizing and neutralizing the rest of the components in formulas (Kao et al., 2014).

Besides neutralizing the toxicity of other herbs at higher concentrations, we wonder if Gan also contributes to the efficacy if the concentration of the herbal drink is lower. To further clarify the difference that Gan makes in an herbal extract, we selected Ju, Jin, and Xia, which all have a cytotoxicity over 0.1 mg/ml (**Figure 2**). The result showed that, the higher the concentration of Gan, the lower the ICAM-1 expression on the surface of HMEC-1 cells (**Figures 4F–H**). The group of 0.1 mg/ml Gan and 0.1 mg/ml Ju significantly suppressed ICAM-1 expression, compared to 0.1 mg/ml extract of Ju only. The same result was achieved when 0.2 mg/ml Gan and 0.1 mg/ml Xia as a group was tested against 0.1 mg/ml Xia alone. In terms of ICAM-1 reduction, the synergistic effect of Jin and Gan were marginal. Jin, Ju, or Xia separately lowered the expression of IL-8 and MCP-1. Because Gan detoxicated other herbs, we tested the combination of Gan with Jin, Xia, and Ju. It showed that Jin and Gan, or Xia and Gan, but not Ju and Gan, exhibited significant synergism (**Figure 4**).

Quantification of Herbal Shang-Huo Reducing Capability *In Vivo*

The anti-inflammation herbal extracts were tested in a mouse model. Systemic inflammation in C57BL/6 mice was induced by

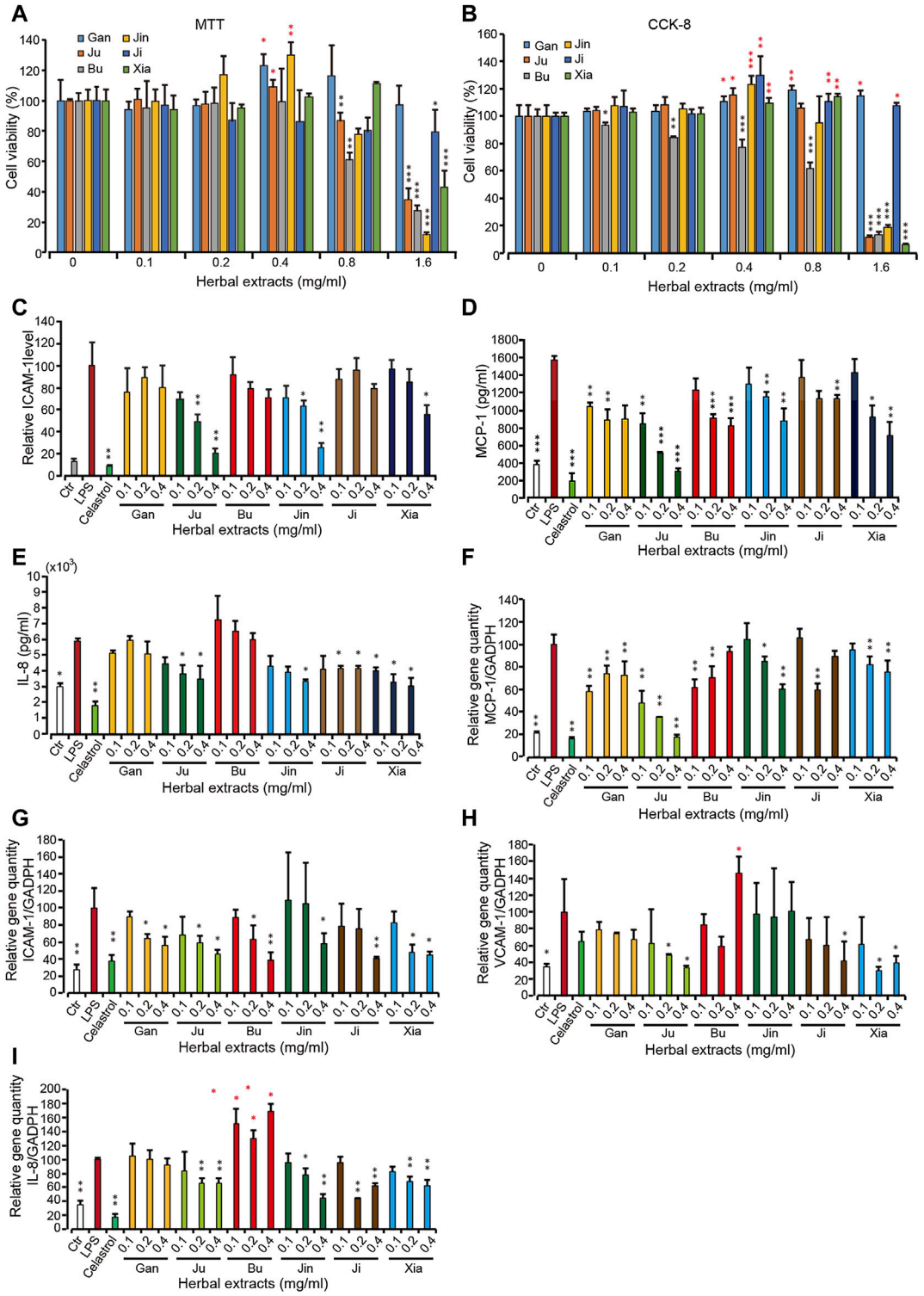

FIGURE 2 | The cellular viability and pro-inflammatory protein and gene level upon treatment with 6 herbal extracts of different concentration in inflamed HMEC-1 cells **(A,B)** The assay was done by MTT or CCK-8. **(C–E)** Pro-inflammatory protein expression under treatment of herbal extracts. HMEC-1 cells were treated with different herbal extracts at indicated concentration for 6 h and then LPS was added for 3 h. Cells were then retrieved for ICAM-1 **(C)** and supernatant was used for MCP-1 **(D)** and IL-8 **(E)** quantification. **(F–I)** The quantitative PCR was conducted for gene MCP-1 **(F)**, ICAM-1 **(G)**, VCAM-1 **(H)** and IL-8 **(I)**. The relative gene quantity was normalized with GADPH and cells without any treatment were used as blank control (Ctr). Error bars represent standard deviation, $n = 3$. Single, double or triple asterisks represent $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively (student's *t*-test).

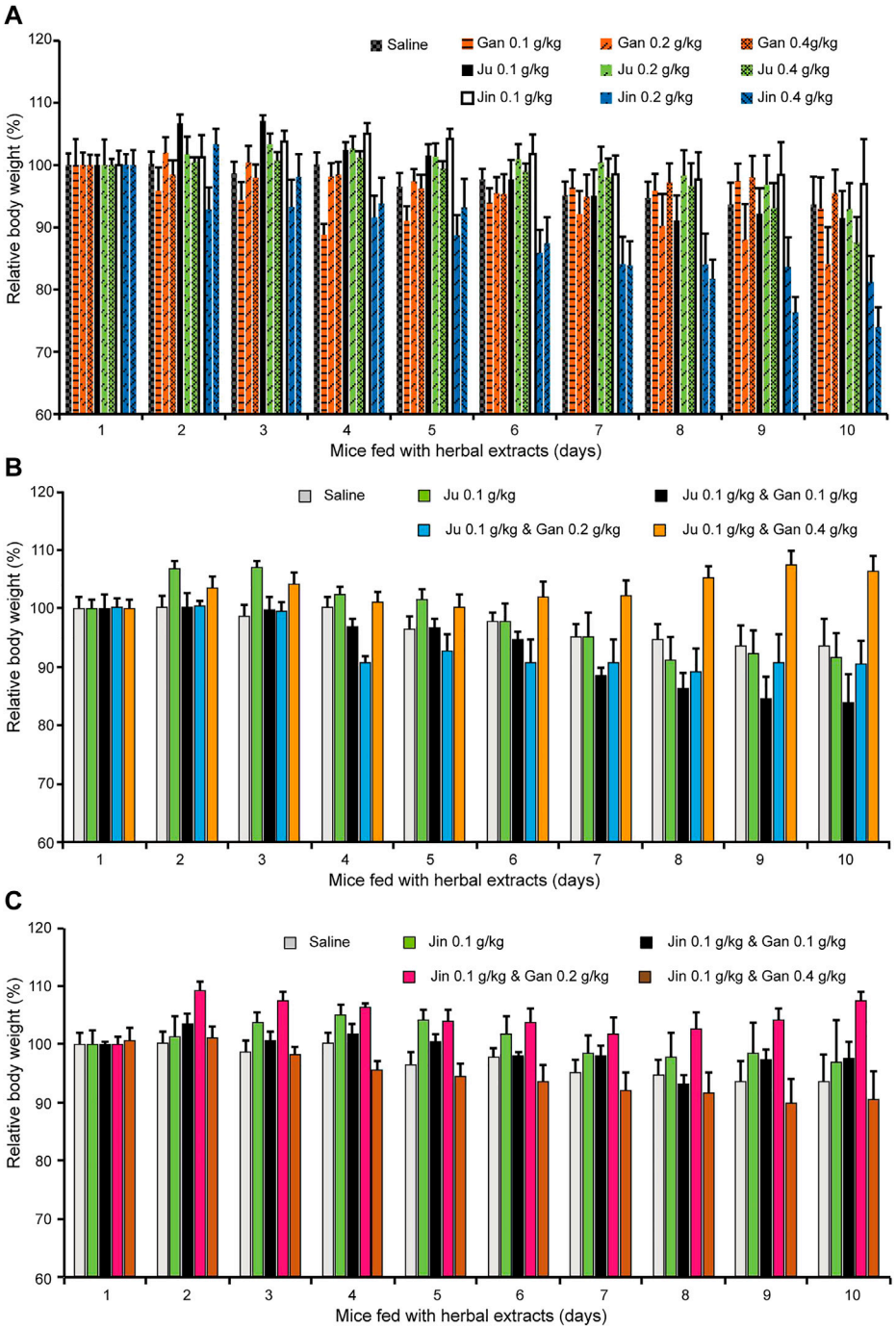

FIGURE 3 | The body weight changed upon oral administration of herbal drink. Mice fed with different combination of herbal drink for 10 days and the body weight of each day was recorded. **(A)** Single herb extract with different concentration was administrated. **(B)** Ju and Gan were co-administrated with different concentration. **(C)** Jin and Gan were co-administrated with different concentration.

LPS with intraperitoneal injection. The quantity of neutrophils and monocytes are subjected to inflammatory stimulation (Hajishengallis and Hajishengallis, 2014; Yang et al., 2014), and thus they represent a status of cellular inflammation. The percentage of neutrophils and monocytes in the peripheral blood was investigated in all treatments. This level was significantly increased by LPS treatment (**Figures 5A,B**). For herbal drinks, all treatments were orally fed three days before LPS. Individual Gan or Ju did not notably alter the percentage of neutrophils. However, the combination of both herbs significantly reduced the level (**Figure 5A**). A similar result was obtained for the percentage of monocytes (**Figure 5B**). The stabilization of the

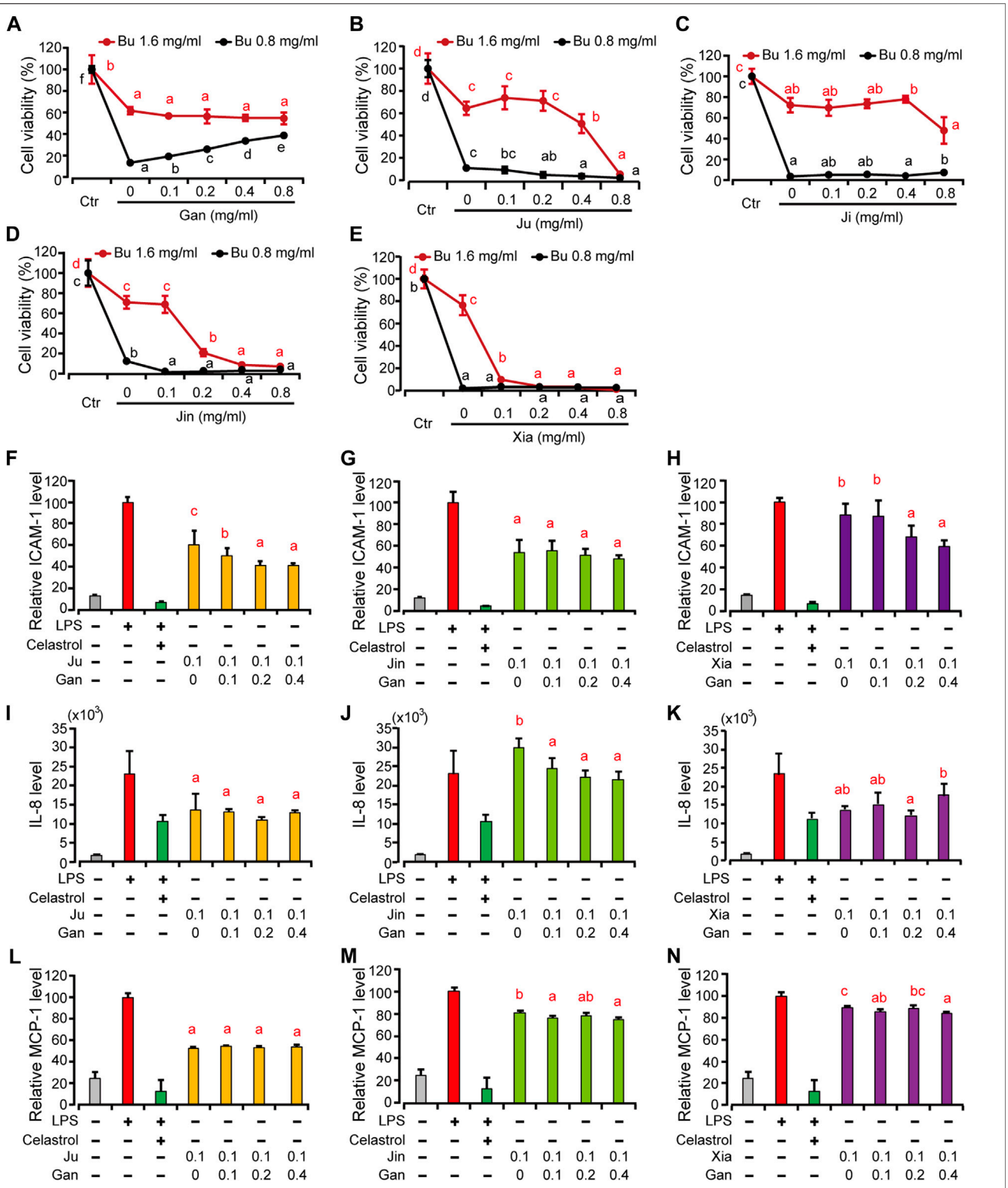

FIGURE 4 | The HMEC-1 viability and pro-inflammatory protein expression treated by different combination of herbal drink treated by Bu and other herbal extracts. **(A–E)** Gan, Ju, Ji, Jin and Xia at different concentration were combined with Bu to treat HMEC-1 cells and the cellular viability was determined with a blank control (Ctr) as 100% **(F–H)**, HMEC-1 cells were treated with different herbal extracts at indicated concentration for 6 h. And then LPS was added for 3 h. Cells were then retrieved for ICAM-1 **(F–G)** and supernatant for MCP-1 **(I–K)** and IL-8 **(L–N)** quantification. Error bars represent standard deviation, $n = 3$. Different letter above the line or bars indicates significant pair-wize differences in LSD post-hoc test ($p < 0.05$).

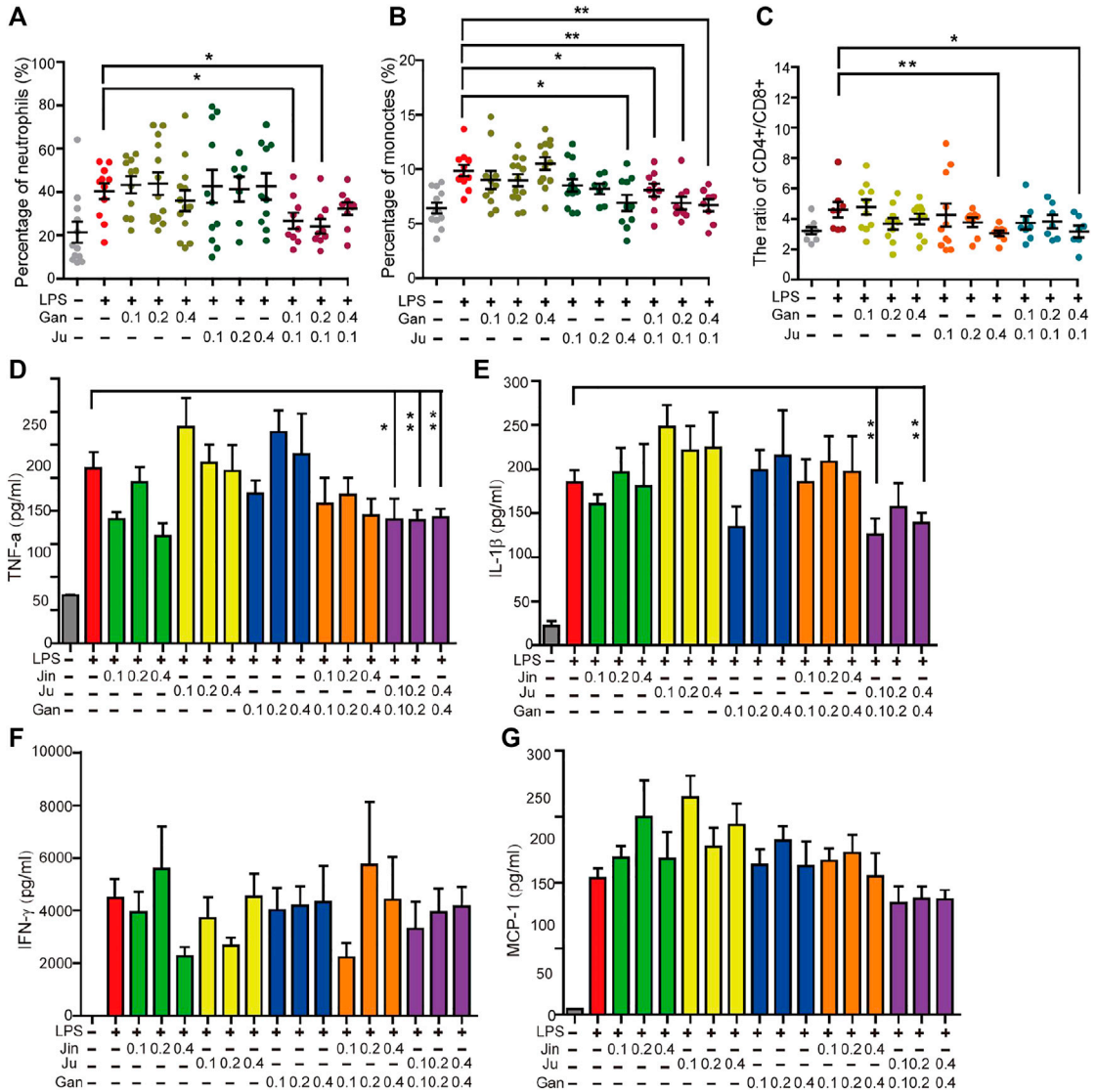

FIGURE 5 | Oral administration of herbal drink changed the mice blood white cells ratio, cytokines and the body weight. The percentage of neutrophils **(A)**, monocytes **(B)** and the ratio of $CD4^+$ and $CD8^+$ T lymphocytes **(C)** in peripheral blood were measured. Cytokines in peripheral blood cells including TNF-α, IL-1β, MCP-1, and IFN-γ were measured **(D–G)**. Single or double asterisks represent $p \leq 0.05$ and $p \leq 0.01$, respectively (student's t-test).

$CD4^+$ to $CD8^+$ T cells was investigated during the treatment. T cells play a major role in cell-mediated immunity. The ratio of $CD4^+$ to $CD8^+$ cells is a marker of HIV progression since it accurately indicates the overall immunity dysfunction imposed by the virus (Mcbride and Striker, 2017). Furthermore, the ratio of $CD4^+$ to $CD8^+$ cells increases in inflammatory diseases (Watkins et al., 2013). The subsets of T lymphocytes were analyzed during herbal extract treatment. Upon LPS induction, the ratio of $CD4^+/CD8^+$ T lymphocytes in the peripheral blood of mice significantly increased compared to the saline group (**Figure 5C**). A single Gan or Ju treatment decreased the ratio of $CD4^+/CD8^+$. When the concentration of the herbal drink went higher, the inhibitory effect was strengthened, however, without statistical significance (**Figure 5C**). The combination of Gan and Ju also lowered the ratio, but there was only a significant difference if the Gan concentration was high (**Figure 5C**).

We investigated a few classical cytokines including TNF-α, IL-1β, MCP-1, and IFN-γ in the serum of mouse peripherical blood. Each of these cytokines was significantly increased by LPS treatment. However, surprisingly, herbal treatment of Jin, Ju, or Gan alone enhanced the level of these cytokines (**Figures 5D–G**). The levels of cytokines were back to normal when Jin or Ju was composed with Gan, indicating that a combination of two herbs helps reduce the inflammatory level.

Mouse body weight was recorded during the treatment (**Figures 3A–C**). The body weight of mice fed Gan or Ju alone had no obvious change. However, it was clearly shown that Jin induced a dose-dependent body weight reduction (**Figure 3A**).

On the other hand, Gan greatly enhanced the body weight when combined with either Ju or Jin (**Figures 3B,C**). However, the most beneficial dosage for each combination was different. Gan assisted Ju (0.1 g/kg) at 0.4 g/kg, but for Jin, it was 0.2 g/kg.

DISCUSSION

Traditional medicine plays an essential role in our life. However, its scientific foundation has always been viewed with skepticism since modern science has dominated biomedical study and practice (Parker, 2003). Facing the strict paradigm of conventional science, the seemingly ambiguous and vague traditional medicine is as a compromise usually called alternative or complementary medicine. However, traditional medicine could be demystified using a modern approach (Gu and Pei, 2017; Li et al., 2018). Herbal drinks are a mild treatment for slight ailments among all means of TCM. This study lent a concept of inflammation from immunology. An abstruse jargon "Shang Huo" could be directly correlated to inflammation by quantitative measurement of inflammation related proteins. This quantification assists in comparing the capability of inhibiting excessive "Huo" between different herbs, which was impossible to differentiate using classic theories of TCM. In a previous study, the endothelial cells HMEC-1 were set up to screen anti-inflammatory chemicals from a library. ICAM-1 was chosen as a marker for the quantitative detection of its ligand and lymphocyte function-associated antigen 1 (LFA-1). LFA-1 is an integrin that is activated and specifically bound to ICAM-1 in inflammation signaling (Luo et al., 2007). With LPS as the positive control, the anti-inflammatory strength of each compound could be quantified (Zhang et al., 2018). The best inflammation-inducing or promoting chemicals were screened by this platform (Zhang et al., 2018). In the current study the system was extended to compare the Huo-clearing capability of each herb, since we deduced that inflammation could be a counterpart of "Huo" in TCM.

Similar to single chemicals, the capability of Shang Huo clearing herbs were also identified and ranked using this method. Most of the herbs tested in this study are used in TCM to clear Huo (Tatsu et al., 2020). However, there was no direct tool to compare their capability. The method allowed us to screen the best ones from a pool of candidates. Although Ju, Jin, Xia, Bu, and Ji are widely used as herbal tea ingredients to clear Huo, their actual potency has not been quantitatively compared in parallel. In this study, we could clearly see that Ju and Jin take the lead over others (**Figures 2**, **5**). Interestingly, two of three major bioactive compounds of Ju and Jin are identical (**Figure 6**). Furthermore, content **1** (chlorogenic acid) and **3** (luteolin-7-O-glucoside) in Ju was less than Jin; while a relatively high content of compound **2** (3,5-O-dicaffeoylquinic acid) was not found in Jin (**Figure 6**). Although the contribution of the anti-inflammatory effect of these compounds was not individually characterized, Ju exhibited better anti-inflammation than Jin (**Figures 2**, **5**). It indicated that two might be a key factor for the better anti-inflammatory effect of Ju than Jin.

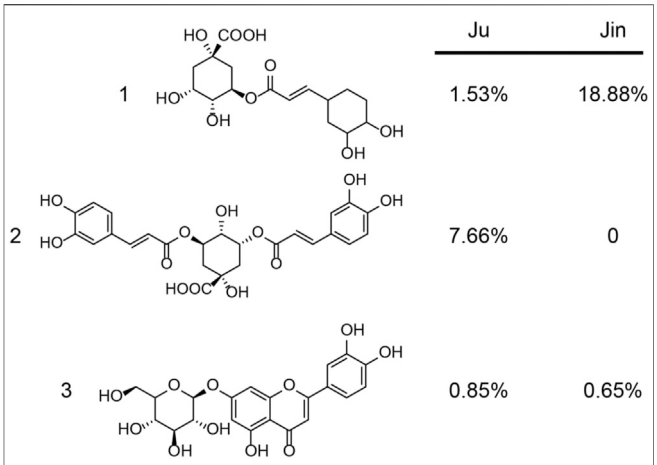

FIGURE 6 | The content of major bioactive compounds from Ju and Jin. The major compounds **1** (chlorogenic acid), **2** (3,5-O-dicaffeoylquinic acid), **3** (luteolin-7-O-glucoside) and their content (w/w) was measured.

Gan is very particular in TCM because it is used in most herbal formulas. Thus, Gan is called the prime minister of TCM (Kao et al., 2014). Glycyrrhizae radices are used in Russian traditional and officinal medicine as n expectorant and emollient, which is related to anti-inflammatory properties (Shikov et al., 2021). Anti-inflammatory features of licorice are utilized in Indian systems of traditional medicine (Pastorino et al., 2018). The philosophy of TCM believes that Gan harmonizes all herbs in most formulas (Hosseinzadeh and Nassiri-Asl, 2015). In this study, Gan showed a mild anti-inflammatory effect (**Figure 2**). However, the importance of Gan was further verified when it neutralized the toxicity of an herb, while other herbs in the group were unable to exhibit similar modes (**Figure 2**). Gan also further increased the anti-inflammation effect (**Figure 4**).

In an effort to explore the formulation principle, the mouse study was conducted. The percentage of neutrophils, monocytes, and CD4/CD8 ratio are all related to inflammation. To check the immune cellular change, mice were fed Gan, Ju, or both. The results clearly showed that Gan or Ju alone did not lower any of these factors but a combination of these two significantly induced a lower ratio, meaning less stress on the immune system.

To our surprise, after herbal extract treatment, all the cytokines were increased when mice were treated by a single herb (**Figure 5**). Obviously, it contradicted the cellular study. One possibility is that in TCM theory, every herb is assigned a profile, which might be hot, neutral, or cold, for treating diseases with these same profiles (Tatsu et al., 2020). However, Gan is neutral while the other herbs in herbal teas all belong to the cold category, which may induce stomach discomfort or inflammation if administrated alone. This could possibly explain why the mice kept losing body weight when fed a single herb (**Figures 3A–C**).

There have been reports of adverse effect due to ingestion of various herbal teas (Kumana et al., 1983; Sewell et al., 1999; Engels

et al., 2013). It has also been indicated that Chinese herbal tea tends to have a negative impact on the spleen and stomach. In the present study, the body weight of mice was monitored after feeding with herbs and it clearly proved that a combination of at least two herbs helped maintain a steady body weight (**Figure 3**). The importance of Gan is reflected by quantitative formulation with Jin and Ju. Again, our experimental results provided testimony to an old TCM principle on the rationality of formulating different herbs into a one-pot drink.

In TCM theory it is difficult to discriminate the difference of Huo-clearing capability and all so-called Huo-clearing herbs. In the current study, linking inflammation in immunology and 'Huo' of TCM enabled comparable quantification of Huo and thus the herbs' contribution to Huo-clearing could be screened and selected. The rationality of mixing different herbs with a certain ratio was also verified with statistical significance *in vitro* and *in vivo*. The synergy principles for herbal mixtures are common for adaptogens (Panossian et al., 2021) and traditional Indian systems of medicine (Mukherjee et al., 2018).

CONCLUSION

There are many kinds of herbal drinks on the market, with various herbs, and different formulas. Although all of these drinks claim a function of Huo-clearing, the lack of an evaluation system makes it difficult for customers to select the correct drink. Our method may aid in future herbs selection and the tea formulations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Ethic Committee of Huazhong Agricultural University (HZAUMO-2018-057).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JW, BZ, XdH, QZ, and XbH. Performed the experiments: JW, XbH, QZ, and XL. Analyzed the data: JW, SH, BZ, SD, MH, JW, JC, JZ, and XbH. Wrote the paper: JW, BZ, SH, AS, and XbH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.654699/full#supplementary-material.

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

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SYSTEMATIC REVIEW
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Efficacy and Safety of Berberine Alone for Several Metabolic Disorders: A Systematic Review and Meta-Analysis of Randomized Clinical Trials

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Background: Metabolic activity is the basic life activity of organisms and the fundamental for maintaining body functions. With the improvement of living standards, the incidence of metabolic disorder is also increasing. At present, most of the clinical treatment strategies and meta-analysis for metabolic disorder uncover that combined medicines with berberine ameliorate several metabolic disorders. However, evidence to disclose the therapeutic effect of berberine treatment alone and the possible factors affecting the efficacy is limited. Therefore, we have formulated strict inclusion criteria and selected more reliable data for meta-analysis through more refined screening strategies to provide evidence and guidance for clinical decision-making and understand the effect of berberine treatment alone and the factors affecting its efficacy.

Methods and results: Using meta-analysis of "Cochrane Handbook for Systematic Reviews of Interventions" as guidelines, we searched PubMed, GeenMedical, Cochrane library, and china national knowledge infrastructure (CNKI) for trials reporting clinical treatment data of berberine. Another 417 trials were included through other sources to increase confidence in results. Among the 1,660 related documents retrieved from the four databases, 18 eligible documents were selected for analysis. Given the differences in trial design and measurement units, we used the standardized mean difference (SMD) method to eliminate the differences and then summarize the data for analysis. The main factors are triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), homeostasis model assessment-insulin resistance (HOMA-IR), and fasting plasma glucose (FPG). Random-effect model analysis was performed: TG (SMD: 0.94; 95%CI: 0.49,1.38; p = 0.00), TC (SMD: 1.06; 95%CI: 0.64, 1.48; p = 0.00), LDL (SMD: 1.77; 95%CI: 1.11,2.44; p = 0.00), HDL (SMD: −1.59; 95%CI: −2.32, −0.85; p = 0.00), HOMA-IR (SMD: 1.25; 95%CI: 0.25,2.24; p = 0.01), and FPG (SMD: 0.65; 95%CI: 0.28,1.03; p = 0.00). This study aimed to conduct a systematic review and meta-analysis of the literature to evaluate the therapeutic effect of berberine singly on metabolic diseases.

Conclusion: Berberine can improve obesity and hyperlipidemia by reducing TG, TC, and LDL and increasing HDL; reduce insulin resistance to improve type II diabetes; and prevent diabetic encephalopathy.

Keywords: meta-analysis, systematic review, metabolic diseases, randomized clinical trials, berberine

INTRODUCTION

The metabolic disorder discussed in this review primarily includes nonalcoholic fatty liver disease (NAFLD), type 2 diabetes, impaired glucose tolerance (prediabetes), polycystic ovarian syndrome (PCOS), and hyperlipidemia. Previous studies have demonstrated that metabolic disorders are prone to diabetic encephalopathy and atherosclerosis (Barenbrock et al., 1995), which will generate Alzheimer's disease and coronary heart disease (Razay et al., 2007). NAFLD is closely related to type 2 diabetes and dyslipidemia (Marchesini and Babini, 2006). Characteristic changes in patients with metabolic disorders include a decrease in serum high-density lipoprotein (HDL) or an increase in serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), fasting plasma glucose (FPG), and homeostasis model assessment-insulin resistance (HOMA-IR).

At present, statins are the widely used lipid-lowering drugs worldwide. Nonetheless, its side effects can cause high blood glucose; thus, it cannot be used alone for hyperlipidemia with diabetes (Sukhija et al., 2009), and a common clinical side effect is memory and cognitive impairment. The effect refers to some unusual swelling in the neurons of patients who take statins, leading to the occurrence of memory and cognitive impairment. This effect has become an independent risk factor for induced cognitive impairment (Liu et al., 2015). At this stage, the common clinical treatment strategy is mostly the combined use of hypolipidemic and hypoglycemic drugs. However, the combined use of such drugs can cause greater risk of side effects compared with the single use of drug alone. The combination of these two drugs may increase the risks of occurrence of cognitive impairment (Suraweera et al., 2016). Therefore, we will summarize various databases on the therapeutic effect of berberine alone on metabolic disorders and conduct a systematic meta-analysis to provide a clinical medication guide and comprehensively understand the therapeutic effect of berberine alone and the factors affecting its therapeutic effect.

Berberine is an isoquinoline alkaloid compound extracted from the traditional Chinese medicine *Coptis chinensis*, and modern research has proven that it has multiple pharmacological activities (Li et al., 2018; Neag et al., 2018; Belwal et al., 2020). Recently, basic research has proven that berberine can be used to lower the blood glucose level (Liang et al., 2019), improve insulin resistance (Lou et al., 2011), improve hyperlipidemia (Li et al., 2016), and prevent mild cognitive impairment (Kumar et al., 2016). This feature improves the shortcomings of the combination of statins and metformin and shows potential as a new first-line treatment drug. This article primarily monitors the changes of HDL, TC, TG, LDL, FPG, and HOMA-IR data of patients to make clinical decision analysis on the effect of berberine, thereby providing a guiding evaluation for clinical treatment.

MATERIALS AND METHODS

Data Source

We used a combination of medical subject headings (Mesh) terminology and keywords to search four databases, namely, PubMed, Cochrane, GeenMedical, and China National Knowledge Infrastructure (CNKI), for the original studies about berberine treatment of type 2 diabetes, hyperlipidemia, and other metabolic disorders for randomized controlled trials (RCTs) without any limitations of time (1927–2021) or language. Meta-analysis was in accordance with the Cochrane Handbook for Systematic Reviews of Interventions. Another 417 additional records were included through other sources to fully incorporate all data (**Figure 1**).

Search Strategy

The Search strategy is (Berberine OR Berberine Alkaloids OR Berberine hydrochloride OR Berberidaceae OR Berberis vulgaris L. OR Coptidis Rhizoma OR Berberis) AND (diabetes OR metabolic syndrome OR hyperinsulinemia OR hyperglycemia OR diabetes mellitus OR insulin resistance OR glycemic OR glycaemic OR hyperlipidemia OR hypertension OR hypercholesterolemia OR hypertriglyceridemia OR lipid metabolism OR dyslipidemia OR overweight OR obese OR obesity OR hepatic adipose infiltration OR low-density lipoprotein cholesterol OR low-density lipoprotein cholesterol OR high-density lipoprotein cholesterol OR LDL-c OR HDL-c OR total cholesterol OR TC OR triglycerides OR TC OR adipokine OR adiponectin OR adiponectins OR leptin OR leptins) AND (human [MeSH Terms] OR Clinical Trial OR Controlled Clinical Trial). A total of 468 articles were retrieved using PubMed, 456 articles were detected by GeenMedical, 256 articles were detected by Cochrane library, and 63 articles were detected by CNKI.

Data Extraction and Study Outcomes

Our inclusion criteria were as follows: 1) study design must be a RCT of placebo controlled or parallel controlled; 2) the patients must have a metabolic disorder; 3) lifestyle intervention must be the same in the control group and trial group; 4) data of monitoring patients before and after HDL\TC\TG\LDL\FPG\HOMA-IR treatment; 5) the drug in the trial group must be berberine without any combination; 6) the drug dose in the trial group must be greater than the minimum effective dose. The exclusion criteria were as follows: 1) repeated papers in each database, 2) combined use

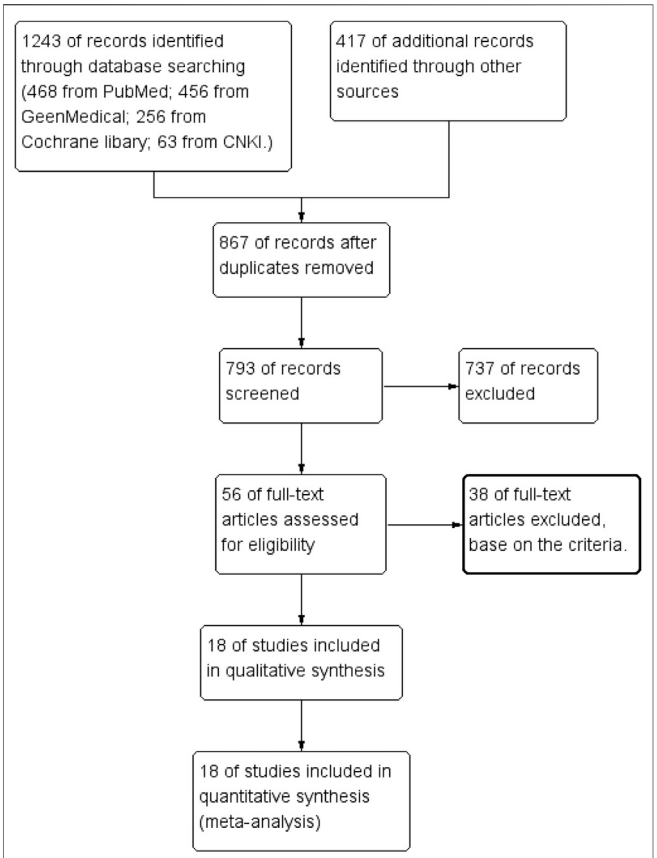

FIGURE 1 | Flow diagram.

of drugs, 3) non-clinical trial papers, 4) literature without data, 5) different lifestyle interventions used in the control and trial groups, and 6) notable deviation of research data with low credibility.

Data Quality Assessment

According to the Cochrane Handbook for Systematic Reviews of Interventions, assessments of the quality of the selected studies involved six facets: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, and selective reporting (Higgins et al., 2011; Yuan et al., 2019).

Data Synthesis and Analysis

The screening and extraction of trial data were independently and simultaneously performed by Yu Ye and Xiufen Liu. The differences in data extraction were resolved through consultation between the two investigators. In general, the baseline and after-treatment data were compared; however, the study design of Wang Li (Wang et al., 2016) and Giuseppe Derosa (Derosa et al., 2013) had a run-in period and washout period before the treatment to 1) eliminate previous treatment drugs that may affect the treatment effect, 2) screen qualified patients to participate in the trial, 3) eliminate the effects of the first phase of the drug, and then perform the second phase of treatment. For these two sets of data, we used wash-out period data as a baseline comparison with after-treatment data. We extracted mean, SD, and sample size (N) from the literature and calculated the mean difference ($mean_{treatment} - mean_{baseline}$), SD difference (square root $(SD_{treatment})^2 + (SD_{baseline})^2 - 2R \times SD_{treatment} \times SD_{baseline}$, presuming a correlation coefficient R = 0.5), and TOTAL = $N_{control} + N_{trial}$ (Yuan et al., 2019). Given the difference in trial design and measurement unit, the data adopted the standard mean difference (SMD) to eliminate the difference (Riley et al., 2011). We used RevMan5.2 software, random-effect models, or fixed-effect models (selection criteria is heterogeneity test $I^2 > 75\%$ for high heterogeneity goes random-effect models, $I^2 < 25\%$ for low heterogeneity goes fixed-effect models) to calculate the SMD and 95%CI. $p < 0.05$ is considered statistically significant, and a forest graph was used to analyze the effect size.

Subgroup Analysis

We conducted the following subgroup analyses to ascertain the causes of heterogeneity and assess the factors that may affect the effectiveness of treatment: 1) treatment period, 2) treatment dose, and 3) study design (placebo/parallel). We grouped all data of TG, TC, LDL, HDL, HOMA-IR, and FPG according to the abovementioned three procedures and then analyzed the data in each subgroup to obtain the following table (**Supplementary Table S1**). Among the factors, TG, TC, LDL, HDL, HOMA-IR, and FPG were random-effect models ($I^2 \geq 75\%$).

RESULTS

Search Result

Screening was performed by passing 1,660 papers through title and abstract review; precluding duplicate papers, non-clinical trial papers, and irrelevant papers; selecting 56 papers for full-text review; and ultimately excluding articles with non-data, drug combination treatment, different life intervention strategy, non-blank, or placebo controlled. The remaining 16 articles met the requirements (**Supplementary Table S2**).

Data Selection

In evaluating the therapeutic effect of berberine on metabolic disorders, we selected four commonly and clinically used blood lipid tests as indicators to measure the effect of berberine in lowering blood lipids. Moreover, FPG and HOMA-IR were selected as indicators to measure the therapeutic effect of berberine in lowering blood sugar and insulin resistance. After full-text review, we extracted 18 TG, 17 TC, 16 LDL, 14 HDL, 6 HOMA-IR, and 15 FPG data from the literature (**Supplementary Table S3**).

Risk of Bias Assessment

All of the 18 included trials have proclaimed a randomized trial; however, only 8 trials proclaimed the method of randomization. Eight trials specified a double-blind study design. Of the 18 included trials, 9 were placebo controlled (lifestyle intervention and berberine vs. lifestyle intervention and placebo), and 9 were

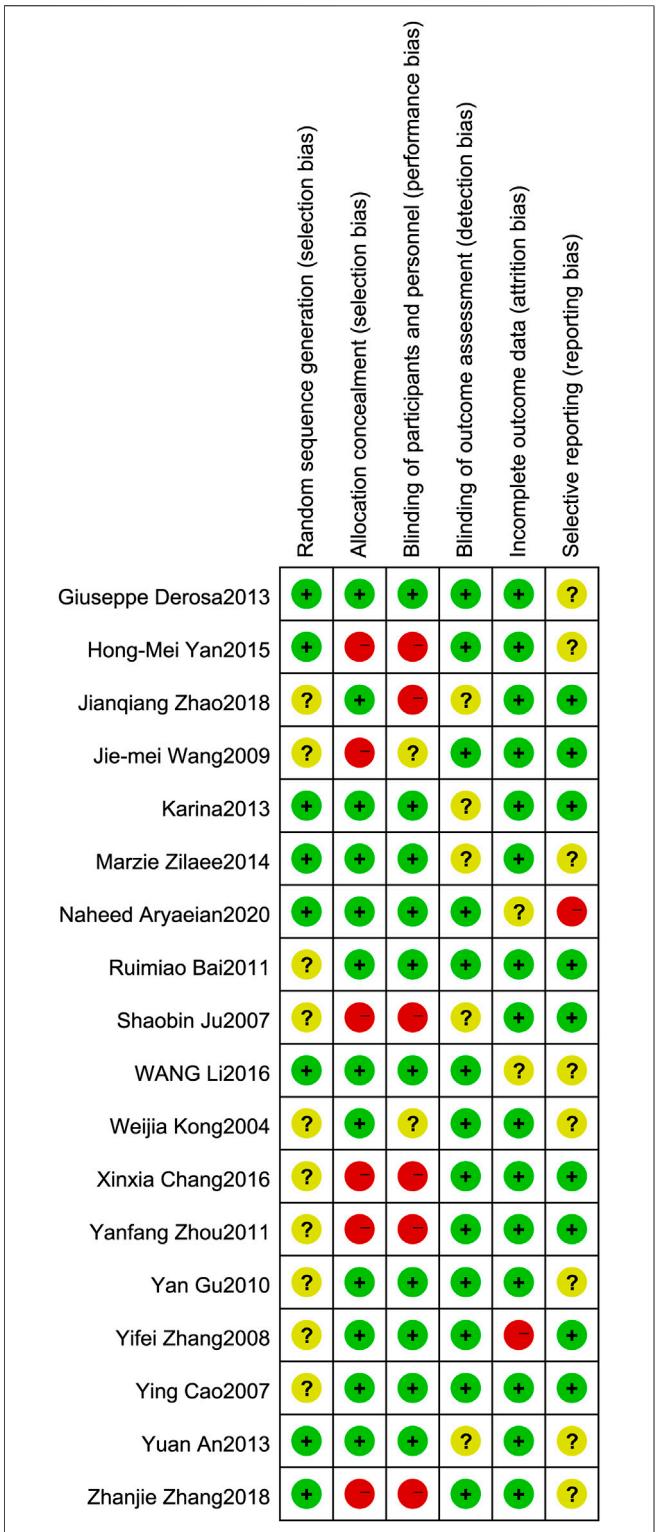

FIGURE 2 | Risk of bias assessment. The details were related to the six domains that contained (1) random sequence generation (selection bias): the literature proclaims that the random sequence method is considered low risk. (2) Allocation concealment (selection bias): explain that allocation concealment or placebo-controlled experiments are considered low risk. (3) Blinding of participants and personnel (performance bias): the literature states (*Continued*)

parallel controlled (lifestyle intervention and berberine vs. lifestyle intervention, **Figure 2**).

Triglyceride

Figure 3 illustrates the efficacy of berberine treatment on TG. It is reported with 704 volunteers in the control group and 745 in the trial group using a randomized model. After standardization, the TG concentration of the trial group decreased by 0.94 (95%CI: 0.49, 1.38) compared with the control group. The I^2 value was 93%, and the *p*-value was 0.00. The data indicate that berberine can reduce the TG level of the patients (**Figure 3**).

Total Cholesterol

Figure 4 illustrates the efficacy of berberine treatment on TC. It is reported with 692 volunteers in the control group and 733 in the trial group using a randomized model. After standardization, the TC concentration of the trial group decreased by 1.06 (95%CI: 0.64, 1.48) compared with the control group. The I^2 value was 93%, and the *p*-value was 0.00. The data demonstrate that berberine can reducethe TC level of the patients (**Figure 4**).

Low-Density Lipoprotein

Figure 5 illustrates the efficacy of berberine treatment on LDL. It is reported with 660 volunteers in the control group and 701 in the trial group using a randomized model. After standardization, the LDL concentration of the trial group decreased by 1.77 (95% CI: 1.11, 2.44) compared with the control group. The I^2 value was 96%, and the *p*-value was 0.00. The chart illustrates that berberine can reduce the LDL level of the patients (**Figure 5**).

High-Density Lipoprotein

Figure 6 illustrates the efficacy of berberine treatment on HDL. It is reported with 580 volunteers in the control group and 613 in the trial group using a randomized model. After standardization, the HDL concentration of the trial group increased by 1.59 (95% CI: −2.32, −0.85) compared with the control group. The I^2 value was 97%, and the *p*-value was 0.00. The chart illustrates that berberine can improve the HDL level of the patients (**Figure 6**).

Homeostasis Model Assessment-Insulin Resistance

Figure 7 illustrates the efficacy of berberine treatment on HOMA-IR. It is reported with 261 volunteers in the control group and 278 in the trial group using a randomized model. After standardization, the HOMA-IR concentration of the trial group decreased by 1.25 (95%CI: 0.25, 2.24) compared with the control group. The I^2 value was 96%, and the *p*-value was 0.01. The chart

FIGURE 2 | that a double-blind study design is considered low risk. (4) Blinding of outcome assessment (detection bias): there is a return visit record or related explanation in the article that is considered low risk. (5) Incomplete outcome data (attrition bias): no outliers during the trial are considered low risk. (6) Selective reporting (reporting bias): no selective report treatment data are considered low risk.

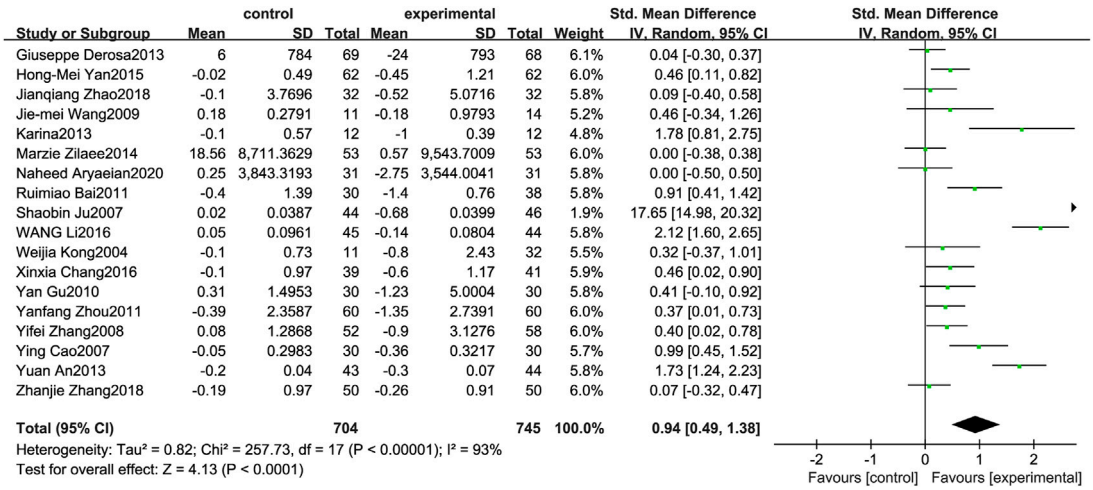

FIGURE 3 | Meta-analysis and migration analysis of the effect of berberine on triglyceride. Forest plot illustrates the differences in changes in triglyceride in adults with metabolic disease who did or did not receive berberine in 15 trials (n = 1,231). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using random-effect Hedges models.

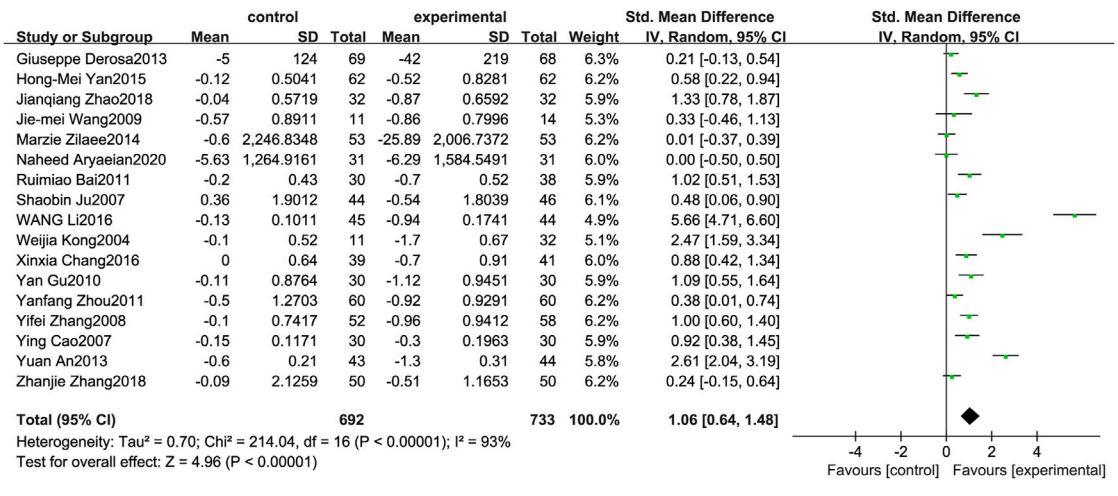

FIGURE 4 | Meta-analysis and migration analysis of the effect of berberine on total cholesterol. Forest plot illustrates the differences in changes in total cholesterol in adults with metabolic disease who did or did not receive berberine in 15 trials (n = 1,297). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using random-effect Hedges models.

illustrates that berberine can reduce the HOMA-IR level of the patients (**Figure 7**).

Fasting Plasma Glucose

Figure 8 illustrates the efficacy of berberine treatment on FPG. It is reported with 580 volunteers in the control group and 600 in the trial group using a randomized model. After standardization, the FPG concentration of the trial group decreased by 0.65 (95%CI: 0.28, 1.03) compared with the control group. The I^2 value was 89%, and the p-value was 0.00. The data shows that berberine can reduce the FPG level of the patients (**Figure 8**).

Subgroup Analysis Result

Drug users have different physiological conditions such as gender, age, individual differences, and dietary structure, and the effects of taking the same drug are different. In addition, the treatment period and treatment dose will affect the drug treatment effect. Our subgroup analysis explored the possible reasons based on three aspects: treatment period, treatment dose, and study design (Table 1).

In TG subgroup analysis, overall differences were found in the therapeutic effects of different treatment periods (p = 0.02), whereas no difference was found in the therapeutic effects of different treatment doses (p = 0.08) and study designs (p = 0.20).

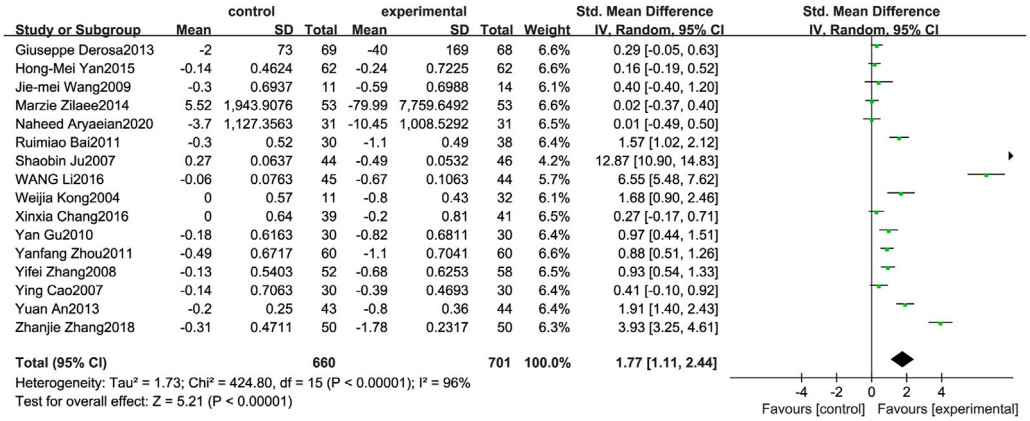

FIGURE 5 | Meta-analysis and migration analysis of the effect of berberine on low-density lipoprotein. Forest plot illustrates the differences in changes in low-density lipoprotein in adults with metabolic disease who did or did not receive berberine in 14 trials (n = 1,233). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using random-effect Hedges models.

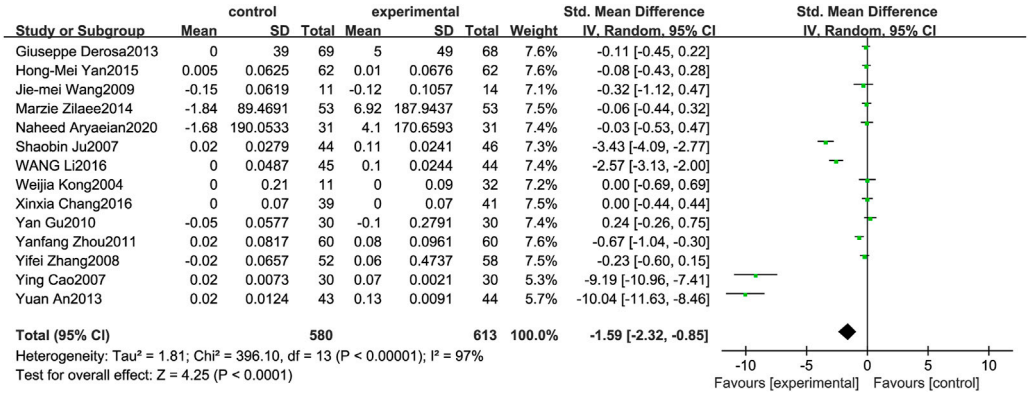

FIGURE 6 | Meta-analysis and migration analysis of the effect of berberine on high-density lipoprotein. Forest plot illustrates the differences in changes in high-density lipoprotein in adults with metabolic disease who did or did not receive berberine in 13 trials (n = 1,133). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using random-effect Hedges models.

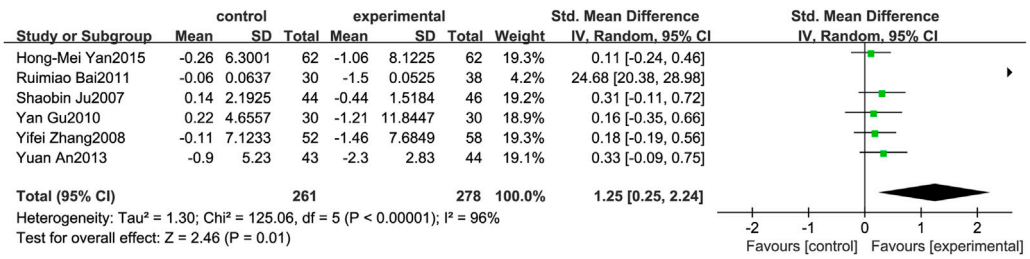

FIGURE 7 | Meta-analysis and migration analysis of the effect of berberine on homeostasis model assessment of insulin resistance. Forest plot illustrates the differences in changes in homeostasis model assessment of insulin resistance in adults with metabolic disease who did or did not receive berberine in 5 trials (n = 471). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using fixed-effects inverse-variance models.

In TC subgroup analysis, overall differences were found in the therapeutic effects of different treatment periods ($p = 0.01$), whereas no difference was found in the therapeutic effects of different treatment doses ($p = 0.60$) and study designs ($p = 0.06$). Treatment period is the main factor causing heterogeneity.

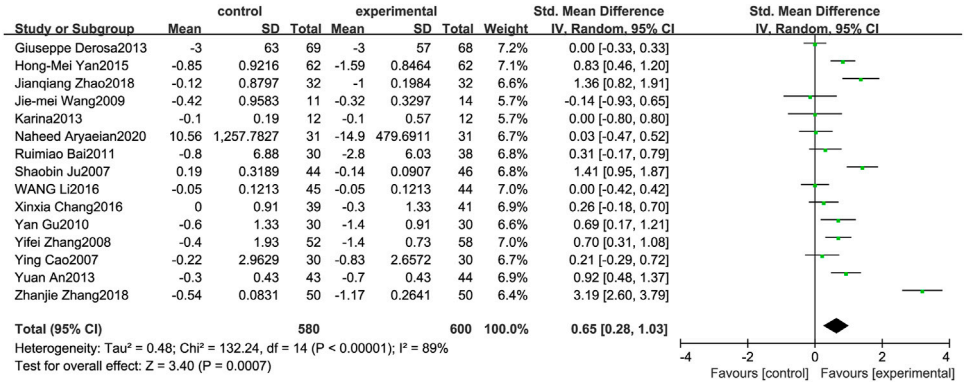

FIGURE 8 | Meta-analysis and migration analysis of the effect of berberine on fasting plasma glucose. Forest plot illustrates the differences in changes in fasting plasma glucose in adults with metabolic disease who did or did not receive berberine in 13 trials (n = 1,052). The mean difference (blue squares), 95% CI (horizontal lines through blue squares), and pooled-effect sizes (green diamonds) were presented using random-effect Hedges models.

In LDL subgroup analysis, differences were observed in the therapeutic effects of different treatment periods (p = 0.04) and treatment doses (p = 0.00), whereas no difference was observed in the therapeutic effects of different study designs (p = 0.30).

In HDL subgroup analysis, no difference was observed in the treatment effect of different treatment periods (p = 0.25), treatment doses (p = 0.08), and study designs (p = 0.42).

HOMA-IR subgroup analysis showed a notable difference in heterogeneity through different treatment periods, treatment doses, and study designs, revealing that more data are needed to enrich credibility.

In FPG subgroup analysis, no difference was observed in the treatment effects of different treatment periods (p = 0.07), treatment doses (p = 0.28), and study designs (p = 0.11).

DISCUSSION

Berberine can be used as an alternative treatment for patients who do not tolerate statins because of its lipid-lowering effects (Banach et al., 2018). The findings of the present meta-analysis demonstrated that berberine alone can reduce TG, TC, LDL, HDL, FPG, and HOMA-IR levels in patients with metabolic disorders, and this effect was observed in healthy participants. This meta-analysis on the efficacy and safety of berberine for several metabolic disorders leads to the propel guidelines of berberine in clinical practice. These effects indicate that berberine has the potential to treat metabolic disorders such as type 2 diabetes complicated with hyperlipidemia. Compared with traditional statins and metformin, berberine also has great benefits in improving mild cognitive impairment, effectively preventing secondary or the occurrence of drug-induced metabolic encephalopathy. In the abovementioned clinical treatment process, only a few patients have occasional abdominal pain, and drug safety is significantly higher than the original treatment measures. Compared with previous studies, we discuss the therapeutic effect of berberine alone in the treatment of metabolic disorders for the first time. Three subgroup analyses were conducted on the basis of the treatment period, treatment dose, and study design (placebo/parallel) to reveal the factors affecting the therapeutic effect. Treatment period and dose are the main factors causing heterogeneity, and study design has almost no effect on heterogeneity. Extending the berberine treatment period (>3 months) will significantly increase the therapeutic effect. Based on the effects of dose and time on different monitoring indicators, subgroup analysis can also be used as a clinical treatment guide. TC is a risk factor for patients with hyperlipidemia, and atherosclerosis dominated by elevated TC is a key factor affecting the treatment effect. The results show that the treatment period is a key factor affecting the treatment effect in patients with metabolic disorders and coronary heart disease dominated by elevated TG. In addition, elevated LDL can affect atherosclerosis formation and cause coronary heart disease. Therefore, as a risk factor for atherosclerosis, LDL can be used to assess the risk of coronary heart disease. Our data indicate that the treatment period and dose for patients with elevated LDL are key factors affecting the therapeutic effect. At present, no sufficient evidence is found to prove that treatment time and treatment dose affect the treatment effect in patients with insulin resistance and/or abnormal blood glucose levels.

Berberine is clinically safe and well-tolerated by the human body. Few adverse reactions are reported, and no negative effect is observed on participants' diet. Research by Yun S. Lee et al. (Lee et al., 2006) showed that berberine can inhibit fat-forming and lipogenic genes of fat, thereby reducing fat production. By increasing the expression of uncoupling protein mRNA in skeletal muscles, heat production and oxygen consumption are increased, and glucose and fat metabolism is accelerated. Berberine is also an AMPK agonist, and it increases energy production and reduces energy storage by activating AMPK. The activation of AMPK can normalize the imbalance of lipid, glucose, and energy and improve the metabolic imbalance caused by metabolic disorders (Srivastava et al., 2012). Activating AMPK can also promote GLUT4 translocation, indirectly speeding up the transport of glucose in the serum (Burcelin et al., 2003) and free fatty acids to the mitochondria by increasing ACC phosphorylation, both of which contribute to the reduction of

glucose and lipids (Zhang et al., 2019). In addition, berberine-mediated AMPK activation has an anti-inflammatory effect, which improves insulin resistance. Berberine can also stabilize LDLR mRNA and prolong half-life (Lou et al., 2011; Yang et al., 2016). The stable expression of LDLR can increase the clearance rate of plasma LDL through receptor-mediated endocytosis, thereby reducing LDL (Kong et al., 2004). Our meta-analysis results correspond to the theory that berberine activates AMPK to regulate metabolism. Berberine activates AMPK to reduce fat production and body fat ratio, improves insulin sensitivity, and promotes glucose transport. Collectively, these effects cause the fat and sugar in patients with metabolic disorders to change from accumulation to decomposition. Furthermore, Asbaghi et al. found that weight loss and anti-inflammatory effects related to berberine intake may play an indirect role in improving the clinical symptoms of metabolic disorders (Asbaghi et al., 2020).

Berberine alone in patients with metabolic disorders can play a good therapeutic effect containing blood lipid-lowering, blood sugar-lowering, and insulin resistance amelioration, and the therapeutic effect becomes conspicuous with treatment time > 3 months. No clear direct evidence is found to prove the effects of age and the severity of the disease on drug efficacy, but evidence supports the use of berberine therapy in older people with metabolic disorders.

Our study still has the following limitations: 1) random errors may be found in the inclusion of the sample; 2) differences are observed in the characteristics of RCT samples, such as gender and age differences; 3) metabolic disorders are often combined with several diseases. Subgroup analysis can be performed in the future to evaluate the therapeutic effect of berberine on a single disease. 4) Life interventions, including diet intervention and exercise intervention of each study design, are not exactly the same. This study does not exclude the possibility that life intervention can improve monitoring indicators. 5) Some articles do not indicate whether the research object is newly diagnosed or has been sick in the past. The frequency of return visits and specific procedures during the experiment are not mentioned. These factors may also lead to the appearance of bias. 6) Differences may be found in the efficacy and metabolism of drugs for different races, and more data are needed to verify the results. 7) The current study is not registered, and it may have a small deviation, but we still strictly follow the steps of a systematic review.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

NW and YY(1st author) performed the screening, extraction of trial data and the manuscript preparation. YH and JW revised and discussed the manuscript. YY(2nd author) and QC participated in its design and coordination. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.653887/full#supplementary-material.

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REVIEW
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Edible and Herbal Plants for the Prevention and Management of COVID-19

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Background: The outbreak of the pandemic coronavirus disease 2019 (COVID-19) has now become a global pandemic spreading throughout the world. Unfortunately, due to the high infectiousness of the novel β-coronavirus, it is very likely to become an ordinary epidemic. The development of dietary supplements and functional foods might provide a strategy for the prevention and management of COVID-19.

Scope and Approach: A great diversity of potential edible and medicinal plants and/or natural compounds showed potential benefits in managing SARS, which may also combat COVID-19. Moreover, many plants and compounds have currently been proposed to be protective against COVID-19. This information is based on data-driven approaches and computational chemical biology techniques. In this study, we review promising candidates of edible and medicinal plants for the prevention and management of COVID-19. We primarily focus on analyzing their underlying mechanisms. We aim to identify dietary supplements and functional foods that assist in managing this epidemic.

Key findings and Conclusion: We infer that acetoside, glyasperin, isorhamnetin, and several flavonoid compounds may prevent and/or be effective in managing COVID-19 by targeting the viral infection, reducing the host cytokine storm, regulating the immune response, and providing organ protection. These bioactive dietary components (used either alone or in combination) might assist in the development of dietary supplements or functional foods for managing COVID-19.

Keywords: COVID-19, virus infection, cytokine storm, immune response, organ protection, dietary supplements, functional foods

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INTRODUCTION

The rapid global spread of coronavirus disease 2019 (COVID-19), which is caused by the novel β-coronavirus SARS-COV-2, poses significant threats to public health. The number of confirmed cases and deaths continues to grow worldwide. COVID-19 can develop rapidly into acute respiratory distress syndrome, resulting in multiple organ dysfunction or death in some cases (Singhal, 2020). Medical resources and experience in treatment are far from sufficient for conquering the virus. Similar to outbreaks of other newly identified viruses, COVID-19 patients are predominately managed with symptomatic therapies such as antiviral drugs (including lopinavir/ritonavir),

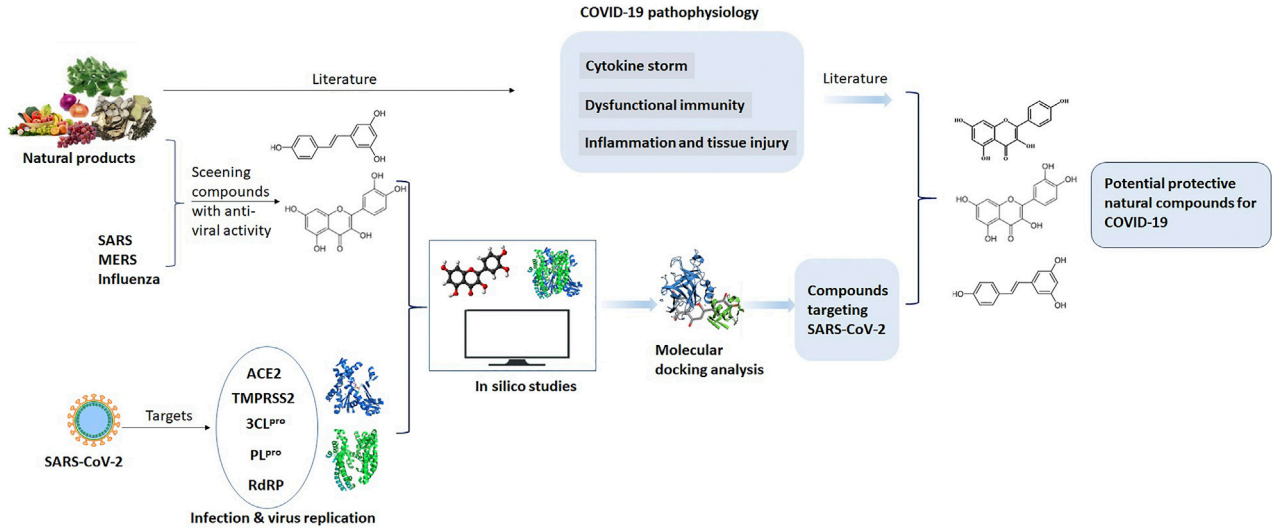

FIGURE 1 | The scheme of this review.

often resulting in an unsatisfactory outcome (Ortiz-Prado et al., 2020). More importantly, due to the high infectiousness of SARS-COV-2, COVID-19 is very likely to become an ordinary epidemic that exists chronically. Therefore, dietary supplements or functional foods to prevent and manage viral infections might be of great importance.

Since the outbreak of SARS (caused by SARS-CoV) in 2003, many plants (including herbal tea and natural compounds) have been assessed for the prevention and treatment of β-coronavirus-associated diseases. Due to the homology in epidemiology, genomic, and pathogenesis of the SARS-CoV and SARS-CoV-2, the effect of edible and medicinal plants on SARS may also assist with managing COVID-19. Increasing evidence indicates that there are many similarities in the pathophysiological processes of the viral infections (SARS-CoV and SARS-CoV-2) in addition to direct lung injury, cytokine storm, dysfunctional immunity, as well as other organ injuries. Benefitting from the current understanding of COVID-19, various data-driven approaches and computational chemical biology techniques (such as molecular docking) have been adopted to screen potential natural compounds for managing COVID-19 (Ren et al., 2020). Within a short period, several large-scale screenings have been performed. A variety of promising natural herbal medicines and dietary bioactive compounds has been identified. In this study, we summarize the edible and medicinal plants that are potential COVID-19 management candidates. **Figure 1** shows the framework of this review. We review the anti-inflammatory and immune-regulatory effects of these dietary bioactive compounds targeting SARS-CoV-2. The aim of this review is to provide the preliminarily research to uncover their molecular mechanism for managing COVID-19. This review provides important insights into the development of dietary supplements and functional foods from natural products for the prevention and management of COVID-19.

CURRENT UNDERSTANDING OF PATHOPHYSIOLOGY OF COVID-19

SARS-CoV-2 is a spherical, enveloped, positive-sense, single-stranded RNA coronavirus that shares approximately 80% similarity with the SARS-CoV genome (Machhi et al., 2020). It has four structural proteins, including spike glycoprotein, membrane glycoprotein, envelope protein, and nucleocapsid protein. The initial infection involves an interaction with a potential host cell. The spike proteins in SARS-CoV-2 are primed by the cellular transmembrane protease serine 2 (TMPRSS2) into S1 and S2 subunits. The S1 subunit specifically binds to the host cell receptors angiotensin-converting enzyme 2 (ACE2) or CD147 for entry, leading to a conformational change in the S2 subunit (Ni et al., 2020). ACE2 is also an entry receptor for SARS-CoV, whereas CD147 is a novel route for SARS-CoV-2 invasion. Functional S2 allows the infusion of viral and cellular membranes, allowing viral RNA to be released into the cytoplasm. Then, the viral genomic RNA begins to express copies of the virus in the host cell. The coronavirus replication involves papain-like protease (PL^{pro}) and 3C-like protease ($3CL^{pro}$), that hydrolyses the viral polyproteins pp1a and pp1ab to generate functional proteins (He et al., 2020). Afterward, the host cell transports copies of the virus to the cell surface, allowing the virus to infect other cells.

After SARS-CoV-2 infection, a well-coordinated and rapid innate immune response is activated (Vabret et al., 2020). The pathogen-associated molecular pattern (PAMP) of the virus is recognized by the pattern recognition receptor (PRR) on the membrane of the host cells, activating innate immune cells (such as macrophages, dendritic cells, monocytes, and neutrophils) to initiate the synthesis and secretion of inflammatory cytokines (Vabret et al., 2020). During viral infections, IL-6 and IL-1β can facilitate inflammation in the alveoli and bronchi. These are considered the major pro-inflammatory cytokines coordinating

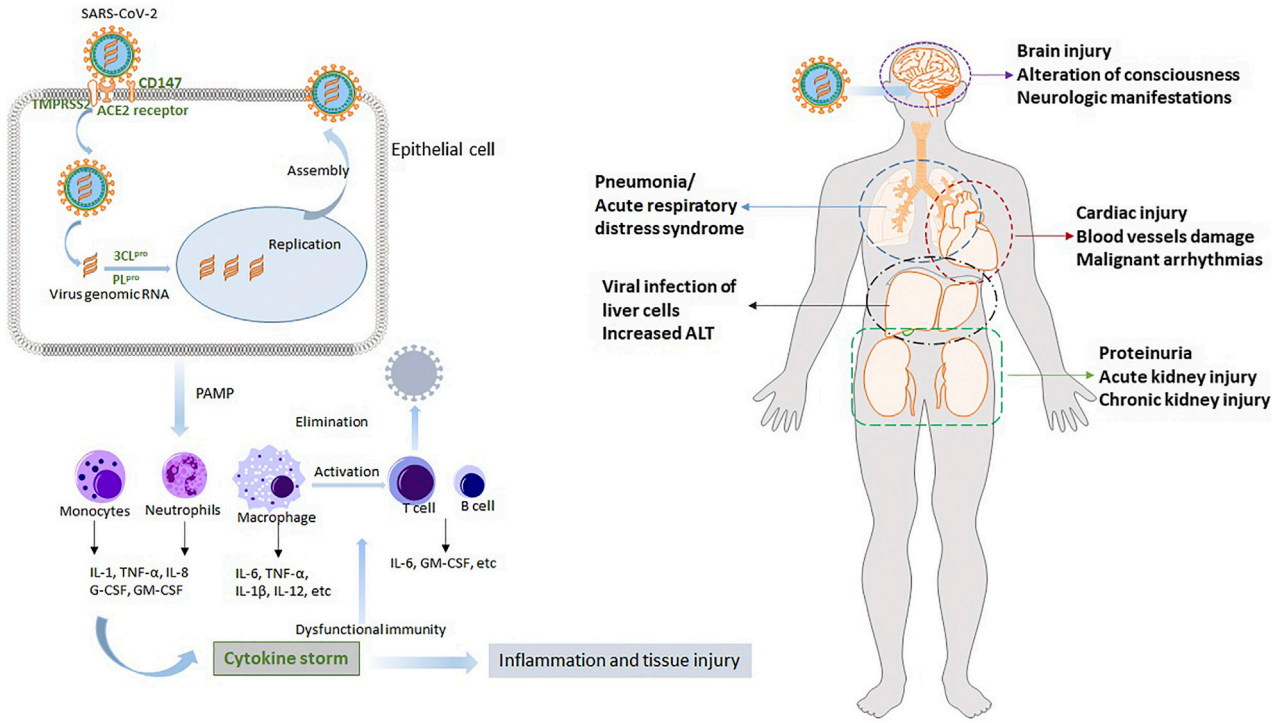

FIGURE 2 | The potential pathogenesis of COVID-19 and its pathophysiology. After SARS-CoV-2 infection, rapid innate immune response is activated. Then uncontrolled inflammatory responses cause cytokines storm, leading to acute lung injury such as severe respiratory failure. Other multiple organs, such as heart, liver and kidney, are also injured due to the virus and cytokines storm.

the local or systemic inflammation in infected individuals. Furthermore, cytokine storms might contribute to the impaired immune system. Ultimately, uncontrolled inflammatory responses cause the cytokine storm, leading to acute lung injury such as severe respiratory failure.

In addition to lung injury, COVID-19 is regarded as a systemic disease involving multiple organs, such as the heart, liver, and kidney (Song et al., 2020; Wu et al., 2020; Zaim et al., 2020). After the initial infection in the respiratory system, SARS-CoV-2 disperses to other vital organs and tissues. This triggers a complicated spectrum of pathophysiological changes and symptoms. Multiple organ dysfunction is partially due to the wide expression of the cellular receptor ACE2 in these organs, and more importantly, results from the cytokine storm. Understanding the potential pathogenesis and pathophysiology of COVID-19 is indispensable for developing effective therapies and protective functional foods. We concisely review the SARS-CoV-2 infection process and the mechanisms underlying cytokine storm and organ injury. The results are provided in **Figure 2**.

POTENTIAL PLANTS AND COMPOUNDS

Many herbal plants, plant preparations, and phytoconstituents have a long history in antiviral therapy and play a vital role in preventing SARS transmission (Ho et al., 2020; Li et al., 2020b). Currently, clinical treatments for COVID-19 patients principally are symptomatic treatments such as anti-viral drugs and ventilator. However, since the physiopathologic processes involved in COVID-19 are complicated, therapies targeting on virus, systematic inflammation, immune response and organ protection together would benefit more for patients. Herbal plants and plant compounds, which are low toxic, cheap and easily available, are considered to treat both principal and secondary aspect of disease in Traditional Chinese Medicine. Many herbal plants are multi-target and multi-component pattern, which are promising for the prevention and management of COVID-19 in the future. The homology in the epidemiology, genomic, and pathogenesis of SARS-CoV and SARS-CoV-2 suggests that studies into effective edible and medicinal plants for SARS might also assist in managing COVID-19. Due to the low toxicity and availability of many natural products, screening plants or active compounds targeting SARS-CoV-2 or the host targets could be a potential strategy for managing COVID-19. In this section, we summarize the frequently used plants and derived natural compounds containing strong binding affinities with COVID-19 related targets.

Edible and Herbal Plants

We analyzed the commonly used herbal formulae proposed for COVID-19. The herbal formulae contain 54 herbs, of which *Radix astragali* praeparata cum melle and *Glycyrrhizae Radix Et*

Rhizoma are most frequently used. Other herbal plants of *Saposhnikovia divaricata (Turcz. ex Ledeb.) Schischk*, *Atractylodes macrocephala Koidz.*, *Lonicera japonica Thunb.*, *Forsythia suspensa* (Thunb.) Vahl, *Atractylodes lancea* (Thunb.) DC., *Platycodon grandiflorus* (Jacq.) A. DC., *Pogostemon cablin* (Blanco) Benth., and *Cyrtomium fortune* J. Sm. are also often used (Luo et al., 2020). In a previous screening study based on data mining, molecular docking, and network pharmacology, 574 herbal prescriptions used for pestilence were obtained from 96,606 classical prescriptions (Ren et al., 2020). Among them, those high-frequency herbal plants were screened, including *Glycyrrhizae Radix Et Rhizoma, Scutellaria baicalensis Georgi, Rhei Radix Et Rhizome*, *Paeonia lactiflora Pall.*, *Citri Reticulatae Pericarpium*, *Bupleurum falcatum* L., *Platycodon grandiflorus* (Jacq.) A. DC, *Atractylodes lancea* (Thunb.) DC, *Angelica sinensis* (Oliv.) Diels, and *Rehmannia glutinosa* (Gaertn.) DC. Notably, *Glycyrrhizae Radix Et Rhizoma*, *Platycodon grandiflorus* (Jacq.) A. DC and *Atractylodes lancea* (Thunb.) DC are regarded as high-frequency herbal plants in these two studies. Particularly, most of those commonly used herbal plants belong to dietetic herbs, such as *Glycyrrhizae Radix Et Rhizoma* (Liquorice), *Lonicera japonica* Thunb., *Citri Reticulatae Pericarpium, Platycodon grandiflorus* (Jacq.) A. DC. and *Angelica sinensis* (Oliv.) Diels, which can be used as foods or sold as herbal tea.

A variety of vegetables and herbal plants has been tested against SARS-CoV. The extract of the tender leaf of the vegetable named Chinese mahogany (*Toona sinensis* (Juss.) M. Roem.), red spider lily (*Lycoris radiata* (L'Hér.) Herb.), and an extract of *Rhizoma Cibotii*, all inhibited SARS-CoV replication in vero cells with the SARS-CoV strain infection model (Panyod et al., 2020). Lianhua Qingwen capsule has been proven to be effective in Influenza A, Influenza B, Avian influenza. It has been selected as a general prescription for the treatment of COVID-19 in different stages that was later promoted to be used nationwide.

Compounds

Due to the limited accessibility of SARS-CoV-2, a diverse array of studies adopted virtual simulation technologies (such as network pharmacology and molecular docking) to predict the potential bioactive component responses from natural products and the possible action mechanisms. These studies demonstrate some common ingredients and action mechanisms used by the edible and medicinal plants in treating SARS-CoV-2 infection.

A total of 166 herbal prescriptions containing 179 medicinal plants that have been proposed for use in treating COVID-19 were analyzed by network pharmacology. *β-Sitosterol*, stigmasterol, and quercetin were screened as the most frequently used compounds that are likely to be related to the antiviral signaling pathway. In another study using a screening system based on data mining, molecular docking, and network pharmacology, 431 chemicals from 35 high-frequency medicinal plants used for pestilence were molecularly docked with the SARS-COV-2 targets, ACE2 and $3CL^{pro}$, using LigandFit (Ren et al., 2020). A total of 48 compounds were docked with ACE2 and 27 compounds were docked with $3CL^{pro}$. The compounds were present in many edible and medicinal plants such as *Glycyrrhizae Radix Et Rhizoma*, *Scutellaria baicalensis* Georgi, *Rhei Radix Et Rhizome*, and *Bupleurum falcatum* L. Notably, acetoside (present in osmanthus flowers) showed the strongest binding activity to $3CL^{pro}$ (Consensus scoring = 7). In another dietetic herb (*Glycyrrhizae Radix Et Rhizoma*), glyasperin had the strongest binding activity to ACE site 1 (Consensus scoring = 6). Isorhamnetin showed the strongest binding ability to ACE site 2 (Consensus scoring = 6). Furthermore, as shown by the constructed compound-target network, quercetin, kaempferol, and baicalein (which are widely distributed in many vegetables, fruits, and medicinal plants) present high interconnection degrees, implying that these compounds regulate multiple disease targets (Ren et al., 2020). Emodin (an anthraquinone compound contained in various plants and several species of fungi) has been shown to suppress the binding of SARS-CoV S protein with ACE2 in a dose-dependent manner (Ho et al., 2007).

Many compounds belonging to flavonoids could target SARS-CoV-2 infection. Flavonoids are rich in many foods, including fruits, vegetables, and other plants. Production of inflammatory cytokines caused by the activation of the NLRP3 inflammasome in activated immune cells leads to respiratory distress syndrome which is associated with SARS coronaviruses (Chen et al., 2019). Various flavonoids have been shown to interfere with NLRP3 inflammasome signaling (such as wogonoside, baicalin, kaempferol, luteolin, myricetin, quercetin, and apigenin) and alleviate the inflammatory response to SARS-CoV infection (McKee et al., 2020). These compounds have also been demonstrated to be effective against various other viruses through multiple mechanisms. These compounds could be used as nutraceutical supplements at daily doses ranging from 100 to 500 mg. Resveratrol, a well-known natural polyphenol that is particularly abundant in grapes and sprouted peanuts, suppresses MERS-CoV infection and facilitates cellular survival after virus infection by inhibiting nucleocapsid protein (Lin et al., 2017). This suggests that these flavonoids might be promising health supplements or medical agents against SARS-CoV-2 infection.

The main constituent of *Nigella sativa*, thymoquinone, showed remarkable anti-oxidant, anti-inflammatory, anti-tumor, and antimicrobial activities (Seo et al., 2014; Ulasli et al., 2014; Ahmad., et al., 2020c). Notably, extract of *Nigella sativa* and thymoquinone have been demonstrated to be effective against avian influenza virus (H9N2 AIV) and in a murine cytomegalovirus infection model (Ahmad., et al., 2020c). Cells pre-treated with Nigella sativa extract reduced the replication of the virus when infected with coronavirus (Ahmad., et al., 2020c). Moreover, gene expression analysis of the transient receptor potential proteins (TRPs) indicated that Nigella sativa treatments decrease virus loads, thus reducing coronavirus survival inside cells. Thymoquinone has been demonstrated a remarkable anti-sepsis and immunomodulatory activities (Ahmad et al., 2013; Alkharfy., et al., 2015b; Raish et al., 2017; Alkharfy et al., 2018). It regulates the production of nitric oxide (NO) and reactive oxygen species (ROS), and prevented from multiple organ dysfunction syndrome (MODS) (Alkharfy., et al., 2015b;

TABLE 1 | The anti-inflammation and immune-regulation pathways mediated by compounds with strong ability to target on SARS-CoV-2 predicted by *in silico* studies (OB and Caco are used to evaluate the druggability of compounds; ↓ down-regulated or reduce; ↑ up-regulated or increase).

Compound	Representive plants or foods	Acts on SARA-CoV-2	Anti-inflammation		Immune-regulation		Bioavailability/ OB/Caco	Refs
			Effects	Mechanisms	Effects	Mechanisms		
Isorhamnetin	*Glycyrrhizae Radix Et Rhizoma, Bupleuri Radix,* yellow onion, berry, grape	Binding with ACE2 and the main protease $3CL^{pro}$	Ameliorated LPS-induced inflammatory response	↓NF-κB signaling	Maintained immune regulation	JAK/STAT pathway	49.60/0.31	Sun et al., (2020b)
Quercetin	*Glycyrrhizae Radix Et Rhizoma etc.* grapes, berries, cherries, apples, citrus fruits, onions	Binding with ACE2 and the main protease $3CL^{pro}$	Reduced inflammation *in vivo* and *in vitro* induced by LPS and high-fat dies	↓TLR4/MyD88/ PI3K; ↓NF-κB signaling	↑ Phenotypic expression of IFN-γ cells and decreased IL-4 positive cells	↓JAK/STAT pathway; ↓ SphK1/S1P signaling	46.43/0.05	Chen et al., (2016); Li et al., (2016)
Kaempferol	*Armeniacae Semen Amarum,* many fruits and vegetables such as grapes, apples, onions, spinach etc.	Binding ability to $3CL^{pro}$	Reduced the inflammation of LPS-treated macrophages and cardiac fibroblasts	↓ Src, Syk, IRAK1 and IRAK4 as well as activation of NF-κB and AP-1; ↓phosphorylation of PI3K and AKT	Reducing inflammatory cytokines in LPS-treated macrophages	Inactivation of NF-κB, AP-1, and JAK-STAT	41.88/0.26	Tran et al., (2009); Lee et al., (2018); Bian et al., (2019)
Baicalein	*Scutellaria baicalensis,* fresh onion	Inhibit $3CL^{pro}$	↓ TNF-α or IL-6 in mice with LPS-induced lethal endotoxemia	↓NF-κB or ERK1/2	↑CD4+Foxp3+ T cells and enhances intestinal barrier function	↓STAT3/4 in the JAK-STAT signaling pathway in T cells; ↓S1P-STAT3 signaling	33.25/0.63	Mabalirajan et al., (2013); Bae et al., (2016); Cheng et al., (2018); Wang et al., (2018); Xu et al., (2019); Xu et al., (2018)
6-gingerol	*Ginger (Zingiber officinale Roscoe)*	Binding with PL^{pro} in high affinity	↓Pro-inflammatory cytokines such as TNF-α, IL-1, and IL-8	↓I-κBα phosphorylation, NF-κB activation	Regulating the cell balance of Th17/Treg	↓FOXP3	35.64/0.54	(Tjendraputra et al., (2001); Verma et al., (2004)
Geniposide	*Gardenia jasminoides Ellis*	High docking score against TMPRSS2	↓LPS-Induced Mastitis in Mice	Regulating expression of TLR4, thus affecting the downstream NF-κB and MAPK signaling pathways	Geniposide could induce duct cell differentiation	JAK2/STAT3 pathway; ↓activation of SphK1 and S1P signal transduction	14.64/-1.7	Sun et al., (2020b); Song et al., (2014); Sun et al., (2020b); Yao et al., (2015)
β-sitosterol	*Isatis indigotica* root, avocados, pistachio nuts, pistachio nuts, almonds	Binding ability to $3CL^{pro}$	Decreasing inflammation on human aortic endothelial cells	Activation of multiple transcription factors	Reducing damage on macrophages	Inactivation of STAT1 and NF-κB is mediated by the activation of S1P	33.94/-0.44	Agrawal and Awad, (2011)
Stigmasterol	*Ophiopogon japonicas,* various vegetables, legumes, nuts, seeds	Involved in anti-viral pathway	↓Pro-inflammatory and matrix degradation mediators in osteoarthritis-induced cartilage degradation	Inhibition of the NF-κB pathway	Immune response in gastric cancer cells	Inactivate the JAK/STAT signaling pathway	43.83/1.44	Gabay et al., (2010); Li et al., (2018)
Acetoside	*Rehmanniae Radix,* verbena, lemon verbena, and olives	Strong binding activity to $3CL^{pro}$	Relieved LPS-induced acute lung injury	Inhibiting proinflammatory cytokine production and NF-κB activation	↓Inflammatory immune response in osteoarthritis rats	Via JAK/STAT signaling pathway	–/–	Jing et al., (2015); Qiao et al., (2019)

(Continued on following page)

TABLE 1 | (*Continued*) The anti-inflammation and immune-regulation pathways mediated by compounds with strong ability to target on SARS-CoV-2 predicted by *in silico* studies (OB and Caco are used to evaluate the druggability of compounds; ↓ down-regulated or reduce; ↑ up-regulated or increase).

Compound	Representive plants or foods	Acts on SARA-CoV-2	Anti-inflammation		Immune-regulation		Bioavailability/ OB/Caco	Refs
			Effects	Mechanisms	Effects	Mechanisms		
Resveratrol	Grapes and sprouted peanuts	ACE2	Reduced inflammation	↑sirtuin-1, ↓ NF-κB and ↓ activation of Nod-like receptor family pyrin domain containing-3 inflammasome	Enhanced antimicrobial defense	Via S1P signaling of cathelicidin antimicrobial peptide production through an NF-κB→C/EBPα-dependent mechanism	19.07/0.8	Park et al., (2013); Filardo et al., (2020)

Raish et al., 2017; Alkharfy et al., 2018). Thymoquinone has been shown to protect against lung fibrosis and collagen deposition via regulating NF-κB and the antioxidant enzyme nuclear factor 2 heme oxygenase-1 (Nrf2/HO-1) signaling pathway (Ahmad., et al., 2020a).

POSSIBLE MECHANISMS UNDERLYING THE ACTION OF NATURAL PRODUCTS

The possible mechanisms underlying the action of natural products in treating COVID-19 mainly act on SARS-CoV-2, anti-inflammation, immunoregulation, and organ protection. We systematically review the potential action mechanisms of these natural products based on the available evidence. We particularly focus on the natural bioactive compounds that act on SARS-CoV-2 including acetoside, glyasperin, isorhamnetin, quercetin, kaempfero, baicalein, luteolin, and resveratrol (**Table 1**).

Direct Target on SARS-CoV-2

Edible and medicinal plants have unique antiviral advantages. They can affect the virus directly, impede its proliferation, and promote the secretion of IFN, this antiviral activity owes to their multi-component and multi-target pattern (Lin et al., 2014; Ma et al., 2015). Since ACE2 is the critical surface receptor initiating SARS-CoV-2 invasion into the host, excess soluble forms of ACE2 or ACE2 inhibitors could be a possible strategy to treat COVID 19 (Liu et al., 2020). A large number of compounds from edible and medicinal plants have been tested (by molecular docking) for binding with ACE2 (**Table 1**). Isorhamnetin, a compound which is highly concentrated in several vegetables (such as parsley and green bell peppers) shows a strong binding ability to ACE site 2 (Consensus scoring = 6) (Pandey et al., 2020). The two viral proteases, $3CL^{pro}$ and PL^{pro} are responsible for virus replication and packaging in host cells, which are also regarded as the key targets for SARS-CoV-2 infection (Pandey et al., 2020). Research involved the high-throughput molecular docking of 12,541 compounds from the TCMSP database with ACE2 and the main protease $3CL^{pro}$ (Gao et al., 2020). It was found that isorhamnetin and quercetin simultaneously show remarkable binding ability with these two proteins. Baicalin and its aglycon baicalein are flavonoids in many edible plants, which could inhibit $3CL^{pro}$ and demonstrate remarkable antiviral activity in cell-based systems. Several compounds from ginger, such as 8-gingerol, 10-gingerol, and 6-gingerol are found to bind with pL^{pro} in high affinity to inhibit SARS-CoV-2 replication (Dibakar et al., 2020).

Another protease regarded as a potential target due to an ability to block SARS-CoV-2 entry into host cells is TMPRSS2, which plays a critical role in the priming of viral spike proteins. Rahman et al. screened 30,927 natural compounds from a database of NPASS to mine potent inhibitors of TMPRSS2 (Rahman et al., 2020). After the initial physicochemical analysis, 2,140 compounds were recognized as potent candidates for further docking. A total of 85 compounds have binding energies comparable to or lower than the standard inhibitor camostat mesylate. Among them, geniposide (which is an important component of *Gardenia jasminoides* J. Ellis) showed the highest docking score against TMPRSS2 (Rahman et al., 2020).

Anti-Inflammation

Clinical studies have indicated that the levels of IFN-γ, IL-6, TNF-α, IL-2, MCP-1, and other pro-inflammatory cytokines are significantly increased in severe or critical COVID-19 patients. Conversely, individuals infected with SARS-CoV-2 have increased levels of anti-inflammatory cytokines such as IL-10 and IL-4 (Coperchini et al., 2020; Hu et al., 2020). Given the vital role of cytokine storm in the pathogenesis of COVID-19, agents able to reduce the release of many cytokines involved in the initiation and progression of inflammation (such as IFN-γ, IL-6, and TNF-α) may assist in preventing the progression of the disease. Overwhelming evidence shows that many herbal plants, vegetables, fruits, and many natural compounds show remarkable anti-inflammatory abilities (Coperchini et al., 2020; Hu et al., 2020; Jafarzadeh et al., 2020).

The transcription factor NF-κB is a critical regulator in initiating and propagating optimal immune responses (Hayden et al., 2006). The constitutive activation of the NF-κB pathway is implicated in lung inflammatory immunopathology caused by

respiratory viruses, including SARS-CoV (DeDiego et al., 2014). In a study of SARS-CoV-infected macaques (comparing adults with younger macaques) demonstrated increased NF-κB nuclear translocation and stronger host responses in aged macaques (DeDiego et al., 2014). Therefore, NF-κB/TNF-α is extensively targeted for the treatment of inflammation and increasing evidence indicates that many edible and medicinal plants used in COVID-19 alleviated inflammation via the NF-κB/TNF-α signaling pathway.

The major compounds targeting SARS-CoV-2 (such as isorhamnetin, acetoside, quercetin, kaempferol, baicalein, geniposid, gingerol and resveratrol) have demonstrated a remarkable ability in alleviating inflammation. Isorhamnetin is one of the active constituents in the medicinal plant *Hippophae rhamnoides* L., as well as in parsley and green bell peppers and has been demonstrated to possess anti-oxidative stress and anti-inflammatory activities in many chronic inflammatory conditions. It ameliorates the LPS-induced inflammatory response via the inhibition of NF-κB signaling (Shi et al., 2018). Acetoside, which is abundant in many foods such as verbena, lemon verbena, and olives, can relieve LPS-induced acute lung injury by inhibiting proinflammatory cytokine production and NF-κB activation in both *in vitro* and *in vivo* studies (Jing et al., 2015). It also showed a positive effect in suppressing inflammation in osteoarthritic rats via the JAK/STAT signaling pathway (Qiao et al., 2019). Quercetin, is a polyphenol in many foods (such as grapes and onions) and has been shown to produce anti-inflammatory activity *in vitro* and in animal models by inhibiting NF-κB activation (Chen et al., 2016). Kaempferol is the major flavonoid aglycone that is widely distributed in various fruits and vegetables, including grapes, apples, raspberries, tomatoes, peaches, potatoes, broccoli, onions, brussel sprouts, lettuce, cucumbers, squash, green beans, and spinach etc. It produces anti-inflammatory activity *in vitro* and *in vivo* by several mechanisms. Kaempferol could reduce the inflammation of LPS-treated macrophages via direct suppression of Src, Syk, IRAK1, and IRAK4 as well as the activation of NF-κB and AP-1. It inhibits the LPS- and ATP-induced phosphorylation in PI3K and AKT in cardiac fibroblasts to reduce inflammatory injury (Tang et al., 2015). Kaempferol and some of its glycosides significantly decrease the release of NO and TNF-α in LPS-treated RAW 264.7 cells (Tran et al., 2009). Baicalein, a bioflavone component derived from the root of *Scutellaria baicalensis Georgi*, and is also rich in some varieties of onions, possesses various pharmacological properties used in treating many diseases (Cheng et al., 2018). Baicalein decreased the generation of TNF-α or IL-6 and inhibited the activation of NF-κB or ERK1/2 in mice with LPS-induced lethal endotoxemia. In mice with airway inflammation, baicalein can attenuate the symptoms through the inhibition of the NF-κB signaling pathway (Mabalirajan et al., 2013; Wang et al., 2018; Xu et al., 2019).

β-sitosterol, is one of the plant sterols and is also found in foods such as avocados, pistachio nuts and almonds, were effective in alleviating inflammatory reactions induced by immune responses (Paniagua-Perez et al., 2017). It has been demonstrated that the anti-inflammatory effect of *β*-sitosterol on human aortic endothelial cells may be mediated by the activation of multiple transcription factors. Stigmasterol, which is present in various vegetables, legumes, nuts, and seeds, inhibits several pro-inflammatory and matrix degradation mediators typically involved in osteoarthritis-induced cartilage degradation and partly through the inhibition of the NF-κB pathway (Gabay et al., 2010). Geniposide showed the highest docking score against TMPRSS2, it is widely used in Asia for the treatment of inflammatory diseases. Geniposide exerts anti-inflammatory effects by regulating the expression of TLR4, thus affecting the downstream NF-κB and MAPK signaling pathways (Song et al., 2014). Gingerol compounds from ginger bind to PL^{pro} with high affinity to inhibit SARS-CoV-2 replication; it also showed remarkable anti-inflammatory ability by reducing the synthesis of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-8 via the suppression of I-κBα phosphorylation and NF-κB activation (Tjendraputra et al., 2001; Verma et al., 2004; Mashhadi et al., 2013). Therefore, these compounds target SARS-CoV-2 directly or involved in anti-viral-related pathways with prominent anti-inflammatory abilities worthy of further research.

Additionally, an acid component of Ephedra polysaccharide (ESP-B4) alleviated pulmonary inflammation by reducing the generation of IL-6, TNF-α, IL-8, and MMP-9 (Liang et al., 2018). Bupleurum polysaccharides significantly protect the lungs from injury through the inhibition of P-selectin-mediated recruitment of neutrophils in an acute pneumonia model (Tong et al., 2014; Tong et al., 2018). Polysaccharides have been found to treat colitis by inhibiting NF-κB signaling and NLRP3 inflammasome activation (Cui et al., 2019).

Immunoregulation

There is a vital role of the immune response in viral infections; an inappropriate and weak innate immune system response to viruses increases the release of inflammatory cytokines is considered the main factor encouraging COVID-19 (Catanzaro et al., 2020; Garcia, 2020; Tufan et al., 2020). After viral infection, the excessive inflammatory innate defense and impaired adaptive immune response may lead to tissue injury both at the site of viral entry and at the systemic level (Azkur et al., 2020; Li et al., 2020a). In populations with SARS-CoV-2 infection, the cytokine storm reflects widespread uncontrolled dysregulation of the host immune response. The altered immune signaling pathways or relevant molecular cascades triggered by SARS-CoV-2 infection may be developed as therapeutic targets or vaccines for COVID-19 patients (Azkur et al., 2020; Feng et al., 2020; Jamilloux et al., 2020).

Immune Cells

Changes in the innate and adaptive immune system in COVID-19 patients have been highlighted in several studies. A significant reduction in the absolute number of circulating $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and B cells, as well as a decrease in monocytes, basophils, and eosinophils has been observed in patients with severe COVID-19 (Mazzoni et al., 2020; Sun et al., 2020a). In a retrospective clinical cohort study of 452 patients with COVID-19, a remarkably lower number of T helper cells and cytotoxic T cells exist in severe cases (Qin et al., 2020). Lymphocyte counts seem to be directly correlated with disease

severity and mortality. The reason for lymphopenia might partially be attributed to the injured lymphatic organs expressing ACE2 receptors (Hajivalili et al., 2020; Lega et al., 2020).

The influence of some medicinal plants and natural compounds on immune cells has been investigated extensively previously. Herbal formulations can enhance enteric mucosal immune responses in mice with *Bacillus dysenteriae* and *Salmonella typhimurium* induced diarrhea by increasing the $CD4^+$ and $CD8^+$ T cell populations (He et al., 2007). In addition to herbal formulae, compounds such as baicalein and gingerol also regulate the immune cell population. In a mouse model of food allergies, baicalein induces CD4+Foxp3+ T cells and enhances intestinal barrier function (Grosso et al., 1989). One of the major components of ginger, 6-gingerol, showed efficacy in treating mice with dextran sulfate sodium (DSS)-induced colitis by regulating the cell balance of Th17/Treg cells (Sheng et al., 2020). Additionally, ginsenoside Rg3, a red-ginseng-derived compound, ameliorates acute exacerbation of chronic obstructive pulmonary disease (COPD) by suppressing neutrophil migration (Guan et al., 2020).

The IL-6/JAK/STAT Signaling Pathway

Signals transmitted by a large number of cytokines, lymphokines, and extracellular factors are transduced by JAK/STAT signaling to induce biological effects in cells such as hematopoietic and immune cells (Ghoreschi et al., 2009; Seif et al., 2017). IL-6 is one of the major activators of JAK/STAT signaling. The increased IL-6 in COVID-19 patients can bind to the glycoprotein (gp130) receptor and IL-6 receptor, thereby facilitating the downstream activation of JAK/STAT signaling (Catanzaro et al., 2020; Zhang et al., 2020). In turn, the activated JAK/STAT pathway further promotes the generation of IL-6 (Zhang et al., 2020). Drugs targeting IL-6/JAK/STAT signaling may assist in the treatment of COVID-19. In addition to monoclonal antibodies targeting IL-6, JAK inhibitors have been tested in several clinical trials on patients with COVID-19 (clinicaltrials.gov) (Atal and Fatima, 2020).

Recently, network pharmacology and computer-aided drug design in virtual screening studies, several dietary bioactive compounds have been identified as beneficial for COVID-19 treatment due to their regulation of the JAK-STAT signaling pathway, such as kaempferol, quercetin, and luteolin. Kaempferol significantly decreased the release of TNF-α, IL-6, IL-1β, VCAM-1 and ICAM-1 that had been induced by LPS. This result demonstrates Kaempferol's negative mediation in TLR4, NF-κB, and STAT signaling in inflamed rat intestinal microvascular endothelial cells (Bian et al., 2019). In another study, kaempferol 7-O-β-D-glucoside decreased pro-inflammatory mediators through the inactivation of NF-κB, AP-1, and JAK-STAT in LPS-treated RAW 264.7 macrophages (Lee et al., 2018). In cholangiocarcinoma cells, the JAK/STAT pathway activated by the proinflammatory cytokines IL-6 and IFN-γ in CCA cells was inhibited by quercetin treatment. This result was demonstrated by a reduction in the up-regulated phosphorylated-STAT1 and STAT3 proteins in a dose-dependent manner (Senggunprai et al., 2014).

A variety of natural compounds from vegetables and fruits target SARS-CoV-2 using their anti-inflammatory abilities. They have also been shown to regulate the JAK-STAT signaling pathway in many laboratory studies. In a - DSS-induced colitis model, baicalein downregulated the mRNA expression of STAT3/4 in the JAK-STAT signaling pathway in T cells, facilitating its mediation of T cell proliferation (Xu et al., 2018). β-Sitosterol exerts anti-inflammatory effects on macrophages by suppressing STAT1 and NF-κB (Agrawal and Awad, 2011). Acetoside also showed a positive effect by suppressing inflammation in osteoarthritic rats via the JAK/STAT signaling pathway (Qiao et al., 2019). Stigmasterol has also been demonstrated to inactivate the JAK/STAT signaling pathway in gastric cancer cells (Li et al., 2018). Isorhamnetin principally maintained glucose homeostasis in myotubes by activating the JAK/STAT pathway (Jiang et al., 2019). Geniposide could induce duct cell differentiation via activation of the JAK2/STAT3 pathway in exocrine cells isolated from mouse pancreas (Yao et al., 2015). Resveratrol suppressed LPS-induced inflammation through the suppression of NF-κB and JAK/STAT signaling pathways (Filardo et al., 2020).

The Sphingosine-1-Phosphate Receptor One Pathway

Sphingosine-1-phosphate (S1P) is a crucial mediator of the immune response and plays a vital role in lymphocyte trafficking, vascular integrity, as well as the release of cytokines and chemokines (Proia and Hla, 2015; Catanzaro et al., 2020). S1P binds with G-protein coupled receptors one to five to mediate innate and adaptive immunity, including regulating the trafficking and migration of various types of immune cells. Notably, S1P receptor 1 (S1PR1) signaling can significantly attenuate the cytokines induced by influenza virus infection by targeting immune cells. This result suggests that S1P or S1PR1 signaling may be a potential target for COVID-19 treatment.

The anti-inflammatory effect of β-sitosterol on macrophages by the inactivation of STAT1 and NF-κB is mediated by the activation of S1P (Agrawal and Awad, 2011). Quercetin alleviated pulmonary fibrosis by inhibiting sphingosine kinase 1 (SphK1)/S1P signaling, as demonstrated by *in vivo* and *in vitro* studies (Zhang et al., 2018). In DSS-induced colitis mice, baicalein decreased the levels of inflammatory mediators and significantly downregulated the expression of SphK1, S1PR1, and p-STAT3 in the colon. This result implies that S1P-STAT3 signaling is involved in the mechanism underlying baicalein's therapeutic effect on colitis (Yao et al., 2020). Geniposide also suppressed the activation of SphK1 and S1P signal transduction. It can significantly inhibit the level of S1P as well as the expression of S1PR1 and SphK1 in fibroblast-like synoviocytes (FLSs) (Sun et al., 2020b). Resveratrol enhanced antimicrobial defense via S1P signaling of cathelicidin antimicrobial peptide production by a NF-κB-C/EBPα-dependent mechanism. These compounds might mediate the immune response via S1P related signaling in COVID-19.

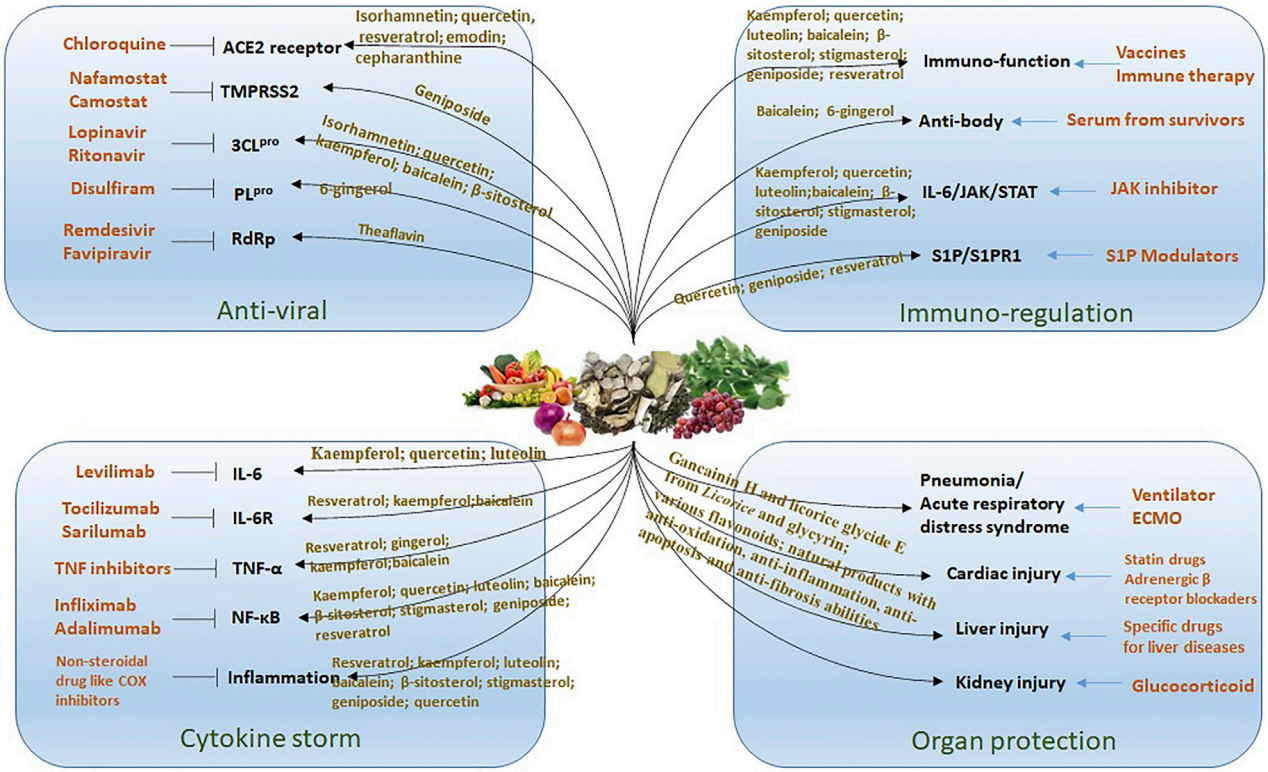

FIGURE 3 | The treatment strategy for COVID-19 by using natural products. There are different drugs (list in orange color) acting on specific target for the treatment of COVID-19. The edible and medicinal plants have multiple compounds that targeting on multiple pathways to against virus infection including anti-viral, reducing cytokine storm, immuno-regulation and organ protection.

Protecting Target Organs

Cardiovascular diseases in COVID-19 patients involves IL-6, ACE2, and angiotensin as the critical mediators that drive the pathological process. The increased IL-6 from activated macrophages and endothelial and smooth muscle cells after SARS-CoV-2 infection promotes the generation of MCP-1, upregulates the expression of cell adhesion molecules, and motivates the proliferation and migration of vascular smooth muscle cells, thus promoting atherogenesis (St Paul et al., 2020). Therefore, the level of circulating IL-6 may be a risk predictor of cardiovascular events in COVID-19. Furthermore, the destruction of lung tissue and the air-blood barrier allows the SARS-CoV-2 to continue to infect other organs via the ACE2 receptor (Zaim et al., 2020). The downregulation of ACE2 expression disrupts the balance between angiotensin I and II, converting them into angiotensin one to nine and one to seven, leading to the over-production of angiotensin II and organ damage. The increased angiotensin II interacts with the angiotensin II receptor type 1. This activates the JAK/STAT pathway to induce the generation of IL-6, forming a positive inflammatory feedback loop and ultimately causing vascular inflammation (Busse et al., 2020; de Abajo et al., 2020).

Natural products are particularly promising for organ protection from COVID-19 due to their multi-component and multi-target patterns. The inhibition of SARS-CoV-2 from binding to ACE2 as well as anti-oxidation, anti-inflammation, anti-apoptosis, and anti-fibrosis properties of natural products contribute to organ protection (Li et al., 2015; Lam et al., 2016). Several studies have indicated that some decoctions of herbal plants possess beneficial effects on many organs, implicating the regulation of TNF, MAPK, PI3K-Akt, Ras, and apoptosis signaling pathways (Chen et al., 2020). The active compounds of another herbal formula may act on ACE2, IL-6, and GM-CSF. Gancainin H and liquorice glycide E (from liquorice) act on ACE2, and glycyrin targets IL-6 and GM-CSF, orchestrating multiple signaling pathways to attenuate inflammation and viral infection, thus preventing lung and heart injury caused by SARS-CoV-2 (Shi-Ying et al., 2020).

CONCLUSION AND PROSPECT

Presently, in managing the spreading epidemic, efforts should include normalizing the control of infection. Providing convincing and promising protective dietary components for people might be a strategy for preventing COVID-19. Evidence from available data, literature analysis, and *in silico* studies indicated that some bioactive compounds from edible and herbal plants are potentially protective against SARS-CoV-2

infection. These compounds are associated with antiviral, anti-inflammatory, immunoregulatory, and organ protection via cooperating multiple targets and pathways using various components (**Figure 3**). However, a limitation of the application of natural products in preventing and treating COVID-19 is due to our current understanding of the action mechanisms is mainly predictive using molecular docking and network pharmacology analysis. The active constituents, potential targets, and pathways predicted in these studies are not always consistent. Rigorous animal studies and trials on people are needed to verify these predictions. In this study, we reviewed the anti-inflammatory and immune-regulatory effects of the compounds predicted to possess a strong ability to target SARS-CoV-2 in experimental studies. Our research provides scientific evidence for their potency in the prevention and management of COVID-19. In conclusion, many dietary components with low toxicity and are easily available, such as flavonoids, acetoside, glyasperin, isorhamnetin, and ginger are promising candidates for the development of food supplements or functional foods for the prevention and management of COVID-19.

AUTHOR CONTRIBUTIONS

YF conceived and designed the study and drafted the manuscript. SL collected the data and drafted the manuscript. CC, CZ, GT, HT, YL and NW commented on and revised the manuscript. All authors read and approved the final version.

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Screening of Anti-Lipase Components of *Artemisia argyi* Leaves Based on Spectrum-Effect Relationships and HPLC-MS/MS

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Pancreatic lipase is a key lipase for triacylglyceride digestion and absorption, which is recognized as a promising target for treatment of metabolic disorders. Natural phytochemicals are hopeful sources for pancreatic lipase inhibitors. The leaves of *Artemisia argyi* H.Lév. and Vaniot (AL) is commonly used as herbal medicine or food supplement in China and other Asian countries for hundreds of years. AL mainly contains essential oils, phenolic acids, flavonoids and terpenoids, which exhibit many pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, analgetic, anti-cancer, anti-diabetes and immunomodulatory effects. However, the anti-lipase activity of AL was lack of study and the investigation of anti-lipase ingredients from AL was also insufficient. In the present study, the anti-lipase activity of AL was evaluated *in vitro* and the potentially pancreatic lipase inhibitors of AL were investigated. High performance liquid chromatography was used to establish fingerprints of AL samples, and fifteen peaks were selected. The anti-lipase activities of AL samples were evaluated by a pancreatic lipase inhibition assay. Then, the spectrum-effect relationships between fingerprints and pancreatic lipase inhibitory activities were investigated to identify the anti-lipase constitutes in AL. As the results, four caffeoylquinic acids, which were identified as neochlorogenic acid, chlorogenic acid, isochlorogenic acid B, and isochlorogenic acid A by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, were selected as potential pancreatic lipase inhibitors in AL. Moreover, anti-lipase activity assessment and molecular docking study of the four compounds were performed to validate the potential lipase inhibitors in AL. The results revealed that the four caffeoylquinic acids in AL as bioactive compounds displayed with anti-lipase activity. The present research provided evidences for the anti-lipase activity of AL, and suggested that some bioactive compounds in AL could be used as lead compounds for discovering of new pancreatic lipase inhibitors.

Keywords: *Artemisia argyi* leaves, anti-lipase activity, spectrum-effect relationships, pancreatic lipase, HPLC-MS/MS

INTRODUCTION

Hyperlipidemia is a chronic, progressive and systemic disease characterized by the lipid metabolism disorders, which is a significant modifiable risk factor for cardiovascular and metabolic diseases (Ray et al., 2019). Drug intervention of lipid metabolism provides a credible method for prevention or treatment of metabolic disorders (Zeng et al., 2018). Pancreatic lipase is a key enzyme responsible for hydrolyzing triacylglycerides in the duodenum, which has been discovered as the crucial target that regulates lipid absorption (Hou et al., 2020b). The pancreatic lipase is secreted from the pancreas, and it has been demonstrated that inhibition on pancreatic lipase and regulation of lipid absorption is an effective approach for discovering new agents for treatment of metabolic disorders (Birari and Bhutani, 2007). Orlistat, which is a hydrogenated derivative of lipstatin, is the only approved pancreatic lipase inhibitor so far. Orlistat exhibits potent anti-lipase effect, but it can cause various side effects, such as oily spotting, fecal incontinence, flatus with discharge and abdominal cramping (Navarro Del Hierro et al., 2021). Thus, discovery and development of safe and effective pancreatic lipase inhibitors are urgently needed.

Recently, there has been a great interest in the screening of potential pancreatic lipase inhibitors from herbal medicines, because that herbal medicines have shown satisfying safety profiles in long-term medical treatments (Sham et al., 2014). Up to now, several herbal medicines, such as Citri Reticulatae Pericarpium and Mori Radicis Cortex, have been demonstrated with strong inhibition on pancreatic lipase for the regulation of lipid metabolism, which are applied to treat hyperlipidemia and other metabolic diseases in clinic (Hou et al., 2018; Zeng et al., 2018). The leaves of *Artemisia argyi* H.Lév. and Vaniot (AL), a common herbal medicine for treatment of hemorrhage, dysmenorrhea, abdominal pain eczema and skinitch (Guo et al., 2019), which is mainly distributed in China, Korea, Mongolia, and Japan (Abad et al., 2012). AL is also used as a food ingredient due to its delicious flavor and characteristic smell (Xiao et al., 2019)., AL is consumed as a condiment and colorant for the traditional food "Qingtuan" in China, and AL is also used as an additive in dietary food to enhance the flavor and nutrition in Japan (Abad et al., 2012). Previous investigations have been reported that AL contains many bioactive compounds such as essential oil, phenols, flavonoids and terpenoids, which possess multiple bioactivities, such as antioxidant, anti-inflammatory, antibacterial, antiviral, analgetic, anti-hypertensive, hypoglycemic, hemostatic and immunoregulatory effects (Huang et al., 2012; Dib and El Alaoui-Faris, 2019; Zhang and Lv, 2019). However, the anti-lipase activity of AL was lack of study and the investigation of anti-lipase compounds from AL was also insufficient.

It is inefficient to discover bioactive compounds from herbal medicines based on the commonly methods of extraction, purification, structure identification and bioassay. (Jiang et al., 2010; Quan et al., 2019; Gong et al., 2020). To overcome this shortcoming, a new and reliable method named spectrum-effect relationships analysis was employed to investigate the correlations between pharmacodynamics and chemical components of herbal medicines (Yang et al., 2016; Liu et al., 2019). Chromatographic fingerprints could characterize the chemical components of herbal medicines, which is an effective method for uniformity and quality evaluation of herbal medicines. Combining with the results of pharmacodynamics research, the chromatographic fingerprint data could be applied to analysis the spectrum-effect relationships to screen out the bioactive compounds from herbal medicines (Jiang et al., 2018; Zhang et al., 2019). In recent years, the spectrum-effect relationships analysis has been used to evaluate the quality and discover bioactive components of herbal medicines as an efficient strategy (Morlock et al., 2014; Xiao et al., 2018).

In this present study, the chromatographic fingerprints of AL samples were established using high performance liquid chromatography (HPLC), and the anti-lipase activities of AL samples were evaluated by the pancreatic lipase inhibition assay. Combined with the data of fingerprints and anti-lipase activities, the spectrum-effect relationships were performed to screen anti-lipase constitutes in AL by Pearson correlation analysis and partial least squares regression (PLSR). The selected pancreatic lipase inhibitors were further identified by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q/TOF-MS). In addition, the anti-lipase activity of the selected compounds was validated and *in silico* molecular docking research was performed to optimize and predict the pancreatic lipase inhibitors in AL.

MATERIALS AND METHODS

Materials and Reagents

Twenty-two batches of AL were collected from different areas in China, and the sample origins are provided in **Supplementary Table S1**. The voucher specimens, identified by Associate Professor Long Guo have been deposited in Traditional Chinese Medicine Processing Technology Innovation Center of Hebei Province, Hebei University of Chinese Medicine.

Reference standards of neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A were purchased from Chengdu Must Bio-Technoligy Co., Ltd. (Chengdu, China). The purities of these compounds were determined to be higher than 98% by high performance liquid chromatography with diode array detector. The chemical structures of the four compounds are shown in **Supplementary Figure S1**. Porcine pancreatic lipase (type II) and 4-methylumbelliferyl oleate were purchased from Sigma-Aldrich (St Louis, MO, United States).

HPLC grade methanol, acetonitrile and formic acid were obtained from Fisher Scientific (Pittsburgh, PA, United States). Ultrapure water was prepared by a Synergy water purification system (Millipore, Billerica, United States). Other chemicals and reagents were of analytical grade.

HPLC-DAD and HPLC-Q/TOF-MS Conditions

The HPLC-DAD analysis was performed on a Shimadzu LC-2030 system comprised an auto-sampler, a binary pump, a thermostatically controlled column apartment and a photo-diode array detector (Shimadzu Seisakusho, Kyoto, Japan). Chromatographic separation was conducted on an Agilent ZORBAX SB C18 column (4.6 × 50 mm, 1.8 μm). The mobile phase consists of 0.1% formic-water (A) and acetonitrile (B) with a gradient elution as follows: 0–5 min, 10% B; 5–10 min, 10–15% B; 10–22 min, 15–22% B; 22–27 min, 22–25% B; 27–37 min, 25–30% B; 37–44 min, 30% B; 44–47 min, 30–42% B; 47–52 min, 42–53%. The flow rate was maintained at 0.4 ml/min, and the column temperature was set at 25°C. The detection wavelength was set at 340 nm.

The HPLC-Q/TOF-MS analysis was performed on an Agilent 1290 UHPLC system coupled with an Agilent 6545 quadrupole time-of-flight mass spectrometer system (Agilent Technologies, Santa Clara, CA, United States). Chromatographic separation was also performed on an Agilent ZORBAX SB C18 column (4.6 × 50 mm, 1.8 μm) and the HPLC chromatographic conditions were the same as HPLC-DAD analysis. The MS acquisition parameters were as follows: drying gas (N_2) temperature, 320°C; sheath gas temperature, 350°C; drying gas (N_2) flow rate, 10.0 L/min; sheath gas flow (N_2) rate, 11 L/min; nebulizer gas pressure, 35 psi; capillary voltage, 3500 V; fragmentor voltage, 135 V; collision energy, 40 eV. The analysis was operated in negative mode with the mass range of m/z 120–1,000 Da. Data acquisition was conducted on MassHunter Workstation (Agilent Technologies, United States).

Sample Preparation

AL samples were powdered and screened through 40 mesh sieves. Each sample powder (0.2 g) was accurately weighed and thoroughly mixed with 75% (v/v) methanol (10 ml), then extracted by ultrasonator for 30 min. The extracted solution was centrifuged at 13,000 r/min for 10 min. For HPLC-DAD and HPLC-Q/TOF-MS analysis, the supernatant was injected into the HPLC instrument. For pancreatic lipase inhibition assay, 600 μl of the supernatant was evaporated to dryness and dissolved in 600 μl Tris-HCl buffer to remove the methanol, since the methanol might inactivated the pancreatic lipase.

HPLC Fingerprints

Method Validation

To ensure the reliability of the HPLC-DAD method used for AL samples analysis, the precision, repeatability and stability of the HPLC method was validated. The precision was determined by the intra- and inter-day variations. For intra-day precision, one AL sample was analyzed for six times within the same day, while for inter-day precision, the sample was examined in duplicates for consecutive three days. For the repeatability test, six replicates of the same AL sample was prepared and analyzed. To confirm the stability, the same AL sample was stored at room temperature and analyzed at 0, 2, 6, 8, 12 and 24 h. The relative standard deviations (RSDs) of peak areas for the fifteen common peaks were used to evaluate the precision, repeatability and stability of the established HPLC method.

Establishment and Evaluation of HPLC Fingerprints

Twenty-two batches of AL samples were analyzed to obtain the chromatograms containing the peak areas and relative retention times, and the chromatograms data were saved as CDF format. Using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version 2004 A), the HPLC fingerprints of AL samples were matched automatically, and the reference fingerprint was formed with the median method by comparison of the chromatograms of twenty-two batches of AL samples. The similarities between the reference fingerprint and the chromatograms of different AL samples were calculated.

Pancreatic Lipase Inhibition Assay

The inhibitory activities of AL samples against pancreatic lipase were investigated using 4-methylumbelliferyl oleate as a substrate, according to the previous method with little modification (Kurihara et al., 2003). The AL samples, pancreatic lipase and 4-methylumbelliferyl oleate were prepared in Tris-HCl buffer solution (13 mM Tris-HCl, 150 mM NaCl, 1.3 mM $CaCl_2$, pH 8.0). 25 μl of AL sample solution (at different concentrations) and 25 μl of pancreatic lipase solution (1 mg/ml) were added into a black bottom 96-well plate. After pre-incubation at 37°C for 10 min, the reaction was started by addition of 50 μl of 4-methylumbelliferyl oleate solution (1 mM). After incubation at 37°C for 20 min, the reaction was stopped by adding 100 μl of 0.1 M citrate buffer solution (pH 4.2). The amount of 4-methylumbelliferone released by the pancreatic lipase was measured with a fluorometrical microplate reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The control sample was prepared by adding buffer solution instead of tested sample. The background sample was prepared by replacing tested sample with the same volume of buffer solution. The blank sample was prepared by adding buffer solution instead of pancreatic lipase solution. All experiments were repeated three times. The inhibition of pancreatic lipase activity was calculated as follows:

$$\text{Inhibition}\,(\%) = \left(1 - \frac{\text{test sample} - \text{background sample}}{\text{control sample} - \text{blank sample}}\right) \times 100$$

The pancreatic lipase inhibitory activity of AL samples were evaluated by the IC_{50} values (the concentration of the sample that inhibited 50% the activity of the pancreatic lipase), and the IC_{50} were calculated by a logarithmic regression curve and expressed as mg/mL methanol extracts equivalents.

To confirm the spectrum-effect relationships analysis result, the pancreatic lipase inhibitory activities of the potential anti-lipase ingredients, including neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A were determined by the above pancreatic lipase inhibition assay, and the IC_{50} value of the four components were also calculated.

Spectrum-Effect Relationship Analysis

Pearson Correlation Analysis

Pearson correlation analysis is a multivariate statistical model, which is applied to extract factors that have the greatest impact on the outcome variables and maximize the relationships between the two sets of variables (Armstrong et al., 2005). Taking the Pearson correlation coefficient as an index, the fifteen common peaks areas in the HPLC fingerprints of AL samples were recognized as one set of variables, and the anti-lipase activities (IC_{50} values) as the other set. The correlations between common peaks and IC_{50} values were analyzed by SPSS 18.0 statistics software (SPSS Inc., Chicago, IL, United States).

Partial Least Squares Regression Analysis

PLSR analysis is a multivariate regression model combining multivariate data fusion and principal component analysis (Svante et al., 2001). In this study, PLSR was used to model the correlation between the common peaks and anti-lipase activities. The fifteen common peak areas were set as the independent X variables, and the anti-lipase activities (IC_{50} values) were set as dependent Y variables. After extraction of principal components, the linear relationships between anti-lipase activities and common peaks were displayed by the PLSR model. The regression coefficients were considered as the index to reveal the relative impact of the predictor variables on the response variable for PLSR model. The PLSR was performed by Simca-P 14.0 software (Umetrics, Umea, Sweden).

Molecular Modeling and Docking Study

An *in silico* protein-ligand docking software AutoDock 4.2 program was performed to analyze binding affinities of ligands to pancreatic lipase and predict the possible binding sites based on the standard procedures (Eswaramoorthy et al., 2021). The structure of pancreatic lipase (PDB ID: 1LPA) was obtained from Protein Data Bank. Unnecessary substructures and water molecules in pancreatic lipase were removed, and hydrogen atoms were added. The gasteiger charges of each atom of pancreatic lipase were calculated. Run AutoGrid to get grid maps. The number of runs was set as 100 by Lamarckian genetic algorithm to give docked conformations.

RESULTS

HPLC Fingerprints

Optimization of HPLC Condition

In order to achieve a rapid and efficient separation of AL samples, several HPLC conditions, including mobile phases (water-methanol, water-acetonitrile, formic acid water-methanol and formic acid water-acetonitrile), flow rates (0.3, 0.4, and 0.5 ml/min), and column temperatures (20°C, 25°C, and 30°C) were optimized. The results indicated that formic acid water-acetonitrile was the best mobile phase for separation of the analystes. The optimal column temperature was 25°C and optimal flow rate was 0.4 ml/min in this present study.

Method Validation

The precision, repeatability and stability of the established HPLC-DAD method were validated. As shown in **Supplementary Table S2**, the intra-day and inter-day precision (RSDs) of peak areas for the fifteen common peaks were less than 2.7 and 2.9%, respectively. The repeatability presented as RSDs was less 2.6%, and the stability was less than 2.8%. The method validation results demonstrated that the established HPLC-DAD method is suitable for analysis of AL samples.

HPLC Fingerprints and Similarity Analysis

Twenty-two batches of AL samples collected from different areas were analyzed by HPLC-DAD using the optimized condition. Then, the HPLC fingerprints were established based on the chromatograms of AL samples by similarity evaluation software (Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine). The chromatographic fingerprints of AL samples are shown in **Figure 1A**, and the reference fingerprint is displayed in **Figure 1B**. Fifteen peaks that existed in all the AL samples with good segregation and resolution were recognized as the common peaks, which indicated the similarity among various samples. The similarities between the HPLC chromatograms of AL samples and the reference fingerprint were compared, and the similarity values were calculated using the correlative coefficient and the cosine value of vectorial angle by the similarity evaluation software. As shown in **Table 1**, the similarity values between HPLC fingerprint of each AL sample and the reference fingerprint were in the range of 0.900–0.997. The similarity analysis results indicated that different batches of AL samples had similar chemical compositions, and the origin might not be the main factor that affects the quality diversity of AL samples.

Anti-Lipase Activity

Most of the bioactive studies of AL are focused on the antioxidant and anti-inflammatory effects (Yun et al., 2016; Han et al., 2017; Xia et al., 2019). To the best of our knowledge, there are no reports about the anti-lipase activities of AL. In this work, the pancreatic lipase inhibitory capacities of twenty-two batches of AL samples collected from different areas were evaluated by pancreatic lipase inhibition assay. The results showed that AL samples inhibited pancreatic lipase in a concentration dependent manner with IC_{50} in the range from 1.92 to 10.29 mg/ml (**Table 2**). The heatmap (**Figure 2**) was adopted to provide the presentation of the common peak areas and anti-lipase activity difference of AL samples from different origins. It could be noted that the anti-lipase activities of AL samples showed significant differences, and the peak areas of bioactive ingredients were also different to some extent. The differences of anti-lipase activities might be due to the presence of various bioactive constituents in AL samples. Therefore, it is necessary to investigate the relationships between the bioactive compounds and anti-lipase activities of AL samples, and find the potential anti-lipase constituents through spectrum-effect relationships analysis.

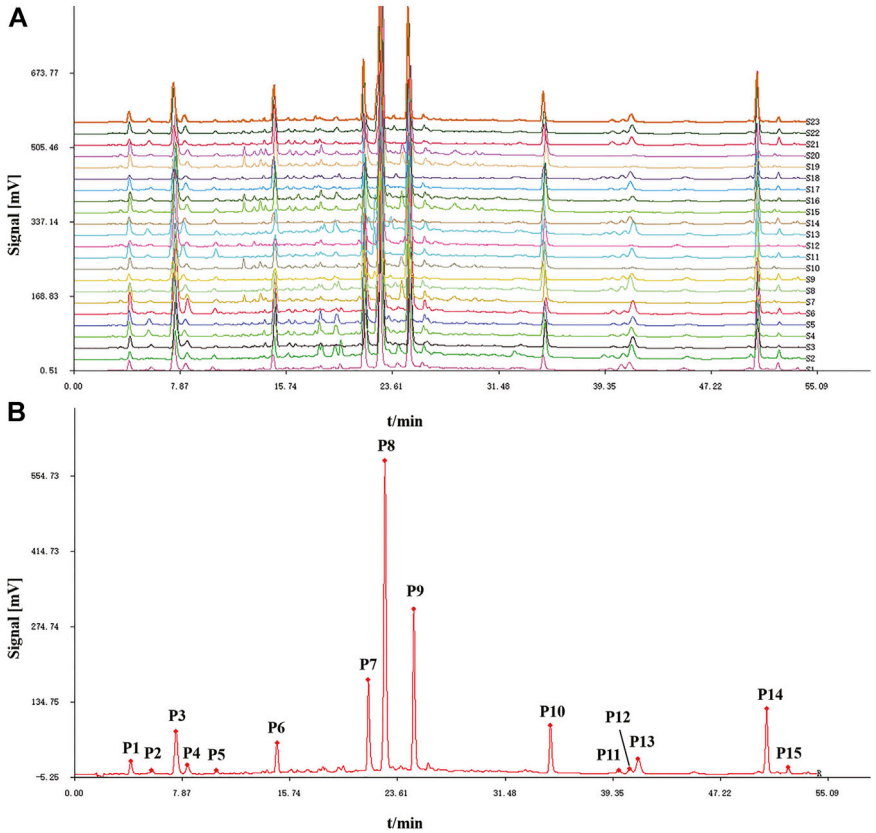

FIGURE 1 | HPLC fingerprints **(A)** and reference fingerprint **(B)** of twenty-two batches (S1-S22) of *Artemisia argyi* leaves.

TABLE 1 | Similarities of twenty-two batches (S1-S22) of *Artemisia argyi* leaves collected from different areas.

NO.	Similarity	NO.	Similarity
S1	0.942	S12	0.931
S2	0.997	S13	0.900
S3	0.970	S14	0.984
S4	0.971	S15	0.985
S5	0.982	S16	0.994
S6	0.977	S17	0.900
S7	0.961	S18	0.979
S8	0.902	S19	0.978
S9	0.980	S20	0.922
S10	0.955	S21	0.968
S11	0.978	S22	0.978

TABLE 2 | Inhibitory effects (IC_{50} values) of twenty-two batches (S1-S22) of *Artemisia argyi* leaves on pancreatic lipase.

NO.	IC_{50} (mg/ml)	No	IC_{50} (mg/ml)
S1	5.99 ± 0.08	S12	4.54 ± 0.12
S2	5.96 ± 0.07	S13	8.50 ± 0.13
S3	4.22 ± 0.06	S14	3.89 ± 0.12
S4	1.92 ± 0.05	S15	5.21 ± 0.10
S5	5.69 ± 0.04	S16	6.66 ± 0.18
S6	4.39 ± 0.02	S17	10.29 ± 0.21
S7	5.36 ± 0.00	S18	4.49 ± 0.07
S8	8.80 ± 0.05	S19	5.75 ± 0.16
S9	5.80 ± 0.11	S20	7.19 ± 0.18
S10	4.77 ± 0.06	S21	5.83 ± 0.01
S11	4.45 ± 0.14	S22	4.45 ± 0.02

Spectrum-Effect Relationship Analysis

Pearson Correlation Analysis

Pearson correlation analysis was firstly applied to study the spectrum-effect relationships between the IC_{50} values of pancreatic lipase inhibitory activities and the fifteen common peak areas of different AL samples. Pearson correlation coefficients of the fifteen common peaks are shown in **Figure 3**. It was observed that ten common peaks, P3, P8, P1, P7, P2, P10, P6, P9, P4 and P5 were negatively correlated to the IC_{50} values, which indicated these common peaks had strong inhibitory effects on pancreatic lipase. The correlation coefficients of P3, P8, P1, P7, P2, P10, P6, P9, P4 and P5 were −0.7920, −0.7690, −0.5820, −0.5710, −0.5610, −0.5240, −0.4860, −0.4730, −0.3960 and, −0.3900, respectively. The higher the absolute value of correlation coefficient was, the stronger anti-lipase effect the common peak had. While peaks P11, P12, P13, P14 and P15 showed positive correlation to the IC_{50} values, indicating that when the areas of these peaks increased the capability on anti-lipase activities would be weaker.

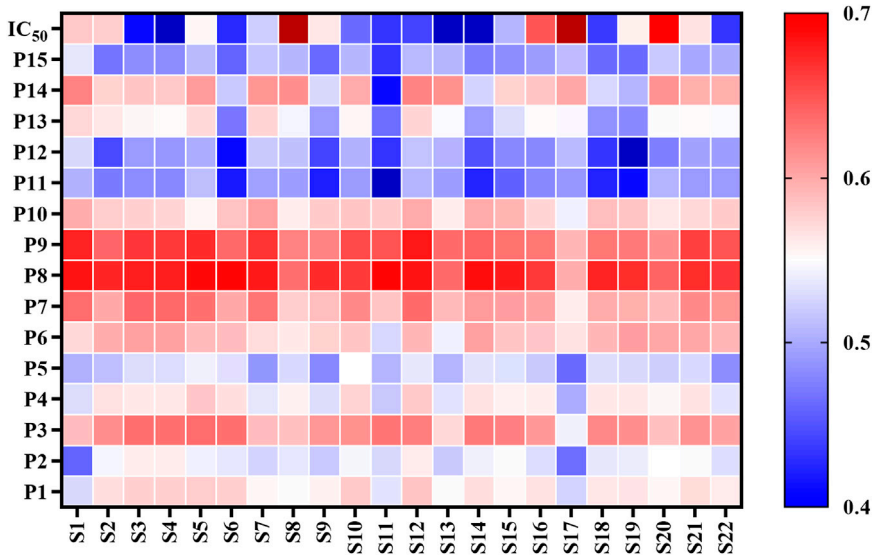

FIGURE 2 | Heatmap analysis of fifteen common peaks (P1-P15) areas and anti-lipase activities (IC_{50} values of pancreatic lipase) of twenty-two batches (S1-S22) of *Artemisia argyi* leaves. Red represents higher contents and blue indicates lower contents.s

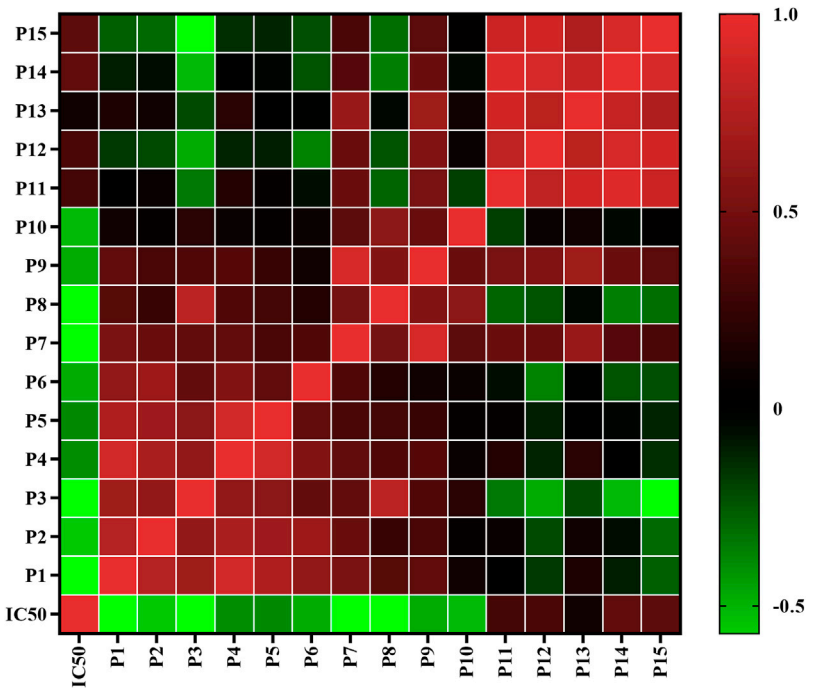

FIGURE 3 | Heatmap analysis of Pearson correlation of fifteen common peaks (P1-P15) areas and anti-lipase activities (IC_{50} values of pancreatic lipase). Red represents positive correlated and green indicates negative correlated.

Partial Least Squares Regression Analysis

PLSR can fully use of data information, and is widely used in spectrum-effect relationships analysis. In this present work, PLSR was further performed to investigate the spectrum-effect relationships between anti-lipase activities and common peaks, and find pancreatic lipase inhibitors of AL samples. As shown in **Figure 4A**, the fifteen common peak areas were set as the independent X variables and the IC_{50} values of pancreatic lipase inhibitory activities of AL samples were taken as the dependent Y variables, and the PLSR models were established

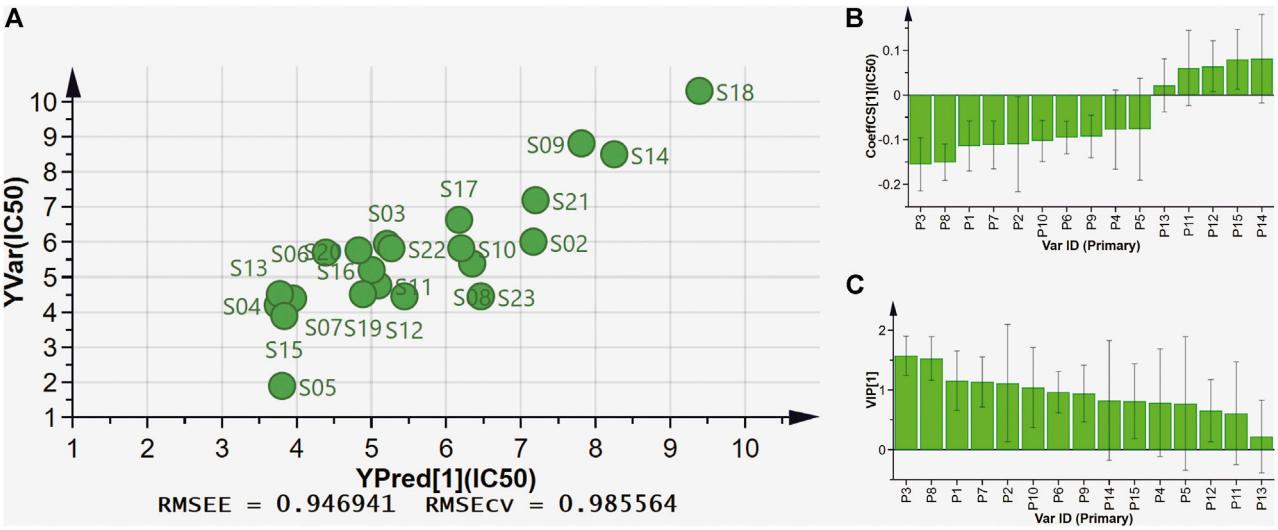

FIGURE 4 | The results of spectrum-effect relationships by PLSR model. **(A)** PLSR linear regression; **(B)** Regression coefficients between fifteen common peaks and anti-lipase activities (IC_{50} values of pancreatic lipase); **(C)** VIP values of fifteen common peaks.

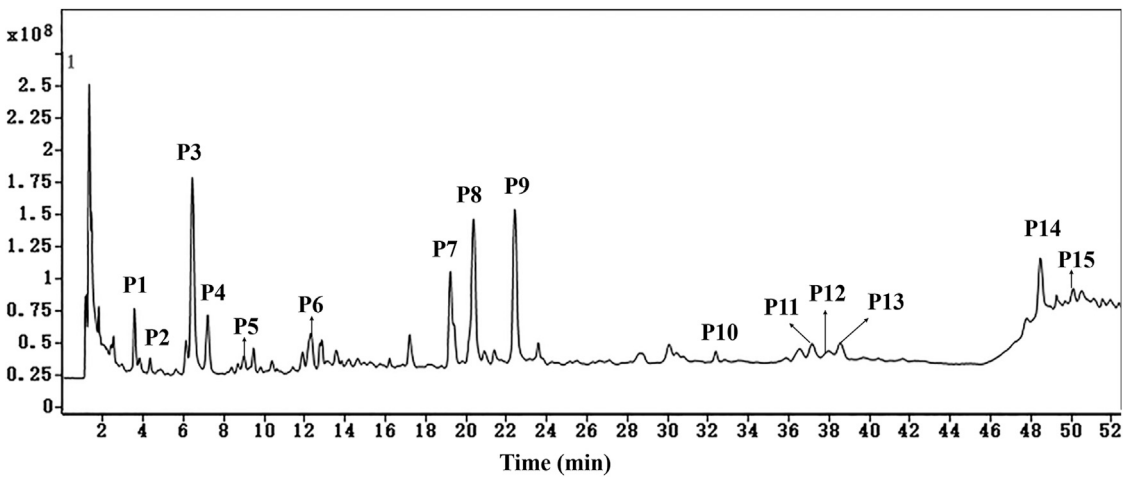

FIGURE 5 | The typical total ion chromatogram of AL sample in negative ion mode. P1, neochlorogenic acid; P2, quinic acid; P3, chlorogenic acid; P4, cryptochlorogenic acid; P5, caffeic acid; P6, 5-feruoyl quinic acid; P7, isochlorogenic acid B; P8, isochlorogenic acid A; P9, isochlorogenic acid C; P10, 3,4,5-tri-*O*-caffeoylquinic acid; P11, hispidulin; P12, centaureidin; P13, jaceosidin; P14, eupatilin; P15, casticin.

sequentially. The regression coefficients of the fifteen common peaks were calculated. As shown in **Figure 4B**, the ten common peaks P3, P8, P1, P7, P2, P10, P6, P9, P4, and P5 showed negative correlation to the IC_{50} values, which indicated that the anti-lipase activity increased with the increasing areas of these common peaks. The inhibitions of the ten common peaks on pancreatic lipase were as the following order: P3 > P8 > P1 > P7 > P2 > P10 > P6 > P9 > P4 > P5, which was consistent with the results obtained by Pearson correlation analysis. The Variable Importance for the Projection (VIP) values of the fifteen common peaks were also calculated. The VIP values represent the importance of the variables, and common peaks with VIP values greater than 1.0 could be considered to be responsible for anti-lipase activity. As shown in **Figure 4C**, the VIP values of P3, P8, P1, and P7 were 1.5742, 1.5279, 1.5778, and 1.1351 respectively, greater than 1.0. Combining the results of Pearson correlation analysis and PLSR, the four common peaks P3, P8, P1, and P7 were considered as potential pancreatic lipase inhibitors of AL.

Identification of Fifteen Common Peaks in *Artemisia argyi* Leaves by HPLC-Q/TOF-MS

The fifteen common peaks (P1-P15) in HPLC fingerprints of AL were further identified by HPLC-Q/TOF-MS, and the typical

TABLE 3 | Identification of fifteen common peaks in *Artemisia argyi* leaves by HPLC-Q/TOF-MS.

Peak	Retention time (min)	$[M-H]^-$ (*m/z*)	Formula	Error (ppm)	Fragment ions (*m/z*)	Identification
P1	3.55	353.0877	$C_{16}H_{18}O_9$	−0.36	191.0560, 179.0347, 161.0241, 135.0452, 127.0400, 111.0450, 93.0344	Neochlorogenic acid
P2	6.44	191.0560	$C_7H_{12}O_6$	−0.62	191.0562, 173.0451, 127.0402, 85.0297, 59.0142	Quinic acid
P3	6.54	353.0875	$C_{16}H_{18}O_9$	−0.85	191.0560, 173.0453, 161.0245, 135.0449, 127.0400, 111.0450, 93.0345	Chlorogenic acid
P4	7.17	353.0875	$C_{16}H_{18}O_9$	−0.89	353.0877, 173.0453, 135.0449, 111.0450, 85.0296, 59.0142	Cryptochlorogenic acid
P5	8.97	179.0348	$C_9H_8O_4$	−0.91	179.035, 135.0450, 134.0369, 107.0503, 79.0551	Caffeic acid
P6	11.83	367.1032	$C_{17}H_{20}O_9$	−0.70	367.1033, 191.0559, 173.0458, 149.0607, 134.0369	5-Feruoyl quinic acid
P7	19.23	515.1194	$C_{25}H_{24}O_{12}$	0.15	515.1191, 353.0874, 335.0768, 299.0558, 191.0559, 173.0453, 161.0241, 135.0448	Isochlorogenic acid B
P8	20.50	515.1192	$C_{25}H_{24}O_{12}$	0.05	515.1190, 353.0873, 335.0770, 191.0560, 179.0345, 161.0241, 135.0442	Isochlorogenic acid A
P9	22.56	515.1195	$C_{25}H_{24}O_{12}$	−0.03	515.1194, 191.0560, 173.0453, 155.0345, 135.0450, 111.0450, 93.0344, 71.0140	Isochlorogenic acid C
P10	32.38	677.1507	$C_{34}H_{30}O_{15}$	−0.96	677.1513, 515.1188, 353.0876, 191.0559, 173.0454, 135.0449	3,4,5-Tri-*O*-caffeoylquinic acid
P11	37.16	299.0560	$C_{16}H_{12}O_6$	−0.39	299.0562, 284.0318, 255.0298, 227.0346, 183.0447, 136.9878, 94.0059	Hispidulin
P12	37.94	359.0771	$C_{18}H_{16}O_8$	−0.56	344.0531, 329.0294, 314.0070, 301.0349, 286.0114, 258.0167, 242.0217, 214.0266	Centaureidin
P13	38.55	329.0666	$C_{17}H_{14}O_7$	−0.37	329.0666, 299.0196, 271.0246, 243.0294, 227.0344, 199.0399, 171.0449, 133.0291, 65.0035	Jaceosidin
P14	48.47	343.0821	$C_{18}H_{16}O_7$	−0.83	343.0823, 313.0351, 298.0115, 285.0401, 270.0165, 242.0216, 214.0267, 163.0035, 147.0447, 132.0215, 65.0035	Eupatilin
P15	50.11	373.0927	$C_{19}H_{18}O_8$	−0.62	373.0929, 343.0453, 328.0223, 312.0987, 285.0037, 257.0089, 213.0190, 185.0237	Casticin

total ion chromatogram of AL sample in negative ion mode is illustrated in **Figure 5**. Fifteen compounds including two organic acids (P2 and P5), seven caffeoylquinic acids (P1, P3, P4, P7, P8, P9, and P10), one feruoylquinic acid (P6) and five methoxylated flavones (P11, P12, P13, P14, and P15) were identified or tentatively characterized. The HPLC-Q/TOF-MS information such as retention time, chemical formula, ppm errors and main fragment ions is summarized in **Table 3**.

The above spectrum-effect relationships results illustrated that the common peaks P3, P8, P1, and P7 were potential pancreatic lipase inhibitory compounds in AL. Peaks P1 and P3 were unambiguously identified as neochlorogenic acid and chlorogenic acid via by comparing the retention time and MS/MS fragmentation pattern with those of *mono*-caffeoylquinic acids and the reference standards (Zhang et al., 2015; Willems et al., 2016). The MS/MS spectra of peaks P1 and P3, and possible fragmentation patterns of caffeoylquinic acids are presented in **Supplementary Figure S2**. These two *mono*-caffeoylquinic acids showed the same deprotonated ion $[M-H]^-$ at *m/z* 353.0877 ($C_{16}H_{18}O_9$), and MS/MS ions at *m/z* 191.0560 ([quinic acid-H]$^-$), 179.0347 ([caffeic acid-H]$^-$) and 135.0452 ([caffeoyl-CO_2-H]$^-$). Similarly, peaks P7 and P8 exhibited the same deprotonated ion $[M-H]^-$ at *m/z* 515.1192 ($C_{25}H_{24}O_{12}$), and MS/MS ions at *m/z* 335.0770 ([M-coffeoyl-H]$^-$), 353.0873 ([caffeoylquinic acid-H]$^-$), and 299.0558 ([M-coffeoyl-$2H_2O$-H]$^-$). Comparing the retention time and MS/MS fragmentation pattern with those of *di*-caffeoylquinic acids and the reference standards (Zhang et al., 2015; Willems et al., 2016), peaks P7 and P8 were unequivocally identified as isochlorogenic acid B and isochlorogenic acid A. The MS/MS spectra of these two *di*-caffeoylquinic acids, and possible fragmentation patterns of caffeoylquinic acids are presented in **Supplementary Figure S3**.

Methoxylated flavonoid is one of the common flavones bearing one or more methoxylated groups on the basic benzo–γ–pyrone (15-carbon, C6–C3–C6) skeleton (Xing et al., 2017). In this present work, five methoxylated flavonoids, including hispidulin (P11), centaureidin (P12), jaceosidin (P13), eupatilin (P14), and casticin (P15) were tentatively identified by comparison of the MS/MS spectras and fragmentation patterns with literatures. Previous studies partially summarized the characteristic fragmentation patterns of polymethoxylated flavones (Ren et al., 2018).

Here, eupatilin (P14) was used as an instance to explain the fragmentation patterns and structural elucidation process of methoxylated flavonoids. The MS/MS spectra and fragmentation patterns of eupatilin are shown in **Supplementary Figure S4**. In high mass range of the MS/MS spectra, the multiple and alternate loss of $\bullet CH_3$ and CO generated several characteristic fragment ions, including *m/z* 328.0587([M-H-$\bullet CH_3$]$^-$), 313.0350 ([M-H-2×$\bullet CH_3$]$^-$), 298.0115 ([M-H-3×$\bullet CH_3$]$^-$), 285.0400 ([M-H-2×$\bullet CH_3$-CO]$^-$), 270.0164 ([M-H-3×$\bullet CH_3$-CO]$^-$) and 242.0217 ([M-H-3×$\bullet CH_3$-2×CO]$^-$). In low mass range of the MS/MS spectra, the fragment ions were generated mainly via RDA cleavage. The fragment ions at 164.0069 and 136.0123 are derived from the RDA cleavage at position 1/3.

In conclusion, the fifteen common peaks in HPLC fingerprints of AL were identified or tentatively identified based on the retention time, MS/MS spectras and fragmentation behaviors by HPLC-Q/TOF-MS. According to the results of qualitative identification, the four potential anti-lipase constitutes in AL

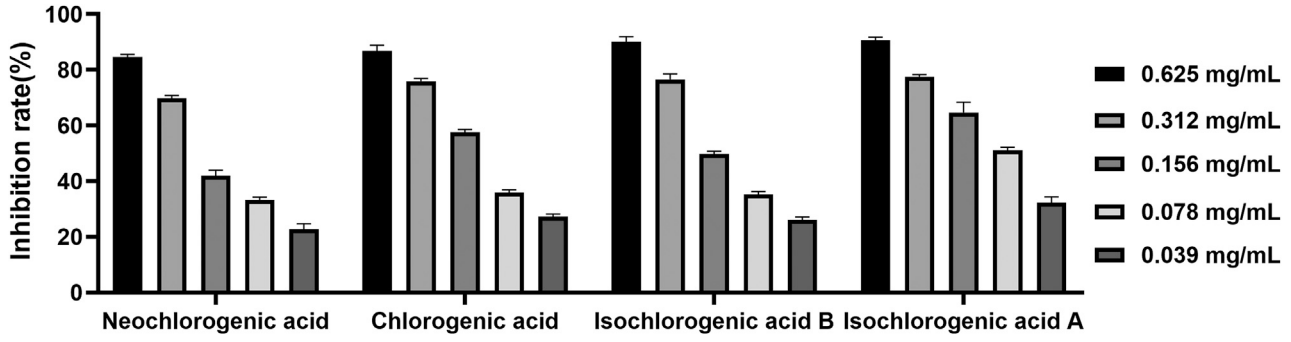

FIGURE 6 | The inhibitory effects of neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A on pancreatic lipase.

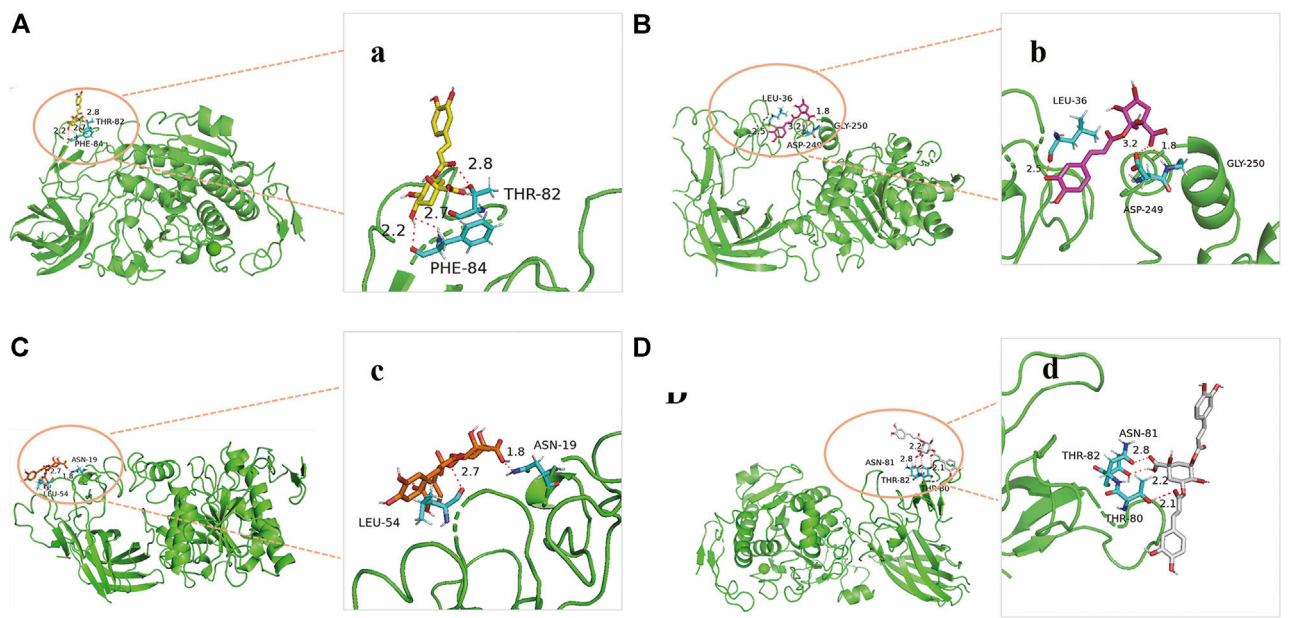

FIGURE 7 | Molecular docking analysis of four anti-lipase compounds with pancreatic lipase. **(A)** Preferred docking position of neochlorogenic acid on pancreatic lipase, **(a)** Interaction of neochlorogenic acid and amino acid residues in pancreatic lipase; **(B)** Preferred docking position of chlorogenic acid on pancreatic lipase, **(b)** Interaction of chlorogenic acid and amino acid residues in pancreatic lipase; **(C)** Preferred docking position of isochlorogenic acid B on pancreatic lipase, **(c)** Interaction of isochlorogenic acid B and amino acid residues in pancreatic lipase; **(D)** Preferred docking position of isochlorogenic acid A on pancreatic lipase, **(d)** Interaction of isochlorogenic acid A and amino acid residues in pancreatic lipase.

represented by peaks P1, P3, P7, and P8 were four caffeoylquinic acids, including neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A, respectively.

Verification of Pancreatic Lipase Inhibitory Activity

Four caffeoylquinic acids, including neochlorogenic acid (P1), chlorogenic acid (P3), isochlorogenic acid B (P7), and isochlorogenic acid A (P8) were selected as potential pancreatic lipase inhibitors in AL. In order to confirm the reliability of the results, the pancreatic lipase inhibitory capacities of the four constituents were determined by pancreatic lipase inhibition assay. Six concentrations of each compound (0.039, 0.078, 0.156., 0.312, and 0.625 mg/ml) were used. As shown in **Figure 6**, the four caffeoylquinic acids were found to possess dose-dependent pancreatic lipase inhibitory activities, and the IC_{50} values of neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A were 0.152 ± 0.020, 0.114 ± 0.025, 0.121 ± 0.022, and 0.081 ± 0.012 mg/ml, respectively. According to the above results, it could be speculated that neochlorogenic acid, chlorogenic acid, isochlorogenic acid B, and isochlorogenic acid A were anti-lipase components of AL.

Molecular Modeling and Docking Study

In order to predict the preferred binding site between pancreatic lipase and the four anti-lipase compounds, and confirm the results of the pancreatic lipase inhibitory experiments described above, an *in silico* molecular docking study was further performed in this present study (**Figure 7**). In the process of interaction between neochlorogenic acid and pancreatic lipase, 3-OH on quinic acid groups and carbonyl on caffeic acid groups of neochlorogenic acid could generate hydrogen bonds with Phe84 and Thr82 in pancreatic lipase. Analogously, 3-OH on caffeic acid groups and carbonyl on quinic acid groups of chlorogenic acid could generate four hydrogen bonds with Leu36, His30, Gly250 and Asp249 in pancreatic lipase. Isochlorogenic acid B docked to pancreatic lipase was stabilized by two hydrogen bonds to Asn19 and Leu54, and isochlorogenic acid A docked to pancreatic lipase was stabilized by three hydrogen bonds to Asn81, Thr82, and Thr80. It has been reported previously that the amino acid residues of Ser153, Asp177, and His264 are the key catalytic sites of pancreatic lipase (Huang et al., 2020). However, the results of molecular docking indicated that all the four pancreatic lipase inhibitors in AL did not bind to the three catalytic amino residues, and there were distances from the preferred binding sites of the four inhibitors on pancreatic lipase to the catalytic sites in space.

DISCUSSION

Hyperlipidemia is a serious public health problem, because hyperlipidemia is closely associated to multiple metabolic diseases, such as diabetes, atherosclerosis and hypertension (Ray et al., 2019). Drug limitation of the intestinal absorption of fats and cholesterol provides a possible approach to prevent or treat hyperlipidemia and other metabolic disorders. Pancreatic lipase is the key enzyme for lipid digestion and absorption, and hydrolysis of triacylglycerols to monoacylglycerols and fatty acids in the duodenum. The pancreatic lipase inhibitors could produce hypolipidemic activity, which could be useful for control or treatment of metabolic disorders (Hou et al., 2020a). Thus, several efforts have been made to discover potential pancreatic lipase inhibiters as anti-lipase agents and a variety of active compounds have been found with inhibitory effects against pancreatic lipase. Among the potential pancreatic lipase inhibiters, orlistat is the most successful case. Orlistat is the only approved pancreatic lipase inhibitor deriving from lipstatin (Nivedita et al., 2018). Although orlistat exhibits good anti-lipase effect, it can lead to several non-negligible gastrointestinal side effects (Pilitsi et al., 2019). The natural phytochemicals existed in some herbal medicines can provide an alternative therapy to reduce the side effects of pharmaceutical drugs (Fabricant and Farnsworth, 2001).

The presence of lipase inhibitors has been reported in several medicinal herbs such as Mori Cortex, Citri Reticulatae Pericarpium Crataegi Fructus and Linderae Radix (Seyedan et al., 2015; Hou et al., 2018; Zeng, et al., 2018). Lots of phytochemicals, especially polyphenols, identified from medicinal herbs exhibited anti-lipase bioactivity (Zheng et al., 2010). As an edible herbal medicine, AL has been widely used in clinic in China and other Asia countries. Our previous studies have shown that polyphenols, including caffeoylquinic acids and flavonoids, are main bioactive constituents in AL, which has been reported to display hypoglycemic and hypolipidemic properties (Guo et al., 2019). However, there is no information about the potential anti-lipase of AL. In this study, the anti-lipase effects of AL were investigated for the first time. The results demonstrated that all the test AL samples exhibited moderate to strong inhibitions on pancreatic lipase with the IC_{50} in the range between 1.92 and 10.29 mg/ml methanol extracts equivalents (**Table 2**). Taking into consideration that pancreatic lipase is a secreted enzyme in the gastrointestinal tract, the pancreatic lipase inhibitory compounds in AL could directly target on pancreatic lipase after oral administration. It is clear that different batches of AL samples showed significant different anti-lipase activities, which could be owing to the variation of bioactive ingredients in AL samples (**Figure 2**). Therefore, the pancreatic lipase inhibition results of AL samples encouraged us to further investigate potential anti-lipase compounds from AL.

At present, the multiple chemical components and targets of herbal medicines pose a challenge in discovering bioactive compounds from herbal medicines. The common research methods usually focus on chemical compounds separation and single components activity, which are time-consuming and cannot reveal the complex roles of multiple components in herbal medicines (Quan et al., 2019; Gong et al., 2020). The spectrum-effect relationship is a new and reliable method that can combine chromatographic fingerprint with pharmacological effects by multiple chemometrics. This method could help to explore the correlations between bioactive components and efficacy, and find the major bioactive ingredients in herbal medicines (Yang et al., 2016; Liu et al., 2019). The spectrum-effect relationship method has been successful used to discover varieties of bioactive compounds in herbal medicines. For instance, Wang *et al.* discovered that sinomenine, magnoflorine, menisperine and stepharanine are the major anti-inflammatory compounds in Sinomenii Caulis, and Zeng *et al.* screened out that polymethoxyflavones are the anti-lipase components in Citri Reticulatae Pericarpium by using spectrum-effect relationship method (Zeng et al., 2018; Wang et al., 2019). In this present work, the spectrum-effect relationships between chromatographic fingerprint peaks and anti-lipase activities of AL samples were explored by Pearson correlation analysis and PLSR models. According to the results (**Figures 3**, **4**), the pancreatic lipase inhibition activities were not dominated by one compound but multiple components in AL. The peaks, P3, P8, P1, P7, P2, P10, P6, P9, P4, and P5 showed positive relationships to pancreatic lipase inhibitions, conversely, peaks P11, P12, P13, P14, and P15 had negative relationships. The chemometrics results revealed that the compounds represented by P3, P8, P1, and P7 played important roles in the anti-lipase activity of AL, which could be regard as the pancreatic lipase inhibitors in AL.

The pancreatic lipase inhibitors represented by peaks P3, P8, P1, and P7 were identified as four caffeoylquinic acids (neochlorogenic acid, chlorogenic acid, isochlorogenic acid B

and isochlorogenic acid A) by HPLC-Q/TOF-MS. Caffeoylquinic acids, the esters of caffeic acid and quinic acid, are characterized as the important polyphenols present in Asteraceae and Lamiaceae families (Spinola and Castilho, 2017). It has been demonstrated that caffeoylquinic acids could regulate lipid metabolism in genetically and healthy metabolic related disorders (Martinez-Lopez et al., 2019). It is speculated that caffeoylquinic acids can display important roles in regulation of lipid and glucose metabolism and use to treat cardiovascular diseases, diabetes, and obesity (Sotillo and Hadley, 2002; Naveed et al., 2018). Previous investigation has reported that neochlorogenic acid, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A exhibited dose-dependent inhibitory activities on pancreatic lipase with the IC_{50} values of 1.12 ± 0.04, 1.09 ± 0.08, 0.32 ± 0.02, and 0.39 ± 0.04 mg/ml, respectively (Narita et al., 2012). In our study, the IC_{50} values of the four caffeoylquinic acids were 0.152 ± 0.020, 0.114 ± 0.025, 0.121 ± 0.022, and 0.081 ± 0.012 mg/ml, which are lower than previous research results. The reason for the differences in the results might be due to the different substrates used in the experiments, we used 4-methylumbelliferyl oleate as the water-insoluble substrate, but Narita et al. used triolein. It has been reported that the inhibitory effects of catechins and theaflavins on the pancreatic lipase-catalyzed hydrolysis of triolein were much weaker than those on the pancreatic lipase-catalyzed hydrolysis of 4-methylumbelliferyl oleate (Kobayashi et al., 2009).

To further predict the possible binding sites between pancreatic lipase and the four caffeoylquinic acids, the molecular docking research was performed. It has been reported previously that the amino acid residues of Ser153, Asp177, and His264 are the key catalytic sites of pancreatic lipase (Hou et al., 2020a). However, the results of molecular docking showed that all the four caffeoylquinic acids did not bind to the three key catalytic sites of pancreatic lipase despite their strong anti-lipase activities. It could be speculated that the four caffeoylquinic acids might bind the pancreatic lipase in noncovalent interactions to exert the pancreatic lipase inhibitory effects. In general noncovalent binding between the enzymes and inhibitors is nonspecific and weak, but multiple noncovalent bingings couldchange the conformation and function of the enzymes (Xu et al., 2019). It has been reported that (−)-Epigallocatechin-3-gallate, an anti-lipase component in green tea, which could inhibit the pancreatic lipase without binding the key catalytic sites (Ser153, Asp177, and His264) of pancreatic lipase (Wu et al., 2013).

CONCLUSION

In the current study, the HPLC fingerprints of different AL samples were established, and fifteen common peaks were selected. The anti-lipase activities of different AL samples were also evaluated by pancreatic lipase inhibition assay. Then, the spectrum-effect relationships between HPLC fingerprints and pancreatic lipase inhibitory activities of AL samples were firstly investigated to discover the anti-lipase compounds. The Pearson correlation analysis and PLSR results showed a close correlation between fingerprints and anti-lipase activity of AL samples. Four caffeoylquinic acids were selected as potential pancreatic lipase inhibitors in AL, which were identified as neochlorogenic acid, chlorogenic acid, isochlorogenic acid B, and isochlorogenic acid A by HPLC-Q/TOF-MS. Moreover, the pancreatic lipase inhibitory activities of the four caffeoylquinic acids were validated experimentally, and the results indicated that the four bioactive compounds significantly inhibited pancreatic lipase with IC_{50} value ranging from 0.081 to 0.152 mg/ml. The molecular docking study was also performed to predict the preferred binding site between pancreatic lipase and the four anti-lipase compounds in AL. The present study demonstrated the reliability of the spectrum-effect relationships analysis and screened out the anti-lipase compounds in AL. The study provided experimental evidence that AL could be potential herbal medicine and food supplement for treatment of hyperlipidemia or other metabolic diseases. The exact mechanisms of the anti-lipase compounds in AL will be studied in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LG and YZ conceived the research subject. YC, DZ, and GY conducted the experiments and analyzed data. YC prepared the first draft of the paper. LG critically read and revised the paper. All authors have read and agreed to the published version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.675396/full#supplementary-material

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

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Integrating Constituents Absorbed into Blood, Network Pharmacology, and Quantitative Analysis to Reveal the Active Components in *Rubus chingii* var. *suavissimus* that Regulate Lipid Metabolism Disorder

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Rubus chingii var. *suavissimus* (S. K. Lee) L. T. Lu (RS)—a sweet plant also known as Tiancha distributed in the south of China where it is used as a beverage—recently gained extensive attention as adjuvant therapy of diabetes and hypertension. Although pharmacological studies indicate that RS has beneficial effects in regulating lipid metabolism disorder characteristics, the active chemicals responsible for this effect remains unclear. The present study aims to predict the effective substances of RS on regulating lipid metabolism disorder through the analysis of the chemical profile of RS, the absorbed prototype components in rat plasma, and network pharmacology. Also, a UPLC method able to quantify the screened potential effective chemicals of RS products was established. First, a total of 69 components—including diterpene, triterpenoids, flavonoids, polyphenols, and lignans—were systematically characterized in RS. Of those, 50 compounds were detected in the plasma of rats administered with RS extract. Through network pharmacology, 9 potential effective components, 71 target genes, and 20 pathways were predicted to be involved in RS-mediated regulation of lipid metabolism disorder. The quantitative analysis suggested that the contents of potential effective components varied among samples from different marketplaces. In conclusion, the presented results provide a chemical basis for further research of *Rubus chingii* var. *suavissimus*.

Keywords: *Rubus chingii* var. *suavissimus*, UPLC-Q/TOF-MS, chemical profile, constituents absorbed into blood, network pharmacology, lipid metabolism disorders, quantification

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INTRODUCTION

Lipid metabolism disorder is an important pathogenic factor of diseases characterized by an abnormal lipid metabolism, such as atherosclerotic vascular disease, type 2 diabetes, and nonalcoholic fatty liver disease (NAFLD) (Guo et al., 2017; Zhang et al., 2018). While statins are widely used as classical chemical medicines in the treatment of lipid metabolism disorder, they have

side effects such as rhabdomyolysis and digestive problems (Jones and Davidson, 2005; Thompson et al., 2016). Recent observations indicating that some ethnomedicines and natural products have lipid-lowering effects with multitarget, multilink, and slight side effect features resulted in the development of combination therapies with western medicines for the treatment of lipid metabolism disorder (Zhang et al., 2018; Li et al., 2020).

Rubus chingii var. *suavissimus* (S. K. Lee) L. T. Lu (Flora of China, 2003) or *Rubus suavissimus* S. Lee (RS) (Li, 1981) also known as Tiancha or sweet tea is a sweet plant distributed in the south of China where it is used as a beverage and the adjuvant therapy of diabetes, the treatment of hypertension, and urinary tract infections (Guangxi Food And Drug Administration, 2011). As an ideal sugar substitute and low-calorie, low-toxic healthcare product, it is comparable with *Siraitia grosvenorii* (Swingle) C. Jeffrey ex Lu et Z. Y. Zhang and *Stevia rebaudiana* Bertoni (Liang et al., 2003) and is mainly used as a tea drink or sweetener with medicinal characteristics. For example, RS was shown to improve blood lipid regulation due to its richness of a variety of active ingredients (Ezure and Amano, 2011; Koh et al., 2011; Wang et al., 2015). Rubusoside—the sweetness compound of RS accounting for up to 5%—was shown to be able to lower serum total cholesterol (TC), triglycerides (TG) levels, and lipid peroxides (Sun et al., 2001; Tian et al., 2001). Our previous study showed that both RS (Jiang et al., 2021) and rubusoside (Li et al., 2020) could alleviate high-fat-diet–induced lipid metabolism disorder and liver injury in a golden hamster model. Besides, RS-derived polyphenols could reduce abdominal fat, triglycerides (TG), and total cholesterol (TC) in animal models of high-fat diet (Liu et al., 2014; Wang et al., 2015). Moreover, RS-derived flavonoids could regulate lipid metabolism disorder by scavenging free radicals and preventing lipid peroxidation (Liu et al., 2017). However, the systematic chemical basis of RS and the identification of the active compounds active in mediating the lipid metabolism disorder should be further evaluated.

Analysis of compounds migrating into the blood proved to be a very useful approach for the identification of bioavailable components of traditional Chinese medicine (TCM) (Zhang et al., 2019). Moreover, the combined analysis of compound characterization and blood absorbent compounds is generally accepted for mining the potential biological active constituents of TCM (Bi et al., 2018). With the development of bioinformatics, network pharmacology has become a novel approach to systematically predict and reveal the active components, molecular targets, and action mechanisms of TCM from the molecular level to the pathway level by establishing a multilevel network model of "components–targets–diseases" (Chen et al., 2016).

Therefore, this study aims to predict the active components and molecular mechanisms underlying the protective action of RS on regulating lipid metabolism disorder. First, an ultra-perfomance liquid chromatography with quadrupole time-of-flight tandem mass spectrometry (UPLC-Q/TOF-MS) method was established to characterize the chemical profile of RS as well as the *in vivo* absorbed components from RS extract-administrated rats. Based on this, a network pharmacology approach was developed to systematically predict the main active components and possible molecular action mechanisms. In addition, the main chemicals predicted to have the potential activity to regulate lipid metabolism disorder were quantified by a validated UPLC method. These results provide a reference for further research and exploration of pharmacodynamics material basis and mechanisms of RS.

MATERIALS AND METHODS

Materials and Reagents

Thirteen batches of plant materials from markets were collected (**Table 3**) and authenticated as the leaves of *Rubus chingii* var. *suavissimus* (S. K. Lee) L. T. Lu by Professor Yi Cai, Guangxi University of Chinese Medicine. The voucher specimen is deposited in the herbarium of School of Pharmacy, Guangxi University of Chinese Medicine.

Gallic acid, caffeic acid, ellagic acid, rutin, hyperoside, isoquercitin, quercitrin, quercetin, kaempferol, rubusoside, steviol, isosteviol, ursolic acid, and oleanolic acid (purity ≥ 98%) were provided by Chengdu PFID Co., Ltd. Formic acid and acetonitrile of LC-MS grade were purchased from Thermo Fisher Scientific. Other chemicals were of analytical grade.

Animals

Male Sprague Dawley (SD) rats (200 ± 20 g) were obtained from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). Animal care and procedures were approved by and conducted according to the standards of the Guangxi University of Chinese Medicine (Nanning, China) (Certificate DW20200713-003). The rats were housed in an animal room (22 ± 2°C, 60 ± 10% relative humidity) on a 12 h dark/12 h light cycle with food and water *ad libitum*. Before experiments, they were fasted for 24 h but with free access to water.

Reference Stock Solutions

Accurately weighted references of caffeic acid, rutin, hyperoside, isoquercitin, quercitrin, quercetin, kaempferol, rubusoside, steviol, isosteviol, ursolic acid, and oleanolic acid were dissolved in methanol, except for gallic acid (in water) and ellagic acid (in DMSO) to get the stock solutions of each reference compound. All reference stock solutions were stored at 4°C.

Chemical Profile of RS by UPLC-Q/TOF-MS

Preparation of Sample Solutions

0.5 g weighed sample powders (RS08) were extracted with 10 mL 60% ethanol at room temperature for 60 min by ultrasonication, then complemented for the weight loss, and centrifuged at 12,000 rpm for 10 min, after which the supernatant fluid was diluted 10 times with 60% ethanol and filtered through a 0.22 μm filter before analysis.

Preparation of Reference Working Solutions

Each reference stock solution was mixed and diluted with methanol to a suitable concentration and then filtered through a 0.22 μm filter before analysis.

Animal Experiment and Plasma Sample Preparation

Preparation of RS Extract for Intragastric Administration to Rats

200.0 g RS powder (RS08) was extracted three times with 1,600 mL 95% ethanol (60 min per time) by ultrasonication, filtered, and recycled the solvent under reduced pressure to obtain the dried extract. It was diluted by 0.5% sodium carboxymethyl cellulose (CMC-Na) to prepare the suspension for animal experiments.

Preparation of Plasma Sample

Ten male SD rats were randomly divided into control group (two) and RS group (eight). Rats in the RS group were gave at a fourfold human equivalent dosage (4 × 2.06 g/kg), and the blood was collected from the orbital veins in heparinized centrifuge tube at 0.17, 0.33, 0.5, 1, 1.5, 2, 4, 6, 8, and 12 h. Rats in the control group received an equal volume of 0.5% CMC-Na orally after which the blood was collected by the abdominal aortic method. The blood samples were centrifuged at 4,000 r/min for 10 min at 4°C to obtain the plasma which was stored at −80°C.

50 μL plasma samples of the above time points were combined (or 500 μL control plasma samples) and loaded onto a pre-equilibrated solid-phase extraction (SPE) column and successively eluted by 3 ml of water and 3 ml of methanol. The methanol eluent was collected and evaporated to dryness under nitrogen at 40°C. The residue was reconstituted in 100 μL methanol and centrifuged at 12,000 rpm for 10 min to obtain the supernatant fluid for analysis.

UPLC-Q/TOF-MS Analysis Conditions

The UPLC-Q/TOF-MS analysis was performed on a Dionex UltiMate3000 coupled with a hybrid quadrupole orthogonal time-of-flight (Q-TOF) tandem mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was carried out on an InertSustain ODS-3 LC column (2 μm, 2.1 × 150mm, GL Sciences Inc.). The mobile phase consisted of 0.1% formic solution (A) and acetonitrile (B) using the following gradient program: 0–1 min, 5% B; 1–6 min, 5–15% B; 6–20 min, 15–24% B; 20–25 min, 24–33% B; 25–30 min, 33–54% B; 30–35 min, 54–80% B; 35–38 min, 80% B; 38–40 min, 80–95% B; 40–43 min, 95% B; 43–45 min, 95–5% B; and 45–50 min, 5% B with a sample injection volume of 5 μL. The solvent flow rate was 0.5 ml/min and the column temperature was set at 40°C.

The ESI source was operated at an optimized capillary voltage of 3.5 V (ESI^-), with a drying gas flow and temperature of 8 L/min at 200°C, a nebulizer pressure of 2.0 bar (=29 psi), a collision RF of 500 Vpp, a transfer time of 60.0 μs, and a prepulse storage of 5 μs. Automatic MS/MS experiments were performed adjusting the collision energy values as follows: *m/z* 50, 10 eV; *m/z* 500, 25 eV; and *m/z* 1,000, 40 eV. Argon was used as collision gas for CID in the MS^E mode. The ion scan ranged from *m/z* 50 to 1,000. Sodium formate solution was used as a calibration solution. The MS data were processed through DataAnalysis 4.3 software (Bruker Daltonics, Bremen, Germany).

Network Pharmacology-Based Analysis

Bioactive Components of RS and Potential Targets

According to the results of the chemical profiling and components absorbed in plasma of RS, the absorbed prototypes in rat plasma were selected as candidate compounds. The molecular targets of RS were identified by PharmMapper (http://www.lilab-ecust.cn/pharmmapper/), STITCH (http://stitch.embl.de/) and SwissTargetPrediction (http://www.swisstargetprediction.ch/). Beyond this, some known molecular targets of identified compounds were collected for comprehensive analysis through literature mining. All targets were combined and the duplicates were removed.

Screening of Putative Therapeutic Targets

The related targets of lipid metabolism disorder were collected from GeneCards (https://www.genecards.org/). Next, the overlapping genes between RS and lipid metabolism disorder were matched by the Venn diagram.

Network Construction of Interactions Between RS and Lipid Metabolism Disorder

To gain insight into the molecular mechanisms underlying the action of RS on lipid metabolism disorder, a bioactive compound–therapeutic target network was constructed with Cytoscape 3.7.1 to depict the interactions between target genes and bioactive compounds. Next, the degree value was calculated by using the NetworkAnalyzer plug-in. Compounds with a high degree value were deliberated as main bioactive RS-derived compounds potentially involved in the RS-mediated alleviation of lipid metabolism disorder. The protein–protein interaction (PPI) network was supported by STRING (https://string-db.org/). For the analysis, the study organism was limited to *Homo sapiens*, while all other settings were kept as default values.

Enrichment of KEGG Pathways

To decipher the possible signaling pathway involved in the intervention of RS on lipid metabolism disorder, the KEGG pathway enrichment analysis was performed with DAVID 6.8 (https://david.ncifcrf.gov/); terms with $p < 0.05$ were considered significant and captured.

Quantitative Analysis of RS by UPLC

Preparation of Sample Solutions

0.5 g weighed sample powders were extracted with 10 ml 60% ethanol at room temperature for 60 min by ultrasonication, then complemented for the weight loss, and filtered through a 0.22 μm filter before analysis.

Preparation of Reference Working Solution

Gallic acid, caffeic acid, rutin, ellagic acid, rubusoside, and kaempferol stock solutions were diluted into suitable working solutions with corresponding solvents before analysis.

UPLC Conditions

UPLC analysis was performed on an Agilent 1290 I system equipped with a 1290 VWD detector, a 1,290 vial sampler, and a 1290 high-speed pump. The column configuration was an InertSustain C_{18} LC column (2 μm, 3.0 × 150 mm, GL Sciences Inc., Japan). The mobile phase consisted of 0.1% phosphoric acid

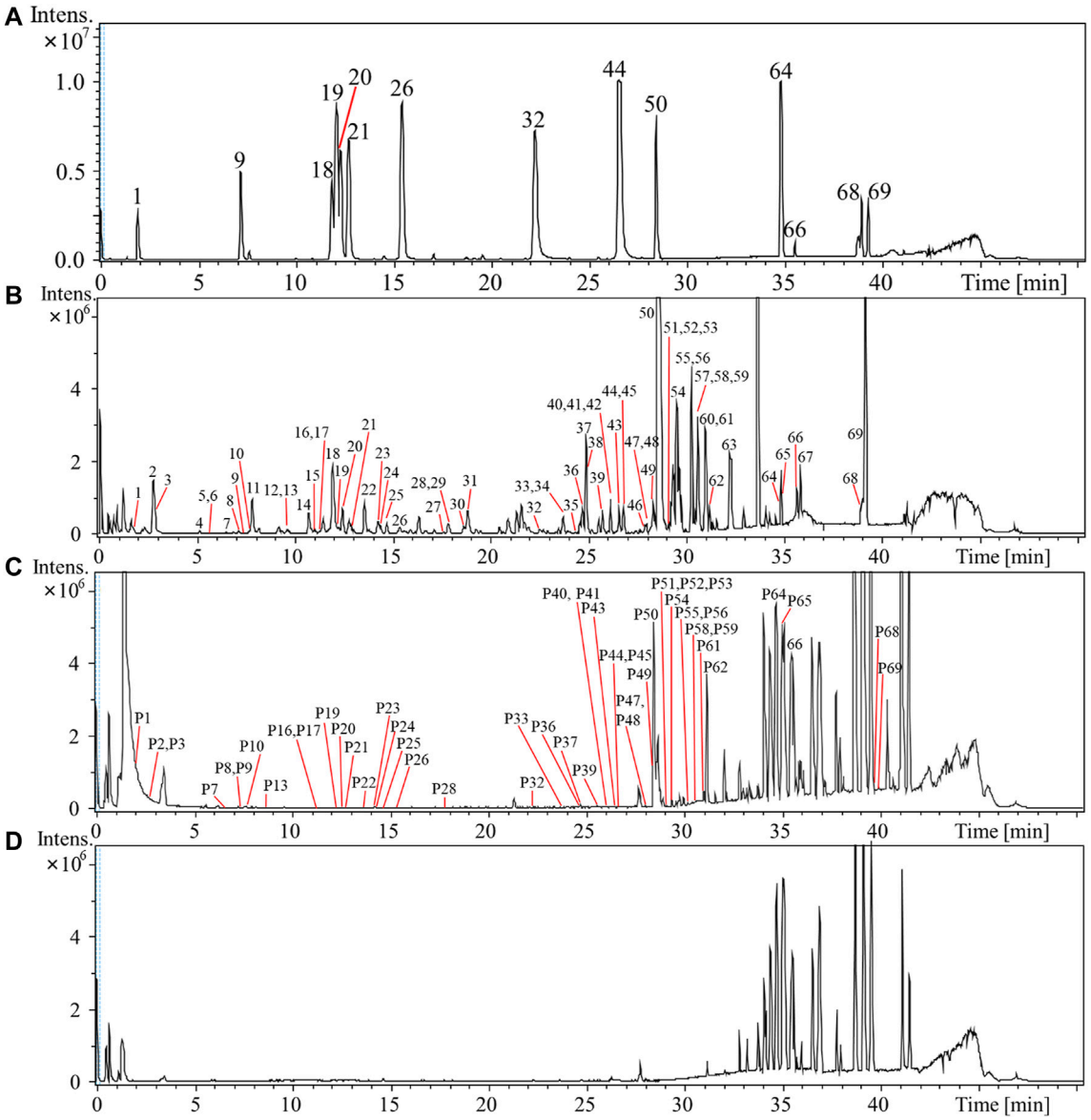

FIGURE 1 | Base peak intensity (BPI) chromatogram of mixed reference compounds **(A)** and 60% ethanol extract sample of *Rubus chingii* var. *suavissimus* (RS08) **(B)**, rat plasma sample **(C)**, and blank rat plasma **(D)** of rats in negative ion mode by UPLC-Q/TOF-MS.

(A) and acetonitrile (B) using a gradient program of 0–6 min, 5–15% B; 6–20 min, 15–24% B; 20–37 min, 24–80% B; 37–38 min, 80–95% B; 38–38.5 min, 95–5% B; and 38.5–40 min, 5% B. The solvent flow rate was 0.35 ml/min and the column temperature was set at 40°C. The detection wavelengths were the following: 205 nm for 0–6 min; 254 nm for 6–23 min; and 205 nm for 23–40 min. An aliquot of 1.0 μL was injected for analysis.

Method Validation

Stock reference solutions of gallic acid, caffeic acid, rutin, ellagic acid, rubusoside, and kaempferol were prepared and diluted to six appropriate concentrations using the corresponding solvent to get the reference working solutions. Six calibration curves were constructed by plotting the content (Y) vs. the peak areas (X) of each analyte. The limit of quantitation (LOD) and limit of detection (LOQ) were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The intraday precision was examined by six repetitive injections within one day, and the interday precision was carried out for three consecutive days. Repeatability was acquired by analyzing six replicates of the same sample solution (RS08). To determine the stability, the same sample solution (RS08) was injected at 0, 1, 2, 4, 6, 8, 12, and 24 h,

TABLE 1 | Characterization of chemical components of *Rubus chingii* var. *suavissimus* by UPLC-Q/TOF-MS.

No.	t_R (min)	Formula	PI pred. (Da)	PI meas. (Da)	Error (ppm)	Major fragment ions	Identification	ClogP	Chemical type
1[a]	**1.9**	**$C_7H_6O_5$**	**169.0142**	**169.0143**	**−0.3**	**125.0260**	**Gallic acid**	–	**P**
2	**2.7**	**$C_8H_8O_5$**	**183.0299**	**183.0298**	**0.3**	**169.0139, 125.0244**	**Methyl gallate**	–	**P**
3	**2.8**	**$C_7H_4O_6$**	**182.9935**	**182.9941**	**−3.4**	**139.0035, 95.0142**	**2-Pyrone-4,6-dicarboxylic acid**	–	**P**
4	5.2	$C_{27}H_{22}O_{18}$	633.0733	633.0733	0.0	481.0601, 300.9988, 257.0076, 238.9998	Corilagin	0.41	P
5	5.7	$C_{34}H_{24}O_{22}$	783.0686	783.0679	0.9	481.0596, 300.9992, 275.0200	Pedunculagin	–	P
6	5.7	$C_{27}H_{22}O_{18}$	633.0733	633.0735	−0.3	300.9983, 257.0086, 229.0129, 169.0137	1-α-Galloyl-2,3-(*S*)-hexahydroxydiphenoyl-*D*-glucose	0.47	P
7	**6.5**	**$C_{15}H_{18}O_9$**	**341.0878**	**341.0878**	**0.0**	**179.0354, 161.0435, 161.0254, 135.0466**	**Caffeic acid-*O*-hexoside**	–	**P**
8	**7.1**	**$C_{27}H_{30}O_{17}$**	**625.1410**	**625.1415**	**−0.8**	**463.0863, 301.0342, 283.0229, 255.0294**	**Quercetin-3,4'-*O*-*D*-β-glucopyranoside**	–	**F**
9[a]	**7.2**	**$C_9H_8O_4$**	**179.0350**	**179.0351**	**−0.9**	**135.0456**	**Caffeic acid**	–	**P**
10[b]	**7.6**	**$C_{21}H_{20}O_9$**	**415.1035**	**415.1038**	**−0.9**	**161.0434, 151.0401, 123.0431**	**Toringin**	–	**F**
11	**7.8**	**$C_{13}H_8O_8$**	**291.0146**	**291.0149**	**−1.0**	**247.0258, 203.0360, 175.0403, 147.0461**	**Brevifolincarboxylic acid**	–	**P**
12	8.6	$C_{21}H_{10}O_{13}$	469.0049	469.0048	0.1	316.9907, 300.9985	Sanguisorbic acid dilactone	–	P
13	**8.6**	**$C_{16}H_{20}O_9$**	**355.1035**	**355.1034**	**0.2**	**193.0511, 161.0457, 161.0239, 133.0296**	**Ferulic acid-*O*-hexoside**	–	**P**
14	10.7	$C_{26}H_{28}O_{16}$	595.1305	595.1310	−0.9	301.0334, 271.0251, 151.0036	Quercetin-3-*O*-xylosyl glucoside	–	F
15[b]	10.8	$C_{27}H_{30}O_{16}$	609.1461	609.1463	−0.3	447.0909, 285.0413	Cyanidin-3-*O*-sophoroside	−1.33	P
16[b]	**11.2**	**$C_{27}H_{30}O_{16}$**	**609.1461**	**609.1466**	**−1.6**	**447.0907, 285.0401**	**Cyanidin-3,5-diglucoside**	**−1.21**	**P**
17[b]	**11.2**	**$C_{16}H_{14}O_4$**	**269.0819**	**269.0816**	**1.2**	**145.0316, 105.0537**	**5-Hydroxy-7-methoxydihydroflavone**	–	**F**
18[a]	**11.9**	**$C_{14}H_6O_8$**	**300.9990**	**300.9995**	**−1.5**	**282.9963, 257.0091, 239.0008**	**Ellagic acid**	–	**P**
19[a]	**12.2**	**$C_{27}H_{30}O_{16}$**	**609.1461**	**609.1469**	**1.4**	**301.0337, 283.0230, 271.0254, 255.0293, 151.0040**	**Rutin**	–	**F**
20[a]	**12.4**	**$C_{21}H_{20}O_{12}$**	**463.0882**	**463.0893**	**−2.4**	**301.0339, 283.0244, 271.0251, 255.0305**	**Hyperoside**	–	**F**
21[a]	**12.7**	**$C_{21}H_{20}O_{12}$**	**463.0883**	**463.0877**	**0.1**	**301.0331, 283.0340, 271.0266, 255.0291**	**Isoquercitrin**	–	**F**
22[b]	**13.6**	**$C_{27}H_{30}O_{15}$**	**593.1512**	**593.1514**	**−0.4**	**447.0905, 285.0399, 255.0306, 151.0041**	**Cyanidin 3-*O*-rutinoside**	**−0.83**	**P**
23	**14.2**	**$C_{20}H_{18}O_{11}$**	**433.0776**	**433.0776**	**0.0**	**301.0337, 271.0253, 255.0300, 151.0042**	**Guaijaverin**	–	**F**
24	**14.3**	**$C_{21}H_{20}O_{11}$**	**447.0933**	**447.0937**	**−0.9**	**285.0406, 255.0303, 151.0048**	**Kaempferol-*O*-hexoside**	**0.26**	**F**
25	**14.6**	**$C_{27}H_{30}O_{15}$**	**593.1512**	**593.1513**	**−0.2**	**285.0407, 255.0304, 151.0056**	**Kaempferol-3-*O*-rutinoside**	**−0.76**	**F**
26[a]	**15.3**	**$C_{21}H_{20}O_{11}$**	**447.0933**	**447.0936**	**−0.7**	**283.0241, 271.0256, 255.0297, 151.0049**	**Quercitrin**	–	**F**
27	17.5	$C_{20}H_{18}O_{11}$	433.0776	433.0784	−1.7	301.0350, 283.0259, 255.0293	Quercetin-3-*O*-α-*D*-ribofuranoside	–	F
28	**17.8**	**$C_{21}H_{20}O_{11}$**	**447.0933**	**447.0931**	**0.4**	**285.0413, 255.0293, 151.0044**	**Luteoloside**	**0.81**	**F**
29	17.8	$C_{30}H_{26}O_{14}$	609.1250	609.1254	−0.7	447.0930, 285.0413, 161.0255	Kaempferol-*O*-caffeoyl-hexoside	–	F
30	18.5	$C_{30}H_{26}O_{15}$	625.1199	625.1201	−0.3	463.0882, 301.0348, 271.0246, 255.0312	Quercetin-3-*O*-sophoroside	−1.86	F
31	18.8	$C_{30}H_{26}O_{15}$	625.1199	625.1299	0.0	463.0888, 301.0349, 255.0291, 161.0258	Quercetin-*O*-caffeoyl-hexoside	0.89	F
32[a]	**22.3**	**$C_{15}H_{10}O_7$**	**301.0354**	**301.0353**	**0.4**	**283.0252, 271.0252, 255.0290, 151.0042**	**Quercetin**	–	**F**
33	**23.6**	**$C_{26}H_{42}O_{10}$**	**513.2705**	**513.2705**	**0.1**	**351.2166, 333.2069,161.0460**	**Glaucocalyxin G**	**0.74**	**D**
34	23.6	$C_{31}H_{38}O_{11}$	585.2341	585.2344	−0.5	497.1587, 493.1896, 221.0471	Erythro-7,8-dihydro-buddlenol B	1.13	L
35	24.2	$C_{30}H_{26}O_{13}$	593.1301	593.1296	0.8	447.0892, 285.0398, 255.0304, 161.0255	Kaempferol-*O*-coumaroyl-hexoside	–	F
36	**24.6**	**$C_{20}H_{30}O_4$**	**333.2071**	**333.2074**	**−0.9**	**315.1978, 289.2153, 271.2063**	**7β-Hydroxysteviol**	**2.11**	**D**
37	**24.7**	**$C_{26}H_{42}O_{10}$**	**513.2705**	**513.2701**	**0.9**	**351.2183, 333.2087, 271.2073, 161.0454**	**Cussovantoside A**	**0.98**	**D**
38	24.8	$C_{31}H_{38}O_{11}$	585.2341	585.2341	0.1	497.1587,493.1924,221.0469	Threo-7,8-dihydro-buddlenol B	1.13	L
39	**25.6**	**$C_{26}H_{42}O_8$**	**481.2807**	**481.2807**	**0.1**	**319.2278, 301.2155, 161.0461**	**Sugereoside**	**1.98**	**D**
40	**26.0**	**$C_{26}H_{40}O_9$**	**495.2600**	**495.2603**	**−0.6**	**333.2063,285.1886**	**7β,17-Dihydroxy-ent-kaur-15-en-19-oic acid 19-*O*-β-*D* -glucopyranoside ester**	–	**D**
41	**26.0**	**$C_{26}H_{42}O_9$**	**497.2756**	**497.2761**	**−0.9**	**335.2231, 317.2139**	**7β,17-Dihydroxy-16β-ent-kauran-19-oic acid 19-*O*-β- *D*-glucopyranoside ester**	**1.95**	**D**

(Continued on following page)

TABLE 1 | (*Continued*) Characterization of chemical components of *Rubus chingii* var. *suavissimus* by UPLC-Q/TOF-MS.

No.	t_R (min)	Formula	PI pred. (Da)	PI meas. (Da)	Error (ppm)	Major fragment ions	Identification	ClogP	Chemical type
42	26.0	$C_{32}H_{52}O_{14}$	659.3284	659.3280	0.6	497.2774, 335.2231	*β-D*-Glucopyranosyl 17-hydroxy-ent-kauran-19-oate-16-*O*-*β*-*D*-glucopyranoside	–	D
43	**26.4**	**$C_{20}H_{32}O_5$**	**351.2177**	**351.2182**	**–1.6**	**333.2055, 303.1952**	**ent-3*α*,16*β*,17-Trihydroxy-kauran-19-oic acid**	–	**D**
44[a]	**26.6**	**$C_{15}H_{10}O_6$**	**285.0405**	**285.0406**	**–0.6**	**285.0409, 255.0306, 151.0029**	**Kaempferol**	–	**F**
45	**26.6**	**$C_{26}H_{44}O_8$**	**483.2963**	**483.2958**	**1.2**	**321.2433, 285.2233, 161.0463**	**Suavisoside-A**	–	**D**
46	27.8	$C_{30}H_{48}O_7$	519.3327	519.3328	–0.2	501.3219, 459.3135, 453.3014	2*β*,3*β*,19*α*,23,24-Pentahydroxy-urs-12-en-28-oic acid	3.84	T
47	**28.0**	**$C_{20}H_{30}O_4$**	**333.2071**	**333.2070**	**0.3**	**315.1976, 289.2179, 271.2068**	**ent-13,17-Dihhydroxy-kauran-15-en-19-oic acid**	**2.87**	**D**
48	**28.0**	**$C_{38}H_{60}O_{18}$**	**803.3707**	**803.3704**	**0.3**	**641.3183, 479.2645, 317.2160**	**13-[(*O*-*β*-*D*-Glucopyranosyl)oxy]ent-kaur-16-en-19-oic acid-2-*O*-*β*-*D*-glucopyranosyl-*β*-*D*-glucopyranosyl ester**	–	**D**
49	**28.2**	**$C_{26}H_{42}O_8$**	**481.2807**	**481.2804**	**–0.7**	**319.2235, 301.2261, 161.0448**	**17-*O*-*β*-*D*-Glucopyranosyl-16*α*-ent-kauran-19-oic acid**	**4.40**	**D**
50[a]	**28.4**	**$C_{32}H_{50}O_{13}$**	**641.3179**	**641.3184**	**–0.9**	**479.2674, 317.2130, 273.2242, 255.2304, 161.0452**	**Rubusoside**	–	**D**
51	**29.1**	**$C_{20}H_{32}O_4$**	**335.2228**	**335.2231**	**–1.1**	**335.2232, 291.2298, 243.2121**	**16*α*,17-Dihydroxy-kauran-19-oic acid**	–	**D**
52	**29.1**	**$C_{30}H_{46}O_7$**	**517.3171**	**517.3180**	**–1.8**	**499.3071, 481.2952, 473.3265, 469.2952**	**Ganoderic acid C2**	–	**T**
53	**29.1**	**$C_{30}H_{48}O_7$**	**519.3327**	**519.3333**	**–1.2**	**501.3205, 471.3101, 459.3106, 441.3022**	**Platycodigenin**	**4.00**	**T**
54	**29.4**	**$C_{30}H_{48}O_6$**	**503.3378**	**503.3388**	**–2.0**	**485.3283, 473.3269, 455.3131, 437.3027, 419.2949**	**Terminolic acid**	–	**T**
55	**30.1**	**$C_{30}H_{48}O_6$**	**503.3378**	**503.3377**	**0.3**	**485.3278, 473.3264, 455.3182, 419.2935**	**2*α*,3*β*,19*α*,23-Tetrahydroxy-urs-12-en-28-oic acid**	**4.51**	**T**
56	**30.1**	**$C_{20}H_{32}O_4$**	**335.2228**	**335.2231**	**–1.0**	**317.2020, 291.2333, 243.2108**	**ent-16*β*,17-Dihdyroxy-kauran-19-oic acid**	–	**D**
57[b]	30.4	$C_{36}H_{56}O_{11}$	633.3750	633.3743	1.1	501.3222, 457.3286, 429.3257	Rubuside J	–	T
58	**30.4**	**$C_{26}H_{42}O_9$**	**497.2756**	**497.2755**	**0.2**	**335.2207, 317.2120, 287.2046**	**Paniculoside** IV	**2.02**	**D**
59	**30.4**	**$C_{26}H_{40}O_8$**	**479.2650**	**479.2654**	**–0.8**	**317.2120, 273.2214, 255.2104**	**ent-Kauran-16-en-19-oic-13-*O*-*β*-*D*-glucoside**	**3.23**	**T**
60	30.7	$C_{30}H_{48}O_6$	503.3378	503.3370	1.7	485.3254, 473.3212, 455.3153	2*α*,3*β*,19*α*,23-Tetrahydroxy-olean-12-en-28-oic acid	4.51	T
61	**30.8**	**$C_{30}H_{46}O_6$**	**503.3222**	**501.3225**	**–0.7**	**483.3121, 467.3145**	**Ilexgenin A**	–	**T**
62	**31.0**	**$C_{26}H_{40}O_8$**	**479.2650**	**479.2640**	**2.2**	**317.2124, 273.2215, 255.2104**	**Cussoracosides E**	**3.45**	**D**
63[b]	32.0	$C_{30}H_{48}O_5$	487.3429	487.3432	–0.6	441.3345, 423.3220	Euscaphic acid	–	T
64[a]	**34.7**	**$C_{20}H_{30}O_3$**	**317.2122**	**317.2116**	**2.0**	**273.2226, 255.2178**	**Steviol**	–	**D**
65	**34.8**	**$C_{20}H_{32}O_3$**	**319.2279**	**319.2270**	**2.7**	**289.2195, 273.2236**	**ent-16*β*, 17-Dihydroxy-kauran-3-one**	–	**D**
66[a]	**35.3**	**$C_{20}H_{30}O_3$**	**317.2122**	**317.2122**	**0.0**	**273.2214, 255.2131**	**Isosteviol**	–	**D**
67	35.5	$C_{30}H_{48}O_4$	471.3480	471.3488	–1.8	471.3476, 453.3369, 435.3142	2*α*,3*β*-Dihdyroxy-urs-12-en-oic acid	–	T
68[a]	**38.7**	**$C_{30}H_{48}O_3$**	**455.3531**	**455.3545**	**–3.1**	**411.3476, 393.3508**	**Ursolic acid**	–	**T**
69[a]	**39.0**	**$C_{30}H_{48}O_3$**	**455.3531**	**455.3534**	**–0.7**	**411.3599, 393.3541**	**Oleanolic acid**	–	**T**

Note: the component absorbed into blood circulation is highlighted in bold.

[a]*The compounds were identified by comparing with reference substances.*

[b]*The compounds were first reported in* Rubus chingii *var.* suavissimus*. F: flavonoids; P: polyphenols; D: diterpenoids; T: triterpenoids; L: lignans.*

respectively. The accuracy of the method was expressed by the recovery. Approximately 100% amount of each reference standard equivalent to those in 0.25 g extracts were added to the same RS sample (RS08), after which the sample solutions were prepared in six replicates. Recoveries were calculated by the following equation: recovery (%) = 100 × (detected amount−original amount)/spiked amount.

Statistical Analysis

All data were expressed as the mean ± standard deviation (SD). T-test was used to compare the two groups. The multigroup comparisons were analyzed by one-way analysis of variance (ANOVA) with SPSS 24.0 (SPSS Inc., Chicago, IL, United States).

RESULTS AND DISCUSSION

Characterization of Chemical Profile of RS by UPLC-Q/TOF-MS

A UPLC-QTOF/MS method was established to characterize the chemical profile. As MS and fragmentations were most informative in the negative ion mode, all analyses were conducted in the negative ion mode. The base

peak intensity (BPI) chromatogram of RS in the negative ion mode is shown in **Figure 1**. The identification data are summarized in **Table 1**, and the temporarily identified structures are shown in **Supplementary Figure S1**. The obtained retention time, quasimolecular ions, and the MS^n fragments data were compared with reference compounds, a self-built chemical database, and reported literature. In addition, a ClogP value was calculated by ChemDraw 11.0 (CambridgeSoft, United States) to estimate the retention time of the isomers.

In total, 69 compounds were identified or tentatively characterized, including 20 diterpenes, 13 triterpenes, 19 flavonoids, 15 polyphenols, and 2 lignans. Compounds 1, 9, 18, 19, 20, 21, 26, 32, 44, 50, 64, 66, 68, and 69 were identified as gallic acid, caffeic acid, ellagic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, kaempferol, rubusoside, steviol, isosteviol, ursolic acid, and oleanolic acid through the comparison of the retention time and quasimolecular ions with reference compounds, respectively. Compounds 4 (corilagin), 19 (rutin), and 50 (rubusoside) were taken as examples for explaining the fragmentation process (**Supplementary Figure S2**).

Kaurane-type diterpenoids, oleanane-type triterpenoids, ursane-type triterpenoids, flavone, dihydroflavone, anthocyanidins, and flavonol—all widely described in RS—are sometimes present in the form of glycoside with one or several sugar moieties (Hirono et al., 1990; Lv and Wang, 2007; Ohtani et al., 1992; Peng et al., 2019; Wang and Lv, 2008). Upon analysis of the MS/MS spectra of kaurane-type diterpenoids and oleanane, or ursane-type triterpenoids, it was observed that a series of diagnostic ions was caused by the loss of $-H_2O$, $-OCH_2$, $-CO_2$, and $-C_6H_9O_5$ (glucose unit). The main characteristic fragmentation behaviors of RS-flavonoids were the loss of glycosyl, elimination of small neutral fragments such as H_2O and CO, and retro Diels–Alder (RDA) cleavage. RS-derived polyphenols are mainly galloyl-oxygen-diphenyl-type ellagitannins and gallotannins of which the fragmentation patterns could mainly be summarized as successive elimination of the galloyl group and lactonization (Nakano et al., 2017; Schulza and Chim, 2019).

Noteworthy, based on the reported chemicals of *Rubus L.* species or other plants (Zuo et al., 2008; Li et al., 2009; Li et al., 2010; Zhang et al., 2018; Schulza and Chim, 2019), the current study is the first to report the presence of toringin (10), cyanidin-3-*O*-sophoroside (15), cyanidin-3,5-diglucoside (16), 5-hydroxy-7-methoxydihydroflavone (17), cyanidin 3-O-rutinoside (22), rubuside J (57), and euscaphic acid (63) in *Rubus chingii* var. *suavissimus*.

TABLE 2 | Top 71 directly relevant targets of *Rubus chingii* var. *suavissimus* in lipid metabolism disorder based on GeneCards.

No.	Target	Score	No.	Target	Score
1	LPL	114.478	37	XDH	54.72941
2	PPARγ	112.1502	38	APOA2	54.1526
3	TNF	99.24533	39	OTC	54.0331
4	COMT	96.67764	40	GSTP1	53.983
5	CYP2D6	96.11501	41	CYP19A1	52.922
6	ALB	93.87442	42	ALDH2	52.37643
7	PPARα	88.45335	43	AR	52.34957
8	ACE	83.09168	44	CFTR	51.83479
9	CYP3A4	80.83278	45	TLR4	51.41657
10	SLC6A3	72.99746	46	MPO	50.38863
11	MAOA	71.15596	47	G6PD	49.77814
12	HMGCR	71.08429	48	PTGS2	49.11167
13	APP	70.99548	49	CYP1B1	49.00745
14	SERPINE1	69.47766	50	MTOR	48.67168
15	ESR1	68.98495	51	GSR	47.65023
16	SNCA	66.55721	52	FABP2	46.94385
17	TP53	66.24002	53	VEGFA	45.86672
18	INSR	66.18439	54	NR3C2	45.62914
19	DRD4	66.08241	55	CYP17A1	45.42647
20	CYP2C9	64.10754	56	HSD11B1	44.66399
21	CYP1A2	63.93973	57	NR1H3	44.37109
22	DRD2	63.63854	58	PIK3CA	44.30278
23	F2	62.81121	59	SOD2	44.21383
24	SREBP1	62.42875	60	CES1	43.65325
25	AKT1	61.84262	61	MAPK1	43.44899
26	MAPT	61.83129	62	TTR	43.18982
27	NR1H2	61.45977	63	ACC1	43.12664
28	JAK2	61.03323	64	PAH	43.05243
29	CNR1	58.8269	65	ALOX5	42.59163
30	GBA	58.24603	66	FAS	42.50793
31	NOS3	58.20886	67	FTO	42.29441
32	ABCB1	57.0409	68	SCD	42.01818
33	VDR	56.72137	69	PTPN11	41.92701
34	NR3C1	55.69765	70	SELE	41.70531
35	NR1H4	55.29206	71	aP2	41.33099
36	PPARD	54.7986	–	–	–

Analysis of *in Vivo* Absorbed Components in Plasma of RS Extract-Administered Rats

Optimization of the Methods to Collect and Pretreat Plasma

Considering that the time to being absorbed into the blood is different for each RS extract-derived component, plasma samples were first collected at 10 different time points (0.17 ~ 12 h) after the administration of RS extract and then finally combined. An SPE column was used for protein precipitation and separation.

Identification of Absorbed Components in Rat Plasma Samples after Oral Administration of RS Extract

Based on reference compounds and the chemical profile of RS and by comparing pre- and postdose rat plasma, an *in vivo* absorbed prototype component profile of the plasma of RS extract-administrated rats was obtained and analyzed.

As shown in **Figure 1** and **Table 1**, a total of 50 compounds—including 19 diterpenoids, 8 triterpenoids, 13 flavonoids, and 10 polyphenols—were identified or tentatively identified in rat plasma samples after the oral administration of RS extract. The detailed identified information, including observed mass values of quasimolecular ions, mass error, and MS^n fragments data, is listed in **Supplementary Table S1**. For example, based on the observation that P50 exhibited a parent $[M-H]^-$ ion at *m/z* 641 which yielded characteristic fragment ions at *m/z* 479 $[M-H-glucose]^-$, 317 $[M-H-2glucose]^-$, and 273 $[M-H-2glucose-CO_2]^-$, it could be identified as rubusoside through comparison of the MS behavior to the reference.

Most diterpenoids and triterpenoids existed as unchanged prototypes in rat plasma and displayed high peak intensity in the BPI chromatogram. Compared to flavonoid aglycones, more flavone glycosides were detected. The fact that RS-polyphenols displayed low peak intensity or were even hard to detect might be due to their bioavailability and the liver first-pass effect (D'Archivio et al., 2010; Zhang et al., 2016).

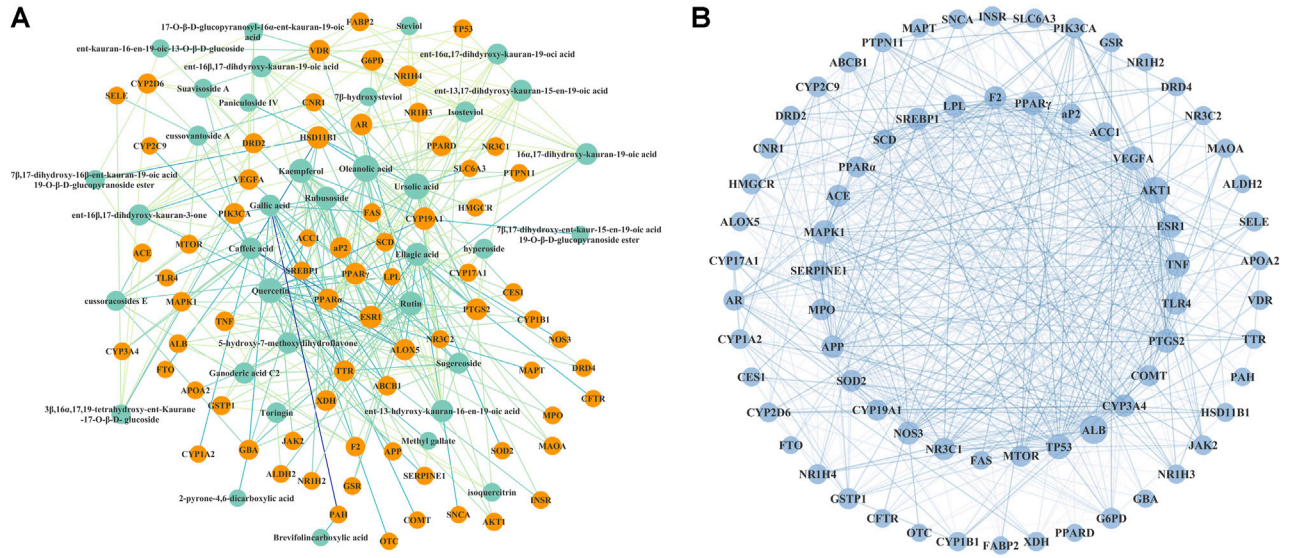

FIGURE 2 | Interaction network of *Rubus chingii* var. *suavissimus* on lipid metabolism disorder. **(A)** Bioactive compound–therapeutic target network. 36 green nodes represent bioactive compounds in RS, and 71 orange nodes represent the lipid metabolism disorder-related targets of RS. **(B)** Protein–protein interaction network of the picked 71 target genes.

Network Pharmacology-Mediated Prediction of Effective Component and Molecular Mechanisms Underlying the Beneficial Effect of RS on Lipid Metabolism Disorder

Candidate Compounds and Target Genes in RS

First, 50 candidate compounds were selected based on the *in vivo* absorbed components profile of RS. Then, a total of 1,882 molecular targets were obtained from PharmMapper, STITCH, and SwissTargetPrediction. Next, the name of the identified targets was converted into an official gene symbol by UniProt (https://www.uniprot.org/). Finally, a target library with 521 target genes was established after integrating the results and removing the duplicates.

Acquisition of Therapeutic Targets of Lipid Metabolism Disorder

1,112 targets (relevance score>22.01) related to lipid metabolism disorder were gathered from GeneCards. Through Venn diagram matching, 213 overlapping target genes between RS and lipid metabolism disorder-related targets were identified. Of note, a target with a higher relevance score as calculated by GeneCards indicated a higher pertinence to the disease. As such, the top 71 targets (**Table 2**) selected by GeneCards were defined as hub genes and retained for subsequent research. Finally, 36 compounds interacting with those previously defined hub genes were identified.

Interaction Network Construction

As shown in **Figure 2A**, a network linking 36 bioactive compounds and 71 target genes was visualized by Cytoscape 3.7.1. The green nodes represent bioactive compounds and the orange nodes represent putative therapeutic targets. Besides, a protein–protein interaction network (**Figure 2B**) illustrates the interconnection of the 71 hub targets.

This network suggested the involvement of the 36 compounds and 71 genes in the beneficial effect of RS on lipid metabolism disorder. In network analysis, a high degree value generally indicates the node is closely connected with other target genes. Based on the calculated results from NetworkAnalyzer, compounds with a degree value ≥ 11 were considered as potential bioactive ingredients. As such, nine compounds including ursolic acid (21.0), oleanolic acid (21.0), quercetin (18.0), kaempferol (16.0), gallic acid (14.0), ellagic acid (14.0), caffeic acid (11.0), rubusoside (14.0), and rutin (16.0) might be the main effective RS-derived compounds involved in the beneficial effect of RS on lipid metabolism disorder.

KEGG Pathway Enrichment Analysis

The top 20 KEGG pathways ($p < 0.01$) are presented in **Figure 3** and **Supplementary Table S2** and include insulin resistance ($p = 7.02\ E^{-08}$, count = 11), the PPAR signaling pathway ($p = 2.18\ E^{-07}$, count = 9), the AMPK signaling pathway ($p = 2.49\ E^{-06}$, count = 10), and the HIF-1 signaling pathway ($p = 3.55\ E^{-06}$, count = 7).

Comprehensive Analysis of the Compounds, Target Genes, and Signaling Pathways Involved in the Beneficial Effect of RS on Lipid Metabolism Disorder

The network pharmacology analysis suggested that nine potential effective components (ursolic acid, oleanolic acid, quercetin, kaempferol, gallic acid, ellagic acid, caffeic acid, rutin, and rubusoside), 71 target genes, and 20 pathways are directly related to lipid metabolism disorder.

Previously, the potential of the above compounds on the regulation of lipid metabolism disorder and antiobesity have

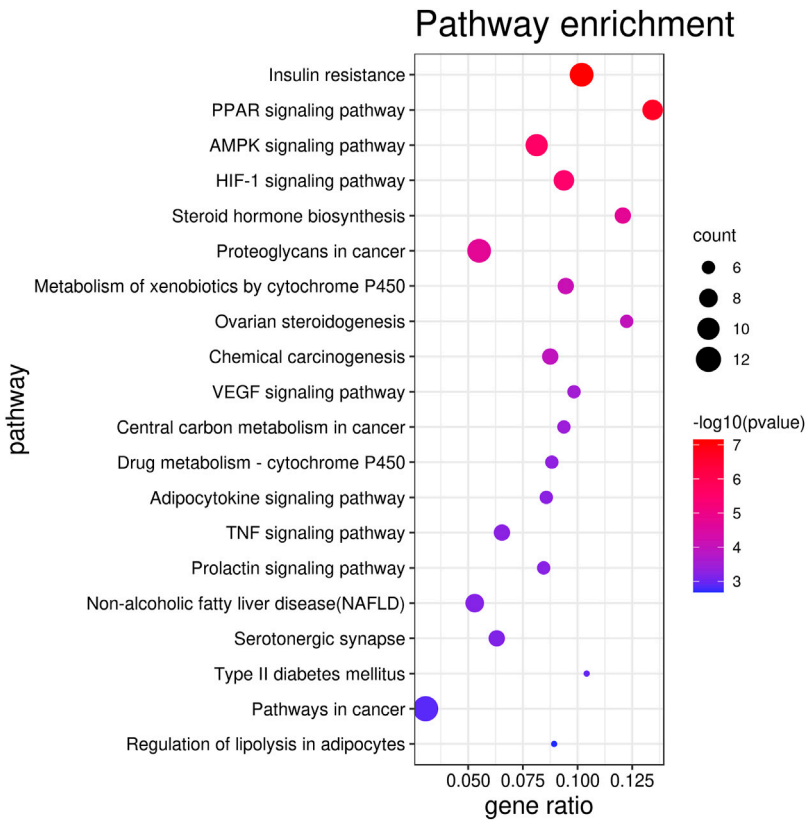

FIGURE 3 | Top 20 KEGG pathways selected based on the p value.

been shown. For example, ursolic acid and oleanolic acid have regulatory effects on the absorption, synthesis, and metabolism of triglycerides and cholesterol (Zhang and Shen, 2015). Also, intake of gallic acid can be beneficial for the suppression of HFD-induced dyslipidaemia, hepatosteatosis, and oxidative stress in rats (Hsu and Yen, 2007). Of note, gallic acid was previously shown to be partially responsible for the antiangiogenic (Liu et al., 2006) and antiobesity (Koh et al., 2011) activities of *Rubus* leaf. Also, caffeic acid ameliorates hepatic steatosis and reduces endoplasmic reticulum stress in obese mice by regulating autophagy (Kim et al., 2018). Rutin, quercetin, and kaempferol can alleviate high-fat diet–induced obesity and fatty liver insulin resistance (Gao et al., 2013; Bai et al., 2014; Wang et al., 2020). Our previous study indicated that rubusoside effectively alleviated liver injury of golden hamsters on a high-fat diet by reversing the metabolic pathway disorder such as amino acid metabolism and synthesis of ketone bodies (Li et al., 2020).

The interaction network indicated that RS might mediate lipid metabolism disorder through a multicomponents and multitargets approach, suggesting a synergistic effect between these compounds; however, this should be further analyzed in future studies.

The pathway enrichment analysis noted that RS might regulate multiple pathways involved in lipid metabolism disorder, such as insulin resistance and PPAR and AMPK signaling pathways. When lipid metabolism disorder occurs, excess free fatty acids (FFA) are deposited primarily in the liver and β-cells are injured potentially resulting in insulin resistance (IR). Meanwhile, IR could promote the transcription of fatty acid synthetase (FAS) (Meex and Watt, 2017). On the other hand, the PPAR signaling pathway is involved in adipocyte differentiation, fatty acid conversion, and lipid synthesis. PPARγ is one of the key transcriptional activators of adipocyte genes such as glucose transporter 4 (Glut4), lipoprotein lipase (LPL), and fatty acid binding protein 2 (aP2). PPARα and AMPK play important roles in regulating the processes of FFA transport and lipid oxidative metabolism (Madrazo and Kelly, 2008; Lopaschuk et al., 2010). Hypoxia-inducible factor-1α (HIF-1α) mainly improves abnormal lipid metabolism by regulating oxidative stress (Ma et al., 2017). Interestingly, LPL, PPARγ, and PPARα—genes which are directly related to the PPAR signaling pathway—are among the top 10 hub targets identified in the present study.

Our previous study showed that RS could alleviate lipid metabolism disorder in a high-fat diet golden hamster model and upregulate the expression of the PPAR pathway mediators PPARα and PPARγ, resulting in the activation of downstream adipocyte genes aP2, Glut4, and LPL (Jiang et al., 2021). These findings verified that RS might regulate lipid metabolism disorder through mediating the PPAR signaling pathway.

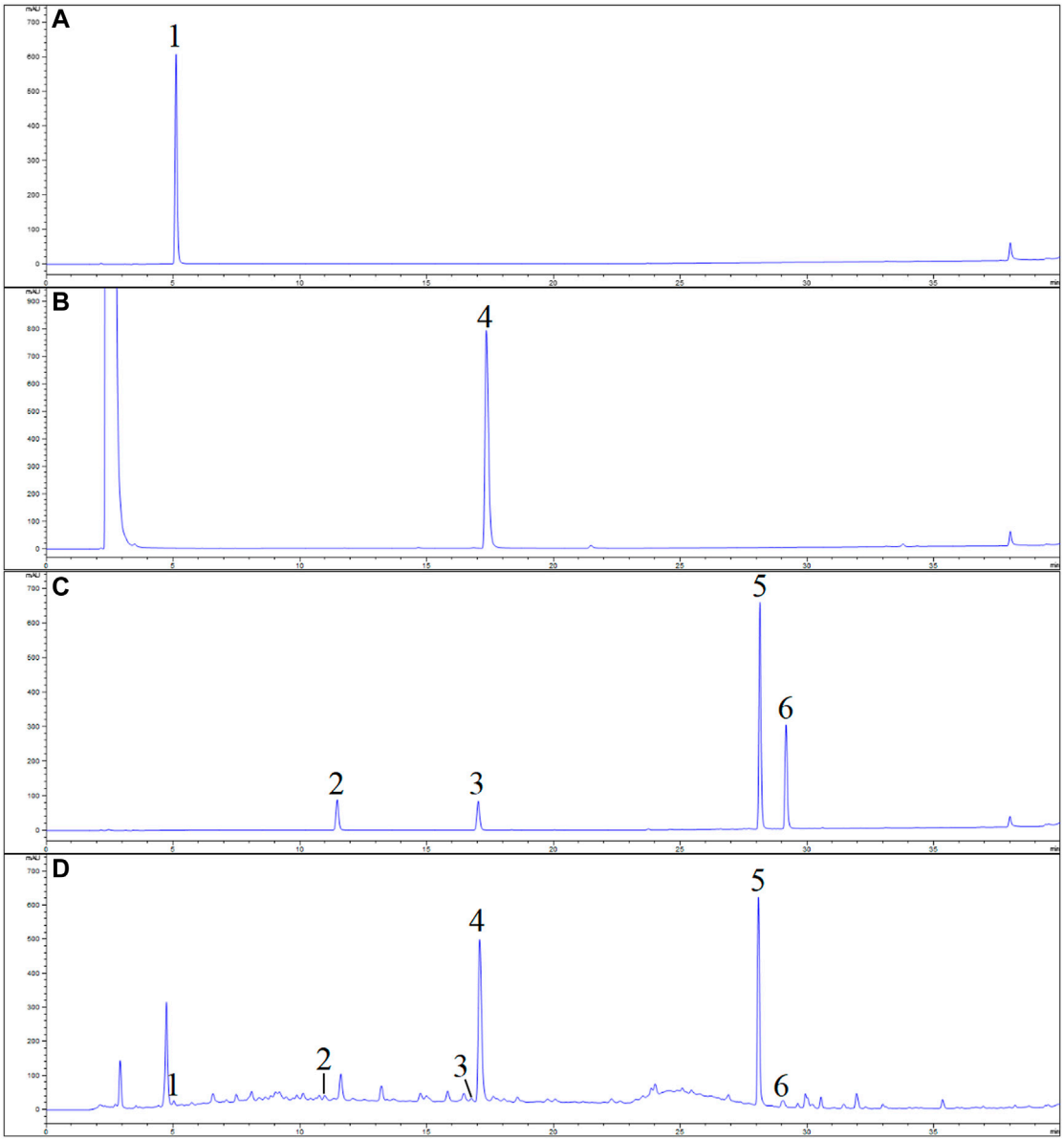

FIGURE 4 | UPLC chromatogram of *Rubus chingii* var. *suavissimus* and the reference solutions **(A)** gallic acid; **(B)** ellagic acid; **(C)** mixed reference compounds; **(D)** sample. 1) Gallic acid, 2) caffeic acid, 3) rutin, 4) ellagic acid, 5) rubusoside, and 6) kaempferol.

Determination of the Content of Compounds with a Potential Effect on Lipid Metabolism Disorder in Different RS Samples by UPLC

In the current study, network pharmacology identified nine compounds—including ursolic acid, oleanolic acid, quercetin, kaempferol, gallic acid, ellagic acid, caffeic acid, rubusoside, and rutin—as potential bioactive ingredients of RS involved in mediating lipid metabolism disorder. However, ursolic acid and oleanolic acid could not be detected in the RS samples due to their weak UV absorptions whereas the content of quercetin was below the detection limit. Therefore, six compounds—gallic acid, caffeic acid, rutin, ellagic acid, rubusoside, and kaempferol—were chosen to determine the content in RS samples obtained from multiple marketplaces (RS01-RS13).

Optimization of Extraction and UPLC Chromatographic Conditions

First, several variables including solvent, extraction methods, extraction time, and number of cycles were optimized to obtain the maximal extraction efficiency. Maximal extraction was achieved through ultrasonication with 20 volumes of 60% ethanol for 60 min once or eight volumes of 95% ethanol for three times in case of intragastric administration to rats.

TABLE 3 | Content of six compounds in 13 batches of *Rubus chingii* var. *suavissimus* samples from markets (μg/g or mg/g, mean ± SD, *n* = 3).

No.	Collecting region	Sample type	Gallic acid (μg/g)	Caffeic acid (μg/g)	Rutin (μg/g)	Ellagic acid (mg/g)	Rubusoside (mg/g)	Kaempferol (μg/g)
RS01	Guilin, Guangxi	Tea bag	68.04±0.69	164.47±3.67	169.49±11.97	0.85±0.00	68.38±0.05	122.02±1.87
RS02	Guilin, Guangxi	Dried tea	42.78±1.29	77.08±2.02	206.33±8.60	0.38±0.00	36.85±1.37	95.45±2.97
RS03	Guilin, Guangxi	Tea bag	65.78±2.10	102.10±4.22	143.60±2.68	0.59±0.08	48.64±0.49	99.80±1.38
RS04	Guilin, Guangxi	Tea bag	97.11±2.80	44.98±3.55	308.58±7.51	1.15±0.01	39.34±0.02	93.28±0.92
RS05	Guilin, Guangxi	Dried tea	89.08±0.92	165.09±44.36	159.66±2.71	0.90±0.08	50.69±1.91	119.09±1.70
RS06	Laibin, Guangxi	Dried tea	91.52±1.05	141.40±4.40	153.33±5.51	0.96±0.02	47.46±0.40	94.83±2.30
RS07	Laibin, Guangxi	Dried tea	67.70±1.47	31.52±0.77	687.29±6.58	0.85±0.01	40.15±0.19	106.88±1.90
RS08	Laibin, Guangxi	Dried tea	101.25±8.10	141.71±11.47	223.89±10.08	2.67±0.16	67.92±1.23	120.68±2.41
RS09	Yongzhou, Hunan	Dried tea	254.38±6.22	195.86±4.71	148.78±5.19	1.40±0.07	46.77±0.33	106.41±3.68
RS10	Hezhou, Guangxi	Dried tea	155.21±0.37	64.74±3.70	287.72±2.35	1.32±0.10	36.74±0.02	110.66±4.42
RS11	Hezhou, Guangxi	Dried tea	113.47±1.40	132.34±7.10	161.06±4.18	1.21±0.06	53.28±2.91	140.45±3.84
RS12	Foshan, Guangdong	Dried tea	53.93±3.06	154.94±4.69	172.32±3.48	0.57±0.02	58.00±0.93	115.37±3.94
RS13	Yongzhou, Hunan	Dried tea	250.79±1.60	174.56±1.10	180.74±8.53	1.74±0.06	44.83±0.61	123.56±1.41

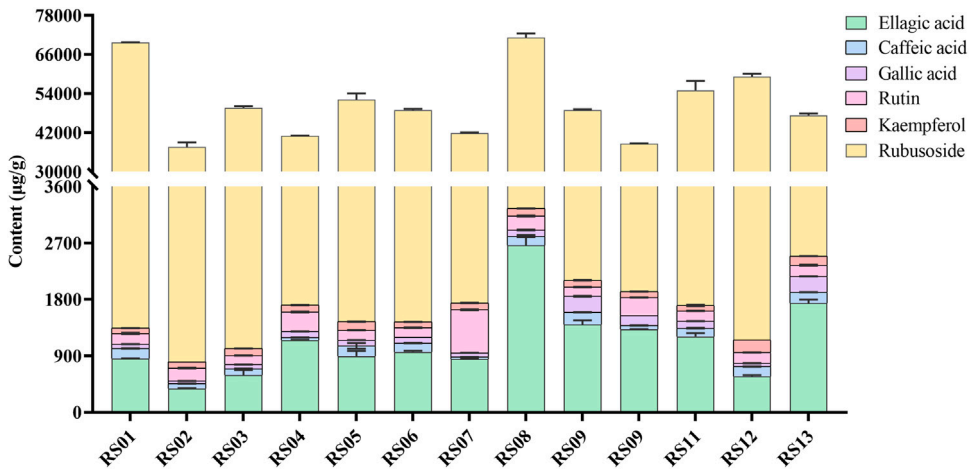

FIGURE 5 | The content of ellagic acid, caffeic acid, gallic acid, rutin, kaempferol, and rubusoside in 13 batches of *Rubus chingii* var. *suavissimus* samples from markets.

Next, the conditions for chromatographic analysis including the type of column, mobile phase gradients, and detection wavelength were optimized. The results showed that optimal separation was obtained by eluting RS samples on an InertSustain C_{18} LC column at 40°C using a linear gradient of 0.1% phosphoric acid and acetonitrile within 40 min. Considering that the maximum absorption wavelength of rubusoside situates at 205 nm while that of the other references situates at 254 nm, a wavelength conversion program was used to obtain a better resolution.

Method Validation of UPLC

The regression equation, linearity range, correlation coefficients, and LODs and LOQs of gallic acid, caffeic acid, rutin, ellagic acid, rubusoside, and kaempferol are summarized in **Supplementary Table S3**. All calibration curves indicated good linearity ($r^2 \geq 0.9993$) within the test ranges while the overall LODs and LOQs were ranged from 0.09 to 1.00 ng and 0.30 to 3.00 ng, respectively. The RSDs of precision, repeatability, stability, and recovery are shown in **Supplementary Table S4**. For six analytes, the RSDs of intra- and interday precisions of these analytes were less than 1.50 and 1.93%, respectively. The reproducibility RSDs were less than 2.94%. The RS sample solution was stable within 24 h with an RSD of less than 1.89%. The recoveries ranged from 97.58 to 100.29%, and the recovery RSDs ranged from 1.21 to 2.24%. Based on these data, the developed UPLC method was considered reliable, accurate, and suitable for the quantification of those components.

Sample Analysis

The obtained peaks in the chromatograms were identified through the comparison of the retention times and on-line UV spectra with those of the standard constituents (**Figure 4**). Retention times of peaks 1–6 were 5.07, 11.03, 16.93, 17.29, 28.15,

and 29.16 min, respectively. The results are summarized in **Table 3** and **Figure 5**. The contents of gallic acid, caffeic acid, rutin, ellagic acid, rubusoside, and kaempferol in the samples ranged from 42.78 to 254.38 μg/g, 31.52 to 195.86 μg/g, 143.60 to 687.29 μg/g, 0.38 to 2.67 mg/g, 36.74 to 68.38 mg/g, and 93.28 to 140.45 μg/g, respectively.

The results showed that the contents of the determined compounds varied in the RS samples obtained from different markets. For example, high levels of ellagic acid and rubusoside were present in all batches. However, even though the samples were purchased from the same origin, the contents could show significant differences. For example, while samples RS06~ RS08 were all purchased from Laibin of Guangxi, the content of rubusoside and ellagic acid in these samples ranged from 40.15 to 67.92 mg/g and 0.85 to 2.67 mg/g, respectively. Similarly, the content of rubusoside and ellagic acid in samples RS01~ RS05—obtained from Guilin—ranged from 36.85 to 68.38 mg/g and 0.38 to 1.15 mg/g, respectively. Importantly, the effectiveness of herbal medicine is highly related to the quality of the raw materials which on itself is affected by a complex set of factors such as the place of origin, growing conditions, medicinal parts, and processing methods. Previously, a higher content of rubusoside in tender tissues of RS (Yin et al., 2008) which was influenced by origin and dosage form (Fan et al., 2012) was shown.

In order to strengthen the quality control of RS, a strict control of the quality of raw materials from the origin and processing procedure is required. Thus, simultaneous determination methods to identify effective bioactive components could be of importance not only to analyze underlying mechanisms but also to establish future quality control of RS.

CONCLUSION

In this study, a total of 69 compounds—including diterpenes, triterpenes, flavonoids, polyphenols, and lignans—were identified or tentatively characterized in *Rubus chingii* var. *suavissimus* by UPLC-Q/TOF-MS. Among them, 50 absorbed prototype components were detected in rat plasma. Next, bioactive compounds–therapeutic target network analysis predicted nine active components (ursolic acid, oleanolic acid, quercetin, kaempferol, gallic acid, ellagic acid, caffeic acid, rutin, and rubusoside), 71 target genes, and 20 pathways to be involved in RS-mediated alleviation of lipid metabolism disorder. Finally, UPLC analysis could show significant variability in the contents of six selected potential active compounds in RS samples obtained from different marketplaces. Taken together, the study provided a basis for further exploration of the mechanism on the regulation of lipid metabolism disorder and the development of *Rubus chingii* var. *suavissimus*–derived therapeutic drugs and healthcare products.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

L-lF and Y-hL conceived and designed the experiment. M-jJ, W-fH, SH, and Y-xL performed the experiments. M-jJ, W-fH, and SH analyzed data. M-jJ, W-fH, and L-lF wrote and revised the manuscript. YH and P-lD prepared plant material and animal samples. LF and Y-hL supervised all work.

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SUPPLEMENTARY MATERIAL

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

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Traditionally Used Plants in the Treatment of Diabetes Mellitus: Screening for Uptake Inhibition of Glucose and Fructose in the Caco2-Cell Model

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The traditional use of plants and their preparations in the treatment of diseases as a first medication in the past centuries indicates the presence of active components for specific targets in the natural material. Many of the tested plants in this study have been traditionally used in the treatment of Diabetes mellitus type 2 and associated symptoms in different cultural areas. Additionally, hypoglycemic effects, such as a decrease in blood glucose concentration, have been demonstrated *in vivo* for these plants. In order to determine the mode of action, the plants were prepared as methanolic and aqueous extracts and tested for their effects on intestinal glucose and fructose absorption in Caco2 cells. The results of this screening showed significant and reproducible inhibition of glucose uptake between 40 and 80% by methanolic extracts made from the fruits of *Aronia melanocarpa*, *Cornus officinalis*, *Crataegus pinnatifida*, *Lycium chinense*, and *Vaccinium myrtillus*; the leaves of *Brassica oleracea*, *Juglans regia*, and *Peumus boldus*; and the roots of *Adenophora triphylla*. Furthermore, glucose uptake was inhibited between 50 and 70% by aqueous extracts made from the bark of *Eucommia ulmoides* and the fruit skin of *Malus domestica*. The methanolic extracts of *Juglans regia* and *Peumus boldus* inhibited the fructose transport between 30 and 40% in Caco2 cells as well. These findings can be considered as fundamental work for further research regarding the treatment of obesity-correlated diseases, such as Diabetes mellitus type 2.

Keywords: plant extracts, traditional use, glucose, fructose, uptake inhibition, Diabetes mellitus, Caco2 cells

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INTRODUCTION

In the last years, not only did the absolute number of people suffering from Diabetes mellitus increase from 108 Mio in 1980 to 422 Mio in 2014 but also the prevalence of this disease rose from 4.7 to 8.5% among adults during this time period worldwide (WHO, 2019). Apart from the estimated annual cost to the world of US$ 827 billion, Diabetes is associated with premature mortality and decreased quality of life (WHO, 2016). Hyperglycemia, as one of the main symptoms for diagnosis, results from decreased insulin secretion and/or reduced insulin action and can lead to long-term damage and dysfunction of different organs (American Diabetes Associa, 2014). Particularly, the most common kind of the disease, Diabetes mellitus type 2 (American Diabetes Associa, 2014), can be prevented or delayed in its progress by a lifestyle, which includes a healthy diet, regular exercise with moderate intensity, and no tobacco use (WHO, 2019).

With a focus on the diet, not only a decrease of energy intake to reduce obesity (Grundy, 1998) but also the selection of the consumed food can improve the health status. Supplements can be used to ensure the adequate intake of certain nutrients and support patient's therapy (Ota and Ulrih, 2017). For example, the reduction of glucose, fructose, and saturated long-chain fatty acids and the increased intake of polyunsaturated, omega-3 fatty acids, such as eicosapentaenoic and docosahexaenoic acid, play a very important role in the prevention and improvement of metabolic disorders by alleviating inflammation processes (Sears and Perry, 2015). Regarding the consumption of plant-based food, secondary metabolites from plants showed hypoglycemic properties by affecting different targets, for example, modulation of intracellular insulin signaling pathways, increase in insulin secretion from β-cells, and inhibition of intestinal enzymes and transporters (Hanhineva et al., 2010; Bahadoran et al., 2013; Ota and Ulrih, 2017). In order to treat obesity as one of the main risk factors for Diabetes mellitus type 2 (Smith, 2007), the reduction of nutritional uptake into enterocytes as the last step before energy overload affects the body is an important therapeutic approach. Furthermore, the inhibition of enzymes and transporters leads to increased concentrations of nutrients in distal sections of the small intestine, which initiates the "ileal brake." This complex mechanism is known to influence the digestive process and ingestive behavior resulting in reduced appetite and food intake (Maljaars et al., 2008). The glucose transport in the small intestine is maintained by the cotransporter SGLT1 (sodium-glucose linked transporter 1) and GLUT2 (glucose transporter 2), which belongs to the GLUT family and facilitates diffusion processes. The intestinal fructose uptake is performed by GLUT5 (glucose transporter 5) and GLUT2 as well (Schreck and Melzig, 2018). Medicinal plants traditionally used in the treatment of diseases have been applied as candidates for drug discovery in the last decades (Fabricant and Farnsworth, 2001). Aspirin, atropine, ephedrine, digoxin, and morphine are examples of effective substances, which owe their discovery to investigative studies of folk medicine (Gilani and Atta-ur-RahmanTrends, 2005). Ethnopharmacology studies show the continuing acceptance of traditionally used plant preparations as a therapy option due to their long-term experience and marginal side effects (Fabricant and Farnsworth, 2001; Gilani and Atta-ur-RahmanTrends, 2005). The plants tested in this study were chosen due to their traditional use in some cultural areas and countries during the last centuries and their hypoglycemic effects, which were discovered in animal or human studies, as listed in **Table 1**. For example, in Jordan, preparations of *Allium sativum*, *Ceratonia siliqua*, *Cuminum cyminum*, *Juglans regia*, *Nigella sativa*, *Olea europaea*, *Sarcopoterium spinosum* have been used in the treatment of Diabetes mellitus (Al-Aboudi and Afifi, 2011), whereas in Congo, *Brassica oleracea* and *Citrus limon* have been prepared as folk medicine to treat Diabetes mellitus (Katemo et al., 2012). The plants *Adenophora triphylla*, *Cornus officinalis*, *Crataegus pinnatifida*, *Eucommia ulmoides*, *Lycium chinense*, *Pueraria lobata*, and *Rosa rugosa* belong to the pharmacopoeia of the Traditional Chinese Medicine (Stöger, 2009). Although *Adenophora triphylla* and *Crataegus pinnatifida* showed hypoglycemic effects in studies, the traditional use as antidiabetic remedy was not confirmed in English-language literature as shown in **Table 1**. According to **Table 1**, some of the plants, such as *Olea europaea* and *Brassica oleracea*, were used in different areas of the world supporting the assumption of efficacy in the treatment of the metabolic disorder. The plants used in this study have been already tested in animal experiments and hypoglycemic properties such as a decrease in blood glucose were confirmed as shown in **Table 1**, but the exact mechanisms still need to be studied. In order to determine the mode of action, methanolic and aqueous plant extracts were screened for their inhibitory activity on intestinal transporters using Caco2 cells, which originally stem from a colorectal adenocarcinoma cell line. After confluence, the cells need 15–21 days to differentiate and fully express intestinal characteristics, such as a brush border membrane with enzymes and transporters. The adenocarcinoma cell line is widely used as a model for intestinal transport studies (Chantret et al., 1988; Hidalgo et al., 1989; Jumarie and Malo, 1991; Sambuy et al., 2005). We have determined the expression of the SGLT1, GLUT2, and GLUT5 transporters in the Caco2 cells during 21 days (Schreck and Melzig, 2019) and adapted the experimental conditions to ensure valuable results.

MATERIALS AND METHODS

Plant Extract Preparations

The methanolic and aqueous extract preparations were performed according to Buchholz (2017) with modifications. The parts of the plants, which were used to prepare the extracts, are listed in **Tables 2**, **3**. Except for the fresh fruits of *Malus domestica*, the botanicals were obtained as dried plant material from the companies. The bulbs of *Allium sativum*, fruits of *Aronia melanocarpa* and *Vaccinium myrtillus*, fruit skin of *Punica granatum*, the herbal part of *Cynara cardunculus* and *Potentilla aurea*, leaves of *Mentha aquatica* and *Olea europaea*, roots of *Panax ginseng* and *Sarcopoterium spinosum*, and seeds of *Nigella sativa* were purchased from Kräuter Schulte aktiv Kräuter Drogerie e.K. (Gernsbach, Germany). The following herbal drugs were obtained from Alfred Galke GmbH (Gittelde, Germany): flowers of *Hibiscus sabdariffa* and *Syzygium aromaticum*; fruits of *Ceratonia siliqua*; fruit skin of *Citrus limon*; leaves of *Brassica oleracea*, *Camellia sinensis*, *Ilex paraguariensis*, *Juglans regia*, *Melissa officinalis*, *Origanum creticum*, *Rosmarinus officinalis*, and *Salvia officinalis*; and seeds of *Cuminum cyminum* and *Vitis vinifera* (pomace). The plant materials used in Traditional Chinese Medicine, such as the bark of *Eucommia ulmoides*; flowers of *Rosa rugosa*; fruits of *Cornus officinalis*, *Crataegus pinnatifida*, and *Lycium chinense*; and the roots of *Adenophora triphylla* and *Pueraria lobata* were acquired from Zieten Apotheke (Berlin, Germany). The fruits of the cucurbitaceae *Momordica charantia* were kindly given by Dr. Serhat Sezai Çiçek (Institute of Pharmacy of Christian-Albrechts-Universitaet Kiel, Germany). The green seeds of *Coffea arabica* were purchased from Ridders Kaffeerosterei (Berlin, Germany). The leaves of *Peumus boldus* were gained from Heinrich Klenk GmbH & Co. KG (Schwebheim, Germany), whereas the leaves of *Artemisia dracunculus* and the fresh fruits of *Malus domestica*

TABLE 1 | Selected plants, which are/were traditionally used in specific countries and their antidiabetic effects measured in studies and discussed active compounds (n.f. = not found in English-language literature for this indication; n.n. = not named).

Scientific plant name	Countries/areas with traditional use of plant preparations as antidiabetic remedy and adjuncts to conventional treatments in the therapy of Diabetes mellitus (reference)	Selected, antidiabetic effects of traditionally used plants measured in studies	Discussed active compounds	Reference for antidiabetic effects and discussed active compounds
Adenophora triphylla (THUNB.) ADC.	n.f.	Decrease of blood glucose, inhibition of intestinal glucose absorption in rats and mice	n.n.	Sanae et al. (1996)
Allium sativum L.	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020), India Rizvi and Mishra (2013), United Kingdom Swanston-Flatt et al. (1991)	Decrease of serum glucose and triglycerides, increase of serum insulin levels in diabetic rats	Allicin-type compounds	Eidi et al. (2006)
Aronia melanocarpa MICHX. ELLIOTT	n.f.	Decrease of serum glucose and lipids, reduced α-glucosidase activity	Anthocyanins	Banjari et al. (2017)
Artemisia dracunculus L.	United Kingdom Swanston-Flatt et al. (1991)	Decrease of elevated blood glucose level and blood insulin concentrations in diabetic mice	Flavonoids (luteolin, apigenin), coumarins (scopoletin), sesquiterpenoid lactones (costunolide), cinnamates	Ribnicky et al. (2006)
Brassica oleracea L.	Congo Katemo et al. (2012), Morocco Idm'hand et al. (2020), United Kingdom Swanston-Flatt et al. (1991)	Decrease of blood glucose in diabetic rats, decrease of blood lipids, and restoration of renal function in rats	n.n.	Kataya and Hamza (2008); Assad et al. (2014); Shah et al. (2016)
Camellia sinensis (L.) KUNTZE (Assam) *Camellia sinensis* (L.) KUNTZE (Darjeeling) *Camellia sinensis* (L.) KUNTZE (gunpowder) *Camellia sinensis* (L.) KUNTZE (Sencha)	Morocco Idm'hand et al. (2020)	Decrease of blood glucose level in rats/mice, reduced α-glucosidase, α-amylase, and lipase activity	Catechins, flavanols, polysaccharides	Gomes et al. (1995); Han et al. (2011); Wang et al. (2017)
Ceratonia siliqua L	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020)	Decrease of blood glucose in rats, reduced α-glucosidase, α-amylase activity, inhibition of intestinal glucose transport	Polyphenolic compounds	Rtibi et al. (2017)
Citrus limon (L.) OSBECK	Congo Katemo et al. (2012), United Kingdom Swanston-Flatt et al. (1991)	Decrease of blood glucose in rats	n.n.	Naim et al. (2012)
Coffea arabica L.	n.f.	Decrease of blood glucose in rats, reduced glucose uptake, stimulated insulin secretion	Caffeine, polyphenolic compounds: chlorogenic acid, quinolactones	Campos-Florian et al. (2013)
Cornus officinalis SIEBOLD and ZUCC.	China Li et al. (2004); Ma et al. (2014)	Decrease of blood glucose in mice, reduced α-glucosidase activity, increased glucose uptake in HepG2	Morroniside, loganin, ursolic acid	He et al. (2016)
Crataegus pinnatifida BUNGE	n.f.	Decrease of hyperglycemia in rats, modulation of insulin regulation, anti-obesity effect, anti-hyperlipidemia effect, reduced rats' α-glucosidase activity	Flavonoids, hyperosid, chlorogenic acid, tripenic acids	Dehghani et al. (2019)
Cuminum cyminum L.	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020)	Decrease of hyperglycemia, oxidative stress, and AGE formation in rats, reduced α-glucosidase activity	Cuminaldehyde, flavonoids	Jagtap and Patil (2010)
Cynara cardunculus L.	Morocco Idm'hand et al. (2020); Tahraoui et al. (2007)	Decrease in blood glucose and serum lipid levels in humans and rats	Flavonoids	Nazni et al. (2006); Heidarian and Soofiniya (2011)
Eucommia ulmoides OLIV.	China, Japan, Korea He et al. (2014)	Decrease in blood glucose and increase in plasma insulin in rats, decreased plasma lipid levels in mice, reduced glycation	Flavonoids	Kim et al. (2004); Lee et al. (2005); Park et al. (2006)
Hibiscus sabdariffa L.	Morocco Idm'hand et al. (2020)		Polyphenolic compounds	Peng et al. (2011)

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TABLE 1 | (*Continued*) Selected plants, which are/were traditionally used in specific countries and their antidiabetic effects measured in studies and discussed active compounds (n.f. = not found in English-language literature for this indication; n.n. = not named).

Scientific plant name	Countries/areas with traditional use of plant preparations as antidiabetic remedy and adjuncts to conventional treatments in the therapy of Diabetes mellitus (reference)	Selected, antidiabetic effects of traditionally used plants measured in studies	Discussed active compounds	Reference for antidiabetic effects and discussed active compounds
		Decreased hyperglycemia, hyperinsulinemia, serum lipids, and AGE formations in rats		
Ilex paraguariensis A. ST.-HIL	South America de Freitas Junior and de Almeida (2017)	Decrease of serum lipids and glucose in mice, modulation of food intake	Polyphenolic compounds, methylxanthines, saponins	Kang et al. (2012)
Juglans regia L.	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020)	Decrease of fasting blood glucose, HbA1c, and fasting blood lipids in humans	Phenolic acids and flavonoids: 3- and 5-caffeoylquinic acids, quercetin-3-galactoside, quercetin-3-arabinoside	Hosseini et al. (2014)
Lycium chinense MILL.	China Li et al. (2004)	Decrease of blood glucose and attenuation of dyslipidemia in rats	Polyphenolic compounds	Olatunji et al. (2017)
Malus domestica BORKH.	Morocco Idm'hand et al. (2020)	Decrease of postprandial blood glucose in mice and humans, decrease of glucose absorption in mouse intestine, inhibition of human SGLT1 in *X. laevis* oocytes, inhibition of lipase *in vitro*, and decrease of plasma triglycerides in mice and humans	Polyphenolic compounds: quercetin, phlorizin, procyanidins	Sugiyama et al. (2007); Schulze et al. (2014)
Melissa officinalis L.	Iran, Turkey Shakeri et al. (2016)	Decrease of plasma glucose levels in rats, reduced α-glucosidase, α-amylase activity, decrease of HbA1c, serum triglyceride and fasting blood glucose levels in humans	Polyphenolic compounds (flavonoids), essential oils	Hasanein and Riahi (2015); Asadi et al. (2019)
Mentha aquatica L.	n.f.	Decrease of fasting blood glucose and lipid levels, nephroprotective, reduced HbA1c, and increase of insulin levels in rats	Polyphenolic compounds (flavonoids, tannins), saponins, volatile oils	Yellanur Konda et al. (2020)
Momordica charantia L.	Asia, South America, East Africa Rizvi and Mishra (2013), United Kingdom Swanston-Flatt et al. (1991)	Decrease of blood glucose and glycosylated haemoglobin and increase of plasma insulin in animal studies, inhibition of intestinal transporters	Charantin, polypeptide-p, momordin ic, oleanolic acid 3-O-monodesmoside, and oleanolic acid 3-O-glucuronide	Grover and Yadav (2004)
Nigella sativa L.	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020)	Decrease of fasting blood glucose, reduced insulin resistance, and improved β-cell function	n.n.	Bamosa et al. (2010)
Olea europaea L.	Jordan Al-Aboudi and Afifi (2011), Morocco Idm'hand et al. (2020), Portugal Neves et al. (2009)	Decrease of serum blood glucose, triglycerides, and cholesterol, increase of serum insulin levels	Oleuropeoside	Eidi et al. (2009)
Origanum creticum L.	Morocco Idm'hand et al. (2020)	Decrease of blood glucose, lipids, and HbA1c in rats, reduced α-amylase activity	n.n.	Prasanna et al. (2017)
Panax ginseng C.A. MEY.	Asia Park et al. (2019)	Decrease of 75 g-OGTT-plasma-glucose indices, fasting plasma insulin, and 75 g-OGTT-plasma-insulin indices in humans	Ginsenosides: PPT, (20R)-PPD, Rg1, Rc, Rd, Re, Rf, Rg2, Rh1, Rb1, and Rb2; peptidoglycan: panaxan B	Vuksan et al. (2008)
Peumus boldus MOLINA	n.f.	Decrease of plasma glucose level, inhibition of α-amylase and lipase	Boldine	Jang et al. (2000); Buchholz and Melzig (2016)
Potentilla aurea L.	n.f.	Inhibition of α-amylase and lipase	n.n.	Buchholz and Melzig (2016)
Pueraria lobata (WILLD.) OHWI	China Li et al. (2004)	Decrease of fasting blood glucose, improved glucose tolerance and insulin sensitivity in mice	Isoflavones: puerarin	Prasain et al. (2012)
Punica granatum L. (*peel*)	Morocco Idm'hand et al. (2020); Tahraoui et al. (2007)	Decrease of fasting blood glucose and serum lipids in rats	Phenolic compounds	Bagri et al. (2009)

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TABLE 1 | (*Continued*) Selected plants, which are/were traditionally used in specific countries and their antidiabetic effects measured in studies and discussed active compounds (n.f. = not found in English-language literature for this indication; n.n. = not named).

Scientific plant name	Countries/areas with traditional use of plant preparations as antidiabetic remedy and adjuncts to conventional treatments in the therapy of Diabetes mellitus (reference)	Selected, antidiabetic effects of traditionally used plants measured in studies	Discussed active compounds	Reference for antidiabetic effects and discussed active compounds
Rosa rugosa THUNB.	Korea Lee et al. (2008)	Decrease of blood glucose, serum insulin, serum lipids, increased insulin sensitivity in rats, reduced α-glucosidase activity	Polyphenolic compounds	Liu et al. (2017)
Rosmarinus officinalis L.	Morocco Idm'hand et al. (2020); Tahraoui et al. (2007)	Decrease of blood glucose and increase of serum insulin in rabbits, reduced α-glucosidase activity	Volatile oils	Bakırel et al. (2008)
Salvia officinalis L.	Morocco Idm'hand et al. (2020); Tahraoui et al. (2007), United Kingdom Swanston-Flatt et al. (1991)	Decrease in 2-h-postprandial blood glucose and cholesterol in humans, decrease in serum glucose and lipids in rats	Flavonoids (rosmarinic acid, phenolic acids, carnosic compounds)	Eidi and Eidi (2009); Behradmanesh et al. (2013)
Sarcopoterium spinosum (L.) SPACH	Jordan Al-Aboudi and Afifi (2011), Israel Bachrach (2007)	Improved glucose tolerance in mice, insulin-like effects, increased insulin secretion *in vitro*, reduced α-glucosidase and α-amylase activity	Polyphenolic compounds: catechin, epicatechin	Smirin et al. (2010); Elyasiyan et al. (2017)
Syzygium aromaticum (L.) MERR. and L.M.PERRY	Morocco Idm'hand et al. (2020)	Decrease of postprandial hyperglycemia in rats, reduced α-glucosidase and α-amylase activity, downregulation of intestinal transporters	Triterpenes (oleanolic acid, maslinic acid)	Khathi et al. (2013)
Thymus vulgaris L.	Morocco Idm'hand et al. (2020), United Kingdom Swanston-Flatt et al. (1991)	Reduced blood glucose and serum lipids in rats	Flavonoids	Ekoh et al. (2014)
Vaccinium myrtillus L.	Europe Helmstaedter (2007)	Decrease of blood glucose and lipids in rats	Polyphenolic compounds	Sidorova et al. (2017)
Vitis vinifera L.	Morocco Idm'hand et al. (2020)	Decrease of postprandial hyperglycemia in mice, reduced α-glucosidase activity	Polyphenolic compounds: flavonoids, anthocyanins	Hogan et al. (2010)

were obtained from a local supermarket. The fresh fruits of *Malus domestica* were washed with water. The fruit's skin was cut into small pieces, dried in a drying cabinet at 40°C for 3 days, and freeze-dried for 48 h. The dried plant material was ground with a M20 mill from IKA®-Werke GmbH & Co. KG (Staufen, Germany). 10.0 g of the plant's powder, adjusted to the drug extract ratio as listed in **Tables 2**, **3**, was extracted in 100 ml. For the preparation of the aqueous extracts, plant material was extracted in water obtained from the ultrapure water system LaboStar from Siemens AG Wasseraufbereitung (Barsbüttel, Germany) for 15 min at 40°C and filtered with a Buechner filter. In some cases, extracts were centrifuged at 4°C first with an Allegra X-30R centrifuge from Beckman Coulter GmbH (Krefeld, Germany) to facilitate the filtration process. The extracts were freeze-dried for at least 48 h with the lyophilization machine Alpha 2-4 LSCplus from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode am Harz, Germany). The powder was stored at −20°C until use. For the preparation of the methanolic extracts, plant material was extracted in methanol purchased from VWR International S.A.S. (Fontenay-sous-Bois, France) for 60 min at 40°C and filtered. In order to obtain a dry powder, they were evaporated to 5 ml with the rotary evaporator RV 10 basic from IKA®-Werke GmbH & Co. KG (Staufen, Germany) and dried in a Petri dish from VWR International GmbH (Darmstadt, Germany) under the exhaust hood overnight. Subsequently, the extracts were freeze-dried to lose residual water and stored at −20°C until use. For application in cell experiments, the aqueous extracts were solved in DPBS (Dulbecco's phosphate-buffered saline) purchased from Lonza Cologne AG (Cologne, Germany), whereas the extracts made with methanol were first dissolved in DMSO (0.3% V/V final well concentration), which was acquired from Fisher Scientific GmbH (Schwerte, Germany), and filled with DPBS until final volume. Extract solutions were filtered with a 0.8 µm and a 0.2 µm sterile filter purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and stored at −20°C until use. Three different extract concentrations (1.0, 0.1, or 0.01 mg/ml) were tested in MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described in the **Supplementary Material** and only the highest concentration, which did not affect cell viability, was used in uptake studies. The GLUT2 inhibitor phloretin, which was purchased from Cayman Chemical (Michigan, United States), and the SGLT1 inhibitor phlorizin applied from Carl Roth GmbH

+ Co. KG (Karlsruhe, Germany) were used with a final well concentration of 100 µM as a positive control for uptake inhibition of glucose.

General Handling of the Cell Line

Caco2 cells were purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and seeded in 24-well plates, which were acquired from Greiner Bio-One GmbH (Frickenhausen, Germany), for every experiment. As general medium DMEM (Dulbecco's modified eagle medium) without phenol red containing 1% UltraGlutamine 1, both acquired from Lonza Cologne AG (Cologne, Germany) and 5 or 20% FBS derived from Bio&SELL GmbH (Nürnberg, Germany) for experiment or mother line, respectively, was used for the handling with Caco2 cells. Cells were washed once with PBS from Lonza Cologne AG (Cologne, Germany) for every medium change and washed twice before Trypsin (TrypLE™ Express without phenol red) from Gibco Life Technologies corporation (NY, United States) was applied to detach the cells for seeding. The cells were incubated at 37°C and 5% CO_2-supply during the whole time. Passages 20–35 were used in the experiments. The buffers and solutions for cell culture were prepared with water obtained from the ultrapure water system LaboStar from Siemens AG Wasseraufbereitung (Barsbüttel, Germany).

Glucose and Fructose Uptake in Caco2 Cells

For uptake studies, cells were seeded in a density of 400,000 cells per well. Confluence appeared 24 h after seeding and was controlled visually. The radiolabeled substrates 1 mCi/ml glucose (specific activity: 30 Ci/mmol) and 1 mCi/ml fructose (specific activity: 5 Ci/mmol) were obtained from Biotrend Chemikalien GmbH (Köln, Germany). Although fructose absorption into cells was determined by using 54 nM solution of radiolabeled substrate as final well concentration, the 2 nM radiolabeled glucose solution was spiked with 0.001 µM non-radiolabeled glucose, which was purchased from Merck KGaA (Darmstadt, Germany). For uptake studies with extracts, cells were used at day three and day fifteen after confluence for glucose and fructose, respectively. On the day of the experiment, cells were washed once with 2 ml buffer containing 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], which was obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and 150 mM NaCl purchased from Fisher Scientific GmbH (Schwerte, Germany). The pH of the buffer was adjusted with sodium hydroxide from Merck KGaA (Darmstadt, Germany) using a 766 Laboratory pH meter from Knick Elektronische Messgeraete GmbH & Co. KG (Berlin, Germany) to 7.4. After 1 h starving incubation with Hepes-NaCl buffer at 37°C, substrate or substrate-extract solution were added to the cells and incubated at 37°C. After 1 h, the buffer was discarded, the cells were washed once with 1 ml ice-cold Hepes-NaCl buffer, and 500 µL Hepes-NaCl solution containing 1% Triton X 100 was added to each well. The samples were taken from the well plates, added to a sufficient volume of scintillation cocktail in scintillation tubes, both purchased from Hidex (Mainz, Germany), mixed for 20 s using a vortexer from Heidolph Instruments GmbH & Co. KG (Schwabach, Germany), and measured with a β-Counter from Hidex (Mainz, Germany). The plant extracts, which showed the strongest inhibition on intestinal glucose transporters, were repeated and tested for their influence on fructose uptake with a focus on methanolic extracts. Randomly, well plates, which were cultivated and treated under the same conditions as cells used for uptake studies, were tested in Hoechst Assay as described in the **Supplementary Material** to determine variations depending on the procedure.

Statistical Evaluation and Use of Software

All statistical tests were performed using Microsoft Excel. Normal distribution was established *via* Shapiro–Wilk test manually before using Excel's tool "Data analysis" for provision of the *F*-test and the *t*-tests. If the data was not normally distributed, Mann–Whitney *U* test was applied for further statistical analysis. IC_{50} data was calculated with GraphPad Prism 5.0.

RESULTS

Uptake Studies with Glucose and Fructose

In order to prevent decreased metabolic activity due to the use of toxic extracts, methanolic and aqueous plant preparations were tested in MTT assay and the concentration, which did not reduce cell viability significantly, was used for uptake studies as shown in **Tables 2**, **3**. According to the results obtained from Hoechst Assay that showed an average coefficient of variation of 21.7% within 14 randomly tested well plates, uptake inhibition of plant extracts was distinguishable from general fluctuations in the procedure for values >25%. In every experiment, a control without extract was running and served as 100% uptake control. After correction of values by subtracting the blank, uptake inhibition was calculated as the difference between control and sample. The obtained values of monosaccharide uptake inhibition in Caco2 cells treated with methanolic and aqueous extracts are reported in **Tables 2**, **3**, respectively. Only the herbal drugs that showed the strongest inhibition rates on glucose uptake were tested for fructose uptake inhibition. For some of the plants, methanolic and aqueous extracts decreased glucose uptake significantly. However, only the extract of a plant that showed stronger inhibition on glucose absorption was repeated and tested for its impact on fructose uptake as described for the following plants. Additionally, the focus for further studies was on methanolic extracts as they naturally contain fewer monosaccharides, which could interfere with the measurement. The methanolic extracts made from the fruits of *Aronia melanocarpa* (uptake inhibition: 57.1%), *Cornus officinalis* (uptake inhibition: 73.8%), *Crataegus pinnatifida* (uptake inhibition: 73.7%), *Lycium chinense* (uptake inhibition: 75.2%), and *Vaccinium myrtillus* (uptake inhibition: 79.9%); the leaves of *Brassica oleracea* (uptake inhibition: 82.8%), *Juglans regia* (uptake inhibition: 52.4%), and *Peumus boldus* (uptake inhibition: 47.5%); and the roots of *Adenophora triphylla* (uptake

TABLE 2 | Results of glucose and fructose uptake studies with methanolic extracts, the used plant part, drug extract ratio, and tested concentration, which did not show any influence on cells in MTT assay. Only the plants that showed the strongest inhibition rates on glucose uptake were tested for fructose uptake inhibition. Regarding methanolic and aqueous extracts, the extract of a plant that showed the stronger inhibition on glucose absorption was repeated and tested for its impact on fructose uptake; mean value ± SD; *significant difference in *U*-test (n = 2 with 12 replicates each; two-sided; α = 0.05); n.d. = not determined.

Scientific plant name	Used part of the plant	Drug extract ratio for methanolic extracts	Used methanolic extract concentration in mg/mL	Uptake inhibition of glucose in %	Uptake inhibition of fructose in %
Adenophora triphylla (THUNB.) ADC.	Root	n.d.	1	64.6* ± 4.5	<25
Allium sativum L.	Bulb	20:1	0.01	<25	n.d.
Aronia melanocarpa MICHX. ELLIOTT	Fruit	n.d.	1	57.1* ± 7.2	<25
Artemisia dracunculus L.	Leaf	9:1	0.1	<25	n.d.
Brassica oleracea L. "*Capitata alba*"	Leaf	3:1	1	82.8* ± 4.3	<25
Camellia sinensis (L.) KUNTZE (Assam)	Leaf	11:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (Darjeeling)	Leaf	5:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (gunpowder)	Leaf	5:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (Sencha)	Leaf	5:1	0.1	<25	n.d.
Ceratonia siliqua L.	Fruit	4:1	1	<25	n.d.
Citrus limon (L.) OSBECK	Fruit skin	7:1	0.1	<25	n.d.
Coffea arabica L.	Green seed	7:1	1	<25	n.d.
Cornus officinalis SIEBOLD and ZUCC.	Fruit	3:1	1	73.8* ± 6.7	<25
Crataegus pinnatifida BUNGE	Fruit	n.d.	1	73.7* ± 4.2	<25
Cynara cardunculus L.	Herb	n.d.	0.1	<25	n.d.
Eucommia ulmoides OLIV.	Bark	n.d.	0.1	<30	n.d.
Hibiscus sabdariffa L.	Flower	4:1	0.1	<25	n.d.
Ilex paraguariensis A. ST.-HIL	Leaf	7:1	0.1	<25	n.d.
Juglans regia L.	Leaf	12:1	1	52.4* ± 1.7	30.2* ± 11.7
Lycium chinense MILL.	Fruit	n.d.	1	75.2* ± 0.5	<25
Melissa officinalis L.	Leaf	n.d.	0.1	<25	n.d.
Mentha aquatica L.	Leaf	16:1	0.1	<25	n.d.
Momordica charantia L.	Fruit	13:1	0.1	<25	n.d.
Nigella sativa L.	Seed	14:1	0.1	<25	n.d.
Olea europaea L.	Leaf	5:1	0.1	<25	n.d.
Origanum creticum L.	Leaf	16:1	0.1	<25	n.d.
Panax ginseng C.A. MEY	Root	9:1	0.1	<25	n.d.
Peumus boldus MOLINA	Leaf	5:1	1	47.5* ± 7.5	32.6* ± 26.1
Potentilla aurea L	Herb	15:1	1	<25	n.d.
Pueraria lobata (WILLD.) OHWI	Root	6:1	0.1	<25	n.d.
Rosa rugosa THUNB.	Flower	6:1	0.1	<25	n.d.
Rosmarinus officinalis L.	Leaf	6:1	0.1	<25	n.d.
Salvia officinalis L.	Leaf	9:1	0.1	<25	n.d.
Sarcopoterium spinosum (L.) SPACH	Root	14:1	0.1	<25	n.d.
Syzygium aromaticum (L.) MERR. and L.M.PERRY	Flower	17:1	0.1	<25	n.d.
Thymus vulgaris L.	Herb	14:1	0.1	<25	n.d.
Vaccinium myrtillus L.	Fruit	2:1	1	79.9* ± 1.1	<25
Vitis vinifera L.	Seed (pomace)	15:1	1	<25	n.d.

inhibition: 64.6%) indicated a strong and reproducible inhibitory potential between 40 and 80% on intestinal glucose transporters as visualized in **Figure 1**. Seven of these extracts showed higher uptake inhibition rates than the two controls phloretin (uptake inhibition: 60.4%) and phlorizin (uptake inhibition: 55.1%). A queous extracts made from the bark of *Eucommia ulmoides* (uptake inhibition: 68.7%) and the fruits of *Malus domestica* (uptake inhibition: 57.5%) inhibited glucose uptake significantly and reproducibly. Additionally, for the methanolic extract of *Brassica oleracea* and the aqueous extract of *Eucommia ulmoides*, IC_{50} was calculated as 0.0904 and 0.4285 mg/ml, respectively. These extracts decreased glucose uptake in a dose-dependent manner. The methanolic extracts of *Juglans regia* (uptake inhibition: 30.2%) and *Peumus boldus* (uptake inhibition: 32.6%) inhibited the fructose uptake significantly.

DISCUSSION

According to the results of the present study, the observed hypoglycemic effects in animal and/or human studies after treatment with preparations of *Adenophora triphylla*, *Aronia melanocarpa*, *Brassica oleracea*, *Cornus officinalis*, *Crataegus pinnatifida*, *Eucommia ulmoides*, *Juglans regia*, *Lycium chinense*, *Malus domestica*, *Peumus boldus*, and *Vaccinium myrtillus* as listed in **Table 1** possibly relate to the inhibition of intestinal transporters. The methanolic plant extracts made from the leaves of *Juglans regia* and *Peumus boldus* decreased glucose as well as fructose uptake possibly related to inhibition of GLUT2, which is able to transport both monosaccharides. These findings show that the traditional use of *Brassica oleracea*, *Cornus officinalis*, *Eucommia ulmoides*, *Juglans regia*, *Lycium chinense*, *Malus domestica*, and *Vaccinium myrtillus* and their preparations

TABLE 3 | Results of glucose and fructose uptake studies with aqueous extracts, the used plant part, drug extract ratio, and tested concentration, which did not show any influence on cells in MTT assay. Only the plants that showed the strongest inhibition rates on glucose uptake were tested for fructose uptake inhibition. Regarding methanolic and aqueous extracts, the extract of a plant that showed the stronger inhibition on glucose absorption was repeated and tested for its impact on fructose uptake; mean value ± SD; *significant difference in *U*-test (n = 2–3; 12 replicates each; two-sided; α = 0.05); n.d. = not determined.

Scientific plant name	Used part of the plant	Drug extract ratio for aqueous extracts	Used aqueous extract concentration in mg/mL	Uptake inhibition of glucose in %	Uptake inhibition of fructose in %
Adenophora triphylla (THUNB.) ADC.	Root	n.d.	1	<40	n.d.
Allium sativum L.	Bulb	2:1	1	<50	n.d.
Aronia melanocarpa MICHX. ELLIOTT	Fruit	3:1	1	<60	n.d.
Artemisia dracunculus L.	Leaf	5:1	1	<25	n.d.
Brassica oleracea L. "*Capitata alba*"	Leaf	2:1	0.1	<60	n.d.
Camellia sinensis (L.) KUNTZE (Assam)	Leaf	6:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (Darjeeling)	Leaf	7:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (gunpowder)	Leaf	5:1	0.1	<25	n.d.
Camellia sinensis (L.) KUNTZE (sencha)	Leaf	6:1	0.1	<25	n.d.
Ceratonia siliqua L.	Fruit	3:1	1	<25	n.d.
Citrus limon (L.) OSBECK	Fruit skin	4:1	1	<40	n.d.
Coffea arabica L.	Green seed	5:1	1	<25	n.d.
Cornus officinalis SIEBOLD and ZUCC.	Fruit	n.d.	1	<65	n.d.
Crataegus pinnatifida BUNGE	Fruit	n.d.	1	<60	n.d.
Cuminum cyminum L.	Seed	5:1	0.1	<25	n.d.
Cynara cardunculus L.	Herb	3:1	0.1	<30	n.d.
Eucommia ulmoides OLIV.	Bark	16:1	1	68.7* ± 13.2	<25
Hibiscus sabdariffa L.	Flower	2:1	0.1	<25	n.d.
Ilex paraguariensis A. ST.-HIL	Leaf	5:1	0.1	<25	n.d.
Juglans regia L.	Leaf	7:1	1	<25	n.d.
Lycium chinense MILL.	Fruit	3:1	1	<75	n.d.
Malus domestica BORKH. "*Gold en delicious*"	Fruit skin	3:1	1	57.5* ± 9.2	<25
Melissa officinalis L.	Leaf	4:1	0.01	<25	n.d.
Mentha aquatica L.	Leaf	6:1	1	<40	n.d.
Momordica charantia L.	Fruit	6:1	1	<25	n.d.
Nigella sativa L.	Seed	4:1	0.1	<25	n.d.
Olea europaea L.	Leaf	5:1	1	<25	n.d.
Origanum creticum L.	Leaf	4:1	0.1	<25	n.d.
Panax ginseng C.A. MEY	Root	3:1	1	<25	n.d.
Peumus boldus MOLINA	Leaf	n.d	1	<40	n.d.
Potentilla aurea L.	Herb	8:1	0.1	<25	n.d.
Pueraria lobata (WILLD.) OHWI	Root	4:1	1	<50	n.d.
Punica granatum L.	Fruit skin	3:1	0.1	<25	n.d.
Rosa rugosa THUNB.	Flower	n.d	0.1	<30	n.d.
Rosmarinus officinalis L.	Leaf	5:1	0.1	<25	n.d.
Salvia officinalis L.	Leaf	5:1	0.1	<25	n.d.
Sarcopoterium spinosum (L.) SPACH	Root	28:1	1	<25	n.d.
Syzygium aromaticum (L.) MERR. and L.M.PERRY	Flower	n.d.	0.1	<25	n.d.
Thymus vulgaris L.	Herb	6:1	1	<25	n.d.
Vaccinium myrtillus L.	Fruit	2:1	1	<80	n.d.
Vitis vinifera L.	Seed (pomace)	27:1	1	<40	n.d.

as antidiabetic remedy and adjuncts to conventional treatments in the therapy of Diabetes mellitus is reasonable. Extracts of these plants show inhibitory activity on intestinal glucose and fructose transporters resulting in lower blood concentrations of the monosaccharides and reduced concomitant health problems.

In literature, there is not much comparable data available, because the inhibition of intestinal transporters by crude plant extracts in Caco2 cells has not been investigated very well (Schreck and Melzig, 2018). Kim et al. determined a moderate inhibition of glucose uptake in Caco2 cells for methanolic extracts of *Adenophora triphylla* (uptake inhibition: 13.4%) and *Cornus officinalis* (uptake inhibition: 16.4%), but other results as for *Crataegus pinnatifida* (no uptake inhibition) are not consistent with our findings (Kim et al., 2011). Zhang et al. confirmed an uptake inhibition of 26.3% for *Eucommia ulmoides* (Zhang et al., 2015). Manzano et al. only tested specific polyphenol-rich fractions of *Malus domestica* (Manzano and Williamson, 2010), which showed inhibitory activity on intestinal glucose transporters, but it is not directly comparable with our work. Discrepancies between results from different studies probably depend on different preparations of the extracts, the use of modified substrates, and/or an insufficient validation of the cell model. We determined the expression of relevant transporters over three weeks in Caco2 cells and adapted our experimental settings in accordance with the ensured presence of functional transporters (Schreck and Melzig, 2019). Buchholz

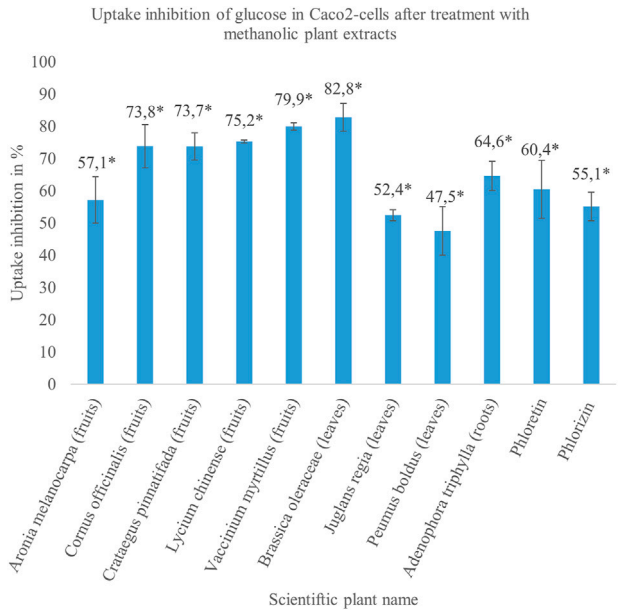

FIGURE 1 | The results of the methanolic plant extracts, which reduced glucose uptake between 40 and 80% in treated cells significantly compared to untreated cells in a concentration of 1 mg/ml. The extracts were made from different parts of the plants enclosed in brackets. The solutions of 100 µM phloretin and 100 µM phlorizin were used as positive controls and inhibited glucose uptake in Caco2 cells by approximately 60%. The illustrated results refer to the data in **Table 2**. Mean value ± SD. * Significant difference in *U*-test (n = 2 with 12 replicates each; two-sided, α = 0.05).

et al. examined whether extracts of the traditionally used plants inhibit cleavage of polysaccharides and lipids by affecting lipase and α-amylase activity (Prasain et al., 2012). The methanolic extracts of *Aronia melanocarpa*, *Peumus boldus*, and *Vaccinium myrtillus* showed strong lipase and α-amylase inhibition as well as inhibition of intestinal monosaccharide transporters in our studies. This multi-target effect of the mentioned plant preparations describes a partial, but multiple drug action and is proposed to exceed drugs with single-target effects. Based on synergistic mechanisms, the plants that affect various targets to alleviate monosaccharide uptake show therapeutic advantages concerning higher intended and lower adverse effects (Csermely et al., 2005). Since the plants, especially the fruits, contain sugars themselves, it cannot be totally excluded that a part of the inhibition is a "pseudo-inhibition" and comes from the displacement of the radioactive substrates by non-radioactive monosaccharides during absorption processes. For this reason, the focus of this work was on methanolic extracts that naturally contain fewer monosaccharides. Commonly, the discussed constituents of the plants with inhibitory effects on intestinal transporters are polyphenolic compounds as shown in **Table 1**. Particularly, the chalcones phlorizin and phloretin (Debnam and Levin, 1975; Zheng et al., 2012; Raja and Kinne, 2015), which are often used as positive controls in transport studies, the flavonoids quercetin, fisetin, and myricetin (Kwon et al., 2007) and the tannins (-)-epigallocatechin gallate, (-)-epigallocatechin, and (-)-epicatechin gallate seem to act as inhibitors of intestinal, monosaccharide transporters (Johnston et al., 2005). Further studies are planned to investigate which fraction of the extracts, or more specifically, which group of compounds are responsible for the inhibition of the intestinal glucose and fructose transporters in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

KS designed the studies, performed experiments, analyzed the data, and drafted the manuscript. MM supervised the performance of the experiments and interpretation of the data. MM proofread the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.692566/full#supplementary-material

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REVIEW
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Panax Ginseng C.A.Mey. as Medicine: The Potential Use of Panax Ginseng C.A.Mey. as a Remedy for Kidney Protection from a Pharmacological Perspective

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Panax ginseng C.A.Mey. has been widely consumed as food/diet supplements from natural sources, and its therapeutic properties have also aroused widespread concern. Therapeutic properties of Panax ginseng C.A.Mey. such as anti-inflammatory, ameliorating chronic inflammation, enhancing the immunity, resisting the oxidation again, and regulating the glucose and lipid metabolism have been widely reported. Recent years, lots of interesting studies have reported the potential use of Panax ginseng C.A.Mey. in the management of DKD. DKD has become the leading cause of end-stage renal disease worldwide, which increases the risk of premature death and poses a serious financial burden. Although DKD is somehow controllable with different drugs such as Angiotensin-Converting Enzyme Inhibitors (ACEI), Angiotensin Receptor Blockers (ARB) and lowering-glucose agents, modern dietary changes associated with DKD have facilitated research to assess the preventive and therapeutic merits of diet supplements from natural sources as medicine including Panax ginseng C.A.Mey. Findings from many scientific evidences have suggested that Panax ginseng C.A.Mey. can relieve the pathological status in cellular and animal models of DKD. Moreover, a few studies showed that alleviation of clinical phenotype such as reducing albuminuria, serum creatinine and renal anemia in DKD patients after application or consumption of Panax ginseng C.A.Mey.. Therefore, this review aims to discuss the effectiveness of Panax ginseng C.A.Mey. as medicine for targeting pathological phenotypes in DKD from a pharmacological perspective. This review will provide new insights into the potential understanding use of Panax ginseng C.A.Mey. in the management of DKD in clinical settings.

Keywords: ginseng, diet supplements, diabetic kidney disease, pathological phenotypes, pharmacological perspective -3 -

INTRODUCTION

An ancient proverb states "Food/Diet Supplements from Natural Sources as Medicine", and this adage is supported by the results of the Global Burden of Disease (GBD) Study 2017, which showed that dietary risk factors and poor diets contributed to 11 million premature deaths and 255 million disability-adjusted life-years. Another study reported that poor diets (such as ultra-processed foods, soft drinks, poultry or fish nuggets and salty snacks are associated with an increased risk of diabetes and its complications (Srour et al., 2020). These staggering data show that suboptimal diets may lead to more deaths and highlight the "Food/Diet Supplements from Natural Sources as Medicine" as a strategy for improving poor diet, combating the burden of non-communicable diseases, including diverse kidney diseases (KD). "Food/Diet Supplements from Natural Sources as Medicine" could potentially have positive effects on kidney protection (KP) (Stenvinkel et al., 2020).

Panax ginseng C.A.Mey. has been widely consumed as food/diet supplements from natural sources, and its therapeutic properties have also aroused widespread concern (**Figure 1**) (Fan et al., 2020a). Therapeutic properties of Panax ginseng C.A.Mey. such as anti-inflammatory, altering the composition and metabolism of the microbiota, ameliorating chronic inflammation, enhancing the immunity, resisting the oxidation again, and regulating the glucose and lipid metabolism have been widely reported (Cao et al., 2019; Jang et al., 2019; Liu et al., 2019, 2020; Xue et al., 2019; Huang et al., 2020; Quan et al., 2020; Xu et al., 2020). Intriguing new data suggest that Panax ginseng C.A.Mey. could minimize renal injury by inhibiting oxidative stress, inflammatory responses, epithelial-mesenchymal transition, and fibrosis (Liu et al., 2020; Shi et al., 2020; Xie et al., 2020; Zhu et al., 2020; Li et al., 2021). This finding is pertinent to the pathologic phenotype of KD, which is characterized by features of destruction of the glomerular filtration barrier involving podocyte, basement membrane and endotheliocyte (Thomas et al., 2015). Further, these pathological changes could contribute to the deterioration of renal function such as proteinuria, increased serum creatinine, changes in glomerular filtration rate and renal anemia (Thomas et al., 2015). Clinically, Panax ginseng C.A.Mey. also has been shown to possess beneficial effects in the treatment of KP (Lang et al., 1998; Zhao et al., 2007).

FIGURE 1 | The photo of Ginseng. The photo of Ginseng collected from Jilin province, one of major production regions of Ginseng in China.

Over the last decade, promising advancements have been made in the mechanisms and clinical outcomes of Panax ginseng C.A.Mey. on KD. Here, we are focusing on a discussion of Panax ginseng C.A.Mey. associating with KP. In addition, the possible active ingredients within Panax ginseng C.A.Mey. responsible for KP are elucidated. **Table 1** summaries the kidney protection performances of ginsenosides in preclinical studies. These contents will be overviewed in detail in the following sections.

POTENTIAL BIO-ACTIVE COMPOUNDS OF PANAX GINSENG C.A.MEY. IN KIDNEY PROTECTION

At present, more than 150 monomers have been identified and isolated from the roots, stems, leaves, flowers, and fruits of Panax ginseng C.A.Mey., of which more than 30 ginsenoside monomers have been identified in Panax ginseng C.A.Mey. as effective ingredients (Chang-Xiao and Pei-Gen, 1992; Nah, 1997; Wong et al., 2015). Panax ginseng C.A.Mey. triol (PT) saponins are also the main representative components of ginsenosides Rg1, Rd, Rb1, which have a high content of active parts and strong activity (Wong et al., 2015). Here, the compounds having potential beneficial effects on kidney protective function are highlighted (**Figure 2**).

PROTECTIVE EFFECT OF PANAX GINSENG C.A.MEY. ON RENAL INNATE CELLS

The preponderance of evidence supports the obvious: Panax ginseng C.A.Mey. can relieve renal innate cells damage and has a protective effect of Panax ginseng C.A.Mey. on the glomerular filtration barrier. The kidney filtration barrier is surrounded by three layers: 1) a fenestrated endothelium, 2) a basement membrane, and 3) the podocytes. The glomerular filtration barrier can effectively prevent albumin and larger molecular weight substances in the plasma from entering the urine. The changes in the structure and function of the glomerular filtration barrier caused by various reasons are the pathophysiological basis of proteinuria (Blaine and Dylewski, 2020). Studies have shown that proteinuria reflects not only kidney damage but also an independent risk factor leading to the progression of kidney disease (Webster et al., 2017). Therefore, understanding the molecular structure and function of the glomerular filtration barrier are essential for delaying the progression of KD. Here, we highlight Panax ginseng C.A.Mey. on kidney protection in the kidney filtration barrier and innate renal cells.

TABLE 1 | Ginsenosides tested in animal or cellular studies for human kidney-related diseases.

Model	Ginsenosides	Animal/cell type	Therapeutic mechanism	References
Targeting podocytes	Panax notoginseng saponins	STZ rats and podocyte cells	Inhibiting the podocyte cell apoptosis *via* reducing oxidative stress	Sun et al. (2011)
	Panax notoginseng saponins	STZ rats	Increasing the mRNA expression of nephrin, α3β1 integrin proteins	Zhou et al. (2014)
Targeting glomerular basement membrane nephritis	Rg1	STZ rats, podocyte cells and basement membrane cells	Inhibiting glomerular basement membrane nephritis through regulating the nuclear factor E2-related factor 2 (Nrf2) pathway	Guo et al. (2019b)
	Rg1	STZ rats, podocyte cells and basement membrane cells	Suppressing inflammation, oxidative stress *via* activating Nrf2 signal pathway	Li et al. (2020c), Alaofi (2020), Li et al. (2020b)
Targeting glomerular endothelial barrier	Rg1	HUVEC	Reducing the heparanase mRNA and transendothelial resistance and transendothelial albumin pass rate	Zhu et al. (2019)
Targeting glomerular mesangial cells	Rg1	HGMC	Inhibition of the TGF-β/Smad signaling pathway and glycosylation end products	Zhao (2018)
	Rb1	HBZY-1	Promoting the proliferation of mesangial cells, inhibiting cell apoptosis and Caspase-3 expression	Tang et al. (2013)
	Rg1	HGMC	Reducing the number of apoptotic cells, the activity of extracellular lactate dehydrogenase and block the cell cycle of human glomerular mesangial cells in G1 phase, and downregulate the mRNA and protein expression level of Cyclin-dependent kinase 4 (CDK4)	Wu and Wang (2015)
Targeting renal tubular cells	Rb1	HK-2	Activating the PI3K/AKT pathway and inhibiting the NF-KB pathway	Ni et al. (2017)
	Rb1	HK-2	Upregulating the expression of proliferating cell nuclear antigen	Yang et al. (2015)
	Rb1	Rats model reduced by cunilateral ureteral obstruction	Inhibiting the transcription and activation of transforming growth factor-β1	Xie et al. (2008), Li et al. (2015a)
Targeting chronic kidney disease	Rd	Rats model reduced by ischemia-reperfusion	Preventing oxygen free radicals from attacking the cell membranes	Yokozawa et al. (1998)
	Rb1	CKD patients	Reducing the levels of inflammatory factors TNF-a and 1L-6, and creatinine level	Xu et al. (2017)
Targeting diabetic kidney disease	Rb1	HGMC	Inhibiting the phosphorylation levels of P38 MAPK, JNK/SAPK and Akt	Park et al. (2010)
	Rb1	Rats model reduced by streptozotocin	reduce serum creatinine and urea nitrogen, mesangial hyperplasia of the glomerulus, and dilatation of renal tubules	Zhang et al. (2008), Zhao et al. (2008), Koizumi et al. (2013)
Targeting acute kidney injury	Rb1	Glycerol-induced acute r enal failure	Activating heme oxygenase (HO-1), Nrf2, and reducing ROS peroxidation	Sun et al. (2013), Sun, (2014)
	Rb1	Unilateral ureteral infarction in rats	Inhibit interstitial fibrous tissue, including tubular tissue damage and collagen deposition *via* reducing TGF-β1, HO-1and 8-OHdG	Xie et al. (2009a)
	Rb1	Renal artery ischemia of white rabbits	Downregulating the expression of Bcl-2 and Bax to inhibit apoptosis	Zhu et al. (2009)
	Rb1	Unilateral ureteral infarction in rats	Reducing the content of MDA and increase the activity of SOD in renal tissue	Sun et al. (2018)
Targeting renal senescence	Rb1	SAMP8 mice	Inhibiting the expression of the fibrinogen TGF-β1 and upregulating the renal protective factor BMP-7	Docherty et al. (2019)
Targeting renal fibrosis	Rg1	UUO animal models	Decreasing a-SMA and E-cadherin expression in the obstructed kidney models and reduce TGF-β1 induced by rat tubular cells	Xie et al. (2008), Xie et al. 2009b, Li et al. (2015a)
	Rg1	UUO animal models	Reverse EMT and renal interstitial fibrosis via targeting the TGF-β1/Smad pathway	Li et al. (2018)
	Ginseng extract	Cyclosporine A animal models; HK-2 cells	Improve renal function and inhibit apoptotic cell death	Doh et al. (2013), Liu et al. (2015)
	Ginseng extract	Cyclosporine A animal models	Targeting the Akt/mTOR pathway	Lim et al. (2014)
	Ginsenosides	Diabetic nephropathy rats	Protect kidney function *via* enhancing SIRT1 and suppressing inflammation	Du et al. (2016)

Improving Podocyte Injury

Podocytes are epithelial cells that regulate the glomerular filtration barrier, which characterized by actin-rich foot processes that reside on the glomerular basement membrane (GBM) (Leeuwis et al., 2010). The disappearance of podocyte foot processes is a sign of podocyte damage and proteinuric kidney disease, accompanied by

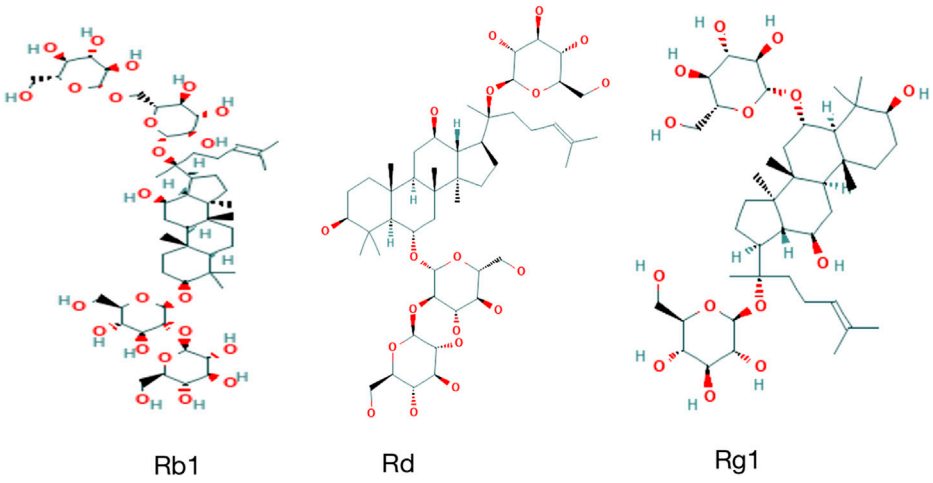

FIGURE 2 | Chemical structure of ginsenosides in this article.

changes in podocyte protein expression and reorganization of the actin cytoskeleton (Fan et al., 2021). Panax notoginseng is the main active ingredient of Panax notoginseng (Burkill) F.H.Chen including Ginsenoside, (Rg1, Rg2, Rb1, Rb2, Rb3, Rc, Rd, Re, Rh, F2), Panax notoginosides (R1, R2, R3, R6, Fa, Fc, Fe, R4). In recent years, the role of PNS in improving podocyte damage has received widespread attention. Sun et al. (2011) reported that in the STZ-induced Diabetic Nephropathy (DN) rat model and the podocyte cell model stimulated by high glucose, the podocyte apoptosis might be related to oxidative stress, and PNS can improve oxidative stress-related indicators *in vivo* and *in vitro*, as well as downregulate the expression of the apoptosis marker protein (caspase-3). In addition, Zhou et al. observed the protective effect of PNS on the podocytes of DN rat. The findings showed that after 10 weeks of PNS intervention, the number of podocytes, mRNA expression of nephrin, α3β1 integrin proteins in the kidney tissue have been significantly improved accordingly (Zhou et al., 2014). These studies demonstrate promising evidence that PNS can improve podocyte damage and delay the progression of DN.

Anti-inflammatory Effects on Glomerular Basement Membrane

The glomerular basement membrane (GBM) plays a key role in the maintenance of the structural integrity of the glomerular capillaries (Kalluri, 2003). Changes in the structural composition and thickness of the glomerular basement membrane can affect the occurrence and development of diverse kidney diseases. Ginsenoside Rg1 inhibits glomerular basement membrane nephritis through regulating the nuclear factor E2-related factor 2 (Nrf2) pathway and reduces the inflammation and apoptosis of podocytes induced by IL-1β, which inhibits the expression of Nrf2. Rg1 can increase the expression of Nrf2 (Guo et al., 2019b). Nrf2 is an important transcription factor that regulates the oxidative stress response of cells, and it is also a central regulator that maintains the intracellular redox homeostasis, reduces cell damage caused by reactive oxygen species and electrophiles, and maintains the body's redox homeostasis (Li et al., 2020c). Nrf2 activation can suppress inflammation, oxidative stress and kidney tissue impairment (Alaofi, 2020; Li et al., 2020b). Thence, Rg1 could be regarded as an activating agent of Nrf2 for prevention strategy for DN progression.

Protection of Glomerular Endothelial Barrier Function

Over the past decade, the preponderance of evidence supports that Panax ginseng C.A.Mey. can improve vascular endothelial function in several diseases (Nabavi et al., 2015). The destruction of the endothelial barrier is critical for vascular complications related to diabetes, and damage to the endothelial glycocalyx has been shown to be involved in this process (Fu et al., 2015). Rg 1 is the main active ingredient extracted from Panax notoginseng and has been widely used to prevent vascular damage. Recent research (Zhu et al., 2019) showed that high glucoscouldan induce endothelial glycocalyx disorders and increase the expression of heparanase mRNA in HUVEC, and Rg1 treatment can reverse this expression. In addition, after high glucose stimulation, Rg1 treatment can reduce transendothelial resistance and transendothelial albumin pass rate. It is worth noting that Rg1 has a protective effect on endothelial barrier dysfunction.

Inhibition Apoptosis of Glomerular Mesangial Cells

Glomerular mesangial cells (MCs) are major components of the glomerular mesangium, hold the capillary arteries and connect them with the juxtaglomerular apparatus. More and more evidences are suggesting the abnormal growth of MCs is an early event in various glomerular diseases (Yoon et al., 2020; Wan et al., 2021). Targeting glomerular mesangial cells as the research object and screening for suitable drugs to repair the damage caused by oxidative stress and inflammation have

important scientific research significance and clinical value. Rg 1 has been demonstrated to have a wide range of pharmacological properties, such as anti-inflammatory, anti-oxidation, anti-ageing, anti-fatigue and anti-tumour activities (Shen et al., 2017; Fan et al., 2020b). Rg1 has an anti-inflammatory effect, and the anti-inflammatory effect may be related to the inhibition of the TGF-β/Smad signaling pathway (Zhao 2018). A certain concentration of ginsenoside Rg 1 can inhibit glycosylation end products (AGEs) caused by TNF-α And cyclooxygenase-2 (COX-2) (Zhao. 2018). Notably, Rg 1 can promote the proliferation of mesangial cells and inhibit cell apoptosis, which may be related to the promotion of Bcl-2 and the inhibition of Caspase-3 expression (Tang et al., 2013). Besides, in the oxidative stress injury model of human glomerular mesangial cells induced by H_2O_2, Rg 3 can reduce the number of apoptotic cells damaged by H_2O_2, the activity of extracellular lactate dehydrogenase (LDH), and the content of malondialdehyde (MDA) as well as block the cell cycle of human glomerular mesangial cells in G1 phase, and downregulate the mRNA and protein expression level of Cyclin-dependent kinase 4 (CDK4) (Wu and Wang, 2015). These evidences reflect that Rg 1 exerts renal protection by inhibiting the apoptosis of glomerular mesangial cells.

Inhibition Epithelial-Mesenchymal Transdifferentiation of Renal Tubular Cells

Epithelial-mesenchymal transition (EMT) is a biological process that directs changes in cell states along the epithelial versus mesenchymal axes, which is a hallmark of tubulointerstitial renal fibrosis (Chen et al., 2020). In addition, the nuclear factor-KB (NF-KB) pathway mainly mediates the inflammatory response, which is associated with activation for phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinase (Akt) pathway (Yang et al., 2019). These signal pathways play a key role in the EMT.

Evidence from several *in vitro* studies has suggested that Rg 1 plays a positive role in improving EMT of renal tubular cells. NI et al. found that ginsenoside Rg l protects the human renal tubular epithelial cell line HK-2 from lipopolysaccharide (LPS)-induced inflammation and apoptosis *via* activating the PI3K/AKT pathway and inhibiting the NF-KB pathway, thereby inhibiting the EMT in renal tubular cells (Ni et al., 2017). This result was confirmed by Yang et al. They used urine protein to induce renal tubular epithelial cell damage and reduce the cell survival rate. After administration of Rg l, the cell survival rate was improved. And the expression of proliferating cell nuclear antigen (PCNA) protein and PCNA mRNA were upregulated (Yang et al., 2015). The results from the *in vitro* studies were confirmed using an *in vivo* model. In a rat model of renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO), Rg1 inhibits the transcription and activation of transforming growth factor-β1 (TGF-β1) and inhibits the transdifferentiation of tubular epithelial myofibroblast (Tubular epithelial myofibroblast transdifferentiation, TEMT) (Xie et al., 2008; Li et al., 2015a). These data provided evidence that the tubular epithelial cells were responding to Rg1treating EMT.

ROLE OF PANAX GINSENG C.A.MEY. IN DIFFERENT NEPHROPATHY

Kidney diseases worldwide prevalence have reached epidemic proportions globally in the last few decades. Several drugs that provide kidney function protection have been widely used. Unfortunately, drug resistance and adverse events have limited their applicability in the clinic. Recently, Panax ginseng C.A.Mey. and Panax ginseng C.A.Mey. extracts have attracted much attention as effective and safe alternative drugs for kidney diseases. Here, we will comprehensively summarize the current studies of Panax ginseng C.A.Mey. in different kidney diseases.

Chronic Kidney Disease

Chronic Kidney Disease (CKD) usually has concealed onset and gets progressively worse, followed by an increasing burden of hypertension, atherosclerosis, calcium and phosphorus metabolism disorders, renal anaemia and other complications, which is a cause of substantial healthcare expenditure (Chen et al., 2019; Tonelli and Dickinson, 2020). Oxidative stress is known as the main mechanism of CKD, and antioxidants have potential value in the treatment of CKD (Podkowińska and Formanowicz, 2020). Most studies have proved that ginsenosides have good anti-oxidation and free radical scavenging functions *in vivo* and *in vitro* (Jung et al., 1998; Kim et al., 2000; Kim et al., 2013). Some scholars investigated the effect of Rd in rats with ischemia-reperfusion. Findings indicate that ginsenoside-Rd could affect cultured proximal tubule cells subjected to hypoxia-reoxygenation, probably by preventing oxygen free radicals from attacking the cell membranes (Yokozawa et al., 1998). This was demonstrated in a preliminary clinical trial by Xu et al. CKD patients on oral Rb1 (500 mg) were found to be more likely to have a lower level of creatinine than among those on the placebo group. Additionally, compared with the placebo group, the of the active Oxygen is significantly improved, and the levels of inflammatory factors TNF-a and 1L-6 are significantly reduced in the Rb1 group (Xu et al., 2017).

Diabetic Kidney Disease

Diabetic kidney disease (DKD) is one of the common and serious long-term complications of hyperglycemia, and long-term glucose metabolic disorder is the main cause of DKD (Barrera-Chimal and Jaisser, 2020). The clinical manifestations are reduced glomerular filtration rate, followed by microalbuminuria, elevated arterial blood pressure, and fluid retention, leading to renal failure (Matoba et al., 2019). In the primary mesangial cell model induced by high glucose, it was found that ginsenoside Rb1 mainly inhibited the phosphorylation levels of $_P$38 MAPK, JNK/SAPK and Akt, and thus suppressed the expression of mesangial fibroconnectin induced by high glucose (Park et al., 2010). At animal levels, Rb1 can improve the quality of life of diabetic nephropathy induced by streptozotocin in rats, reduce serum creatinine and urea nitrogen, mesangial hyperplasia of

the glomerulus, and dilatation of renal tubules, mainly *via* down-regulating mRNA and protein expression of MCP-1 mRNA and TGF-β1 mRNA in kidney tissue (Zhao et al., 2008; Zhang et al., 2008; Koizumi et al., 2013). In addition, clinically, strict glycaemia and blood pressure control can also slow the progression of DKD (Doshi and Friedman, 2017). Panax ginseng C.A.Mey. plays an important role in the improvement of hypertension, hyperglycaemia and lipid metabolism disorders. Rb1 can reduce the complications of diabetic nephropathy by reducing free fatty acids, promoting lipid metabolism, improving insulin resistance in obese mice, inhibiting the levels of TNF-a and IL-6 inflammatory factors, and the decomposition of adipocytes (Wang, 2011). Ginsenosides (Rg1, Rg3, Rb1 and compound K) act on the targets of Caspase-3, Bel-2, MDA, and SOD, reducing the gluconeogenesis, lipid metabolism, inflammatory and oxidation in diabetic nephropathy, which can be used as a potential adjunctive drug for the treatment of diabetes (Bai et al., 2018; Shao et al., 2020).

Acute Kidney Injury

Acute kidney injury (AKI) is a common clinical critical illness, mainly manifested as the accumulation of metabolic substances and declined renal functions (Guo et al., 2019a). Renal ischemia and reperfusion injury (IRI) is a major cause of acute kidney injury (AKI) (Singbartl and Kellum, 2012). Ginsenosides are reported to have antioxidant and anti-inflammatory effects, as well as remit kidney damage caused by intestinal ischemia in mice. Rb1 can activate heme oxygenase (HO-1), Nrf2, and reduce ROS peroxidation damages and protect mitochondrial function to reduce kidney damages (Sun et al., 2013; Sun, 2014). In acute kidney injury mediated by unilateral ureteral infarction in rats, Rb1 can significantly inhibit interstitial fibrous tissue, including tubular tissue damage and collagen deposition *via* reducing TGF-β1, HO-1and 8-OHdG (Xie et al., 2009a). In the renal artery ischemia of white rabbits, Rb1 can downregulate the expression of Bcl-2 and Bax to inhibit apoptosis and reduce kidney damage (Zhu et al., 2009). Similarly, Sun et al. found that Rb1 can reduce the content of MDA and increase the activity of SOD in renal tissue, and then downregulate the expression of Caspase-3 in renal cells, thus alleviating the apoptosis of renal cells induced by ischemia and reperfusion to protect the kidney function (Sun et al., 2018).

Renal Senescence

Progressive renal recession is a common phenomenon in the ageing process, and ageing-related declines in renal function are associated with a progressive loss of functioning nephrons (Fang et al., 2020). Glomerular basement membrane thickening, mesangial matrix hyperplasia, segmental glomerulosclerosis, renal tubular atrophy and tubulointerstitial fibrosis, arterial intimal fibrous thickening are the main histological features of renal ageing (Docherty et al., 2019). Ginsenoside Rbl can inhibit the expression of the fibrinogen TGF-β1 and upregulate the renal protective factor BMP-7 to reduce the abnormal accumulation of ECM components in the process of renal ageing, thereby harnessing tubular interstitial damage and glomerular sclerosis in the kidney ageing in SAMP8 mice (Docherty et al., 2019).

Renal Fibrosis

Chronic kidney disease, as a global public health burden, has attracted great attention. So far, it has faced huge challenges due to the lack of effective treatment strategies (Yang et al., 2020). Renal fibrosis is an important pathological process from chronic kidney disease to end-stage renal diseases (Humphreys, 2018). Ginsenosides have shown to exert a renoprotective effect. Findings from Xie et al. showed that ginsenoside Rg1 could retard interstitial fibrosis in the UUO animal models (Xie et al., 2008). Intriguingly, Rg1 also was reported that Rg1 could decrease a-SMA and E-cadherin expression in the obstructed kidney models and reduce TGF-β1 induced by rat tubular cells, hinting that the potential mechanism might be partly related to the kickbacking of EMT (Xie et al., 2008; Xie et al., 2009b; Li et al., 2015a). Besides this, their results showed that Rg1 could reverse EMT and UUO-induced renal interstitial fibrosis *via* targeting the TGF-β1/Smad pathway (Li et al., 2018).

As a commonly used clinical immunosuppressant, cyclosporine A (CsA) has been widely prescribed for inhibiting rejection after organ transplantation. Many experimental studies showed that long-term use of CsA could contribute to progressive renal interstitial fibrosis, renal cell apoptosis, and immune cell infiltration (Wu et al., 2018; Lin et al., 2019). Findings from Doh's group showed that Panax ginseng C.A.Mey. extract could effectively improve renal function and inhibit apoptotic cell death *in vivo* and vitro (Doh et al., 2013; Liu et al., 2015). Meanwhile, the Akt/mTOR pathway participating in Panax ginseng C.A.Mey. extract for the CsA animal model has been identified, which might be involved in autophagosome formation and autophagic aggregates (Lim et al., 2014). In diabetic nephropathy rats, the protective role of ginsenosides on diabetic nephropathy was also verified *in vivo* experiments. Du et al. suggested that Panax ginseng C.A.Mey. administration could protect kidney function *via* enhancing SIRT1 and suppressing inflammation in diabetic nephropathy rats (Du et al., 2016). In light of the above, ginsenosides were suggested as an important option during the treatment of renal fibrosis.

PANAX GINSENG C.A.MEY. FOR KIDNEY-RELATED DISEASES IN CLINICAL TRIALS

In recent years, accumulating evidence has revealed that Panax ginseng C.A.Mey. has beneficial effects against diabetes, obesity, stroke, and cardiovascular diseases (Kim, 2012; Rastogi et al., 2014; Zhang et al., 2014; Gui et al., 2016; Hong et al., 2020). However, fewer data are available in the field of Panax ginseng C.A.Mey. for kidney-related diseases in clinical trials. Here, we summarized this topic while adding some important updates and focused on the clinical trials of kidney-related diseases. Li et al. implemented a randomized

single-blinded trial with an administration of American Panax ginseng C.A.Mey. compound liquor or American Panax ginseng C.A.Mey. liquor. Their results showed that Panax ginseng C.A.Mey was beneficial to improve microcirculation, reduce whole blood viscosity and decrease urinary albumin so as to retard the progress of DKD (Lang et al., 1998). The results agreed with the findings of CKD patients from Peng and Guo (2010) and Xu et al. (2017). Peng et al. focused on the Panax notoPanax ginseng C.A.Mey. for chronic renal failure (CRF), which the study showed that Panax notoPanax ginseng C.A.Mey. possessed such therapeutic effects as improving the renal function and lowering urine protein (Peng and Guo, 2010). The aim of Xu et al. study (Xu et al., 2017) was to evaluate the effects of Rb1 (500 mg daily oral administration) prospectively in patients with early CKD (stage 2 or 3) for 6 months. Findings showed that GS-Rb1 could present an antioxidant-based approach to slow the progression of CKD at the early stages (Xu et al., 2017). However, some studies concluded the opposite (Stavro et al., 2006; Kulaputana et al., 2007). Panax ginseng C.A.Mey. did not affect serum cystatin C level, 24-h BP and renal function (Stavro et al., 2006), as well as an ergogenic property on aerobic fitness enhancement in well-fit individuals (Kulaputana et al., 2007). The conflicting evidence outlined above might be due to the poor research quality employed. The reason can attribute to the following reasons: 1) the sample size of these studies was relatively small and results in weak statistical power. 2) patients with CKD form heterogeneous study populations, so that it may be more difficult to control for confounding. Especially, DKD population is highly heterogeneous in terms of comorbid illnesses and functional impairments. 3) Under population heterogeneity, selection of the features that affect the drug sensitivity has not been addressed in trials. These factors may have contributed to the inconsistency of these findings.

LIMITATIONS AND FUTURE PERSPECTIVES

Ginsenosides as natural medicine have been widely approved to exert therapeutic effects in kidney-related *in vivo* and vitro. However, human clinical studies present several limitations. There are several existing obstacles concerning the utilization of ginsenosides or Panax ginseng C.A.Mey. before translation into clinical application is made possible. One of the most difficult handicaps is a lack of high-quality, evidence-based medical evidence for Panax ginseng C.A.Mey. treating kidney-related diseases. In respect of study population selection, the current research is still insufficient due to the focusing on a single participant (such as DKD, CKD); thus, further exploration (such as IgA nephropathy, Kidney ageing) is required. In terms of the selection of the indicators, the current study focuses on more serum creatinine levels, proteinuria, urea nitrogen, and physiological and biochemical parameters, less than estimated glomerular filtration rate (eGFR), urine albumin-creatinine ratio (UACR) and the composite of renal outcomes (annual rate of change in GFR, doubling of serum creatinine level or 50% reduction in GFR, end-stage renal disease).

In addition, CKD may have electrolyte abnormalities, proteinuria, and anemia. It is a key role to control these risk factors. However, there is currently a lack of evidence to support that. Future CKD should focus not only on the inherent cells but also on the electrolyte abnormalities, proteinuria, and anemia. Although creatinine levels, proteinuria, urea nitrogen, and physiological and biochemical parameters could reflect renal functions in part, these outcomes might not show a replaceable role in the progression of renal diseases (Luo et al., 2019; Kurth et al., 2020). Therefore, evaluating the composite of renal outcomes in clinical trials is a future direction for researchers. Performing well-designed, randomized, placebo-controlled clinical trials for ginsenosides in humans are very urgent and pivotal.

Furthermore, oral bioavailability has been reported to affect the efficacy of some drugs. It has been demonstrated that the majority of ginsenosides had low oral bioavailability, which limits their long-term efficacy and stability (Kim et al., 2014; Biswas et al., 2017; Chen et al., 2018; Jin et al., 2018). Some scholars tend to explain the factors that lead to low oral bioavailability of ginsenosides, which large molecular weight (Han et al., 2006), low water-solubility (Liu et al., 2009), poor gastrointestinal stability of ginsenosides (Artursson et al., 1993), and intestinal or hepatic first-pass effect, then leading to low oral bioavailability (Yanni, 2007). Future studies should address these issues to fully determine the bioavailability of ginsenosides *via* building effective drug delivery systems, including vesicles (Chen et al., 2014), microsphere (Wei et al., 2007), micelles (Xiong et al., 2008), emulsion delivery systems (Li et al., 2015b), and nanoparticle drug delivery systems (Cai et al., 2014).

The safety of Panax ginseng C.A.Mey. in clinical practices is an issue of widespread concern. Although the efficacy and safety of Panax ginseng C.A.Mey. or ginsenosides have been confirmed in these clinical trials in Table X, the safety should be evaluated with caution due to the low quality and quantity of research. Moreover, natural products are the basis of traditional Chinese medicine, which the most biological function is entirely by identifying their pharmacologically active ingredients. Hence, it is difficult to ensure safety (Li et al., 2020a). In clinical applications, possible side effects need much-weighted attention.

Evidence that ginsenosides have significant effects on anti-oxidative, anti-inflammatory, anti-apoptotic, and anti-fibrosis, with different molecular mechanisms. These processes participate in the regulation of physiological and pathological conditions and are implicated in kidney disease development (Kim et al., 2019; Knoppert et al., 2019; Alicic et al., 2021). However, a comprehensive understanding of the mechanisms that ginsenosides regulate in diverse pathological conditions is lacking. Additionally, more in-depth research should explore the detailed molecular mechanisms of action and pharmacokinetic profile *in vivo*. Careful study will be required to resolve these issues in the future.

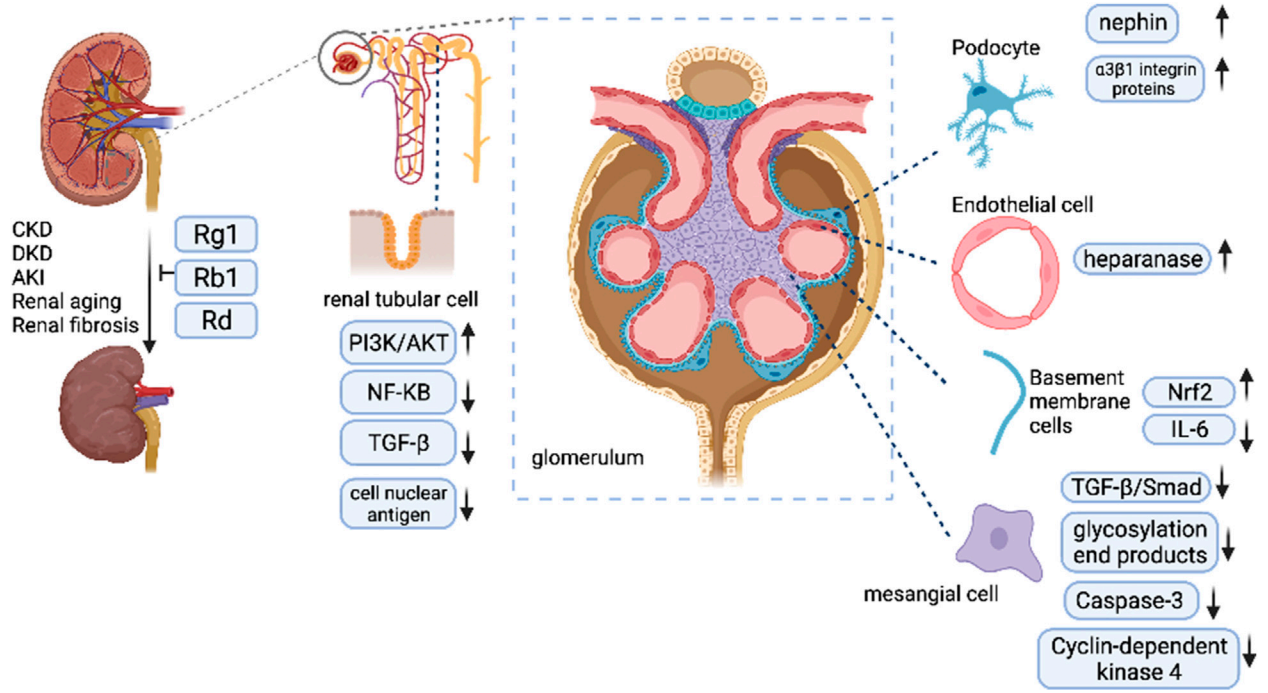

FIGURE 3 | Ginsenosides exert their kidney-protected effects.

CONCLUSION

In this work, we summarize brief information on diverse types of studies *in vitro* and vivo concerning kidney diseases that suggest similar pathological mechanisms, including inflammatory response, apoptosis of innate renal cells, vascular endothelial function, renal senescence, and renal fibrosis. These pathological processes have been identified, all of which might be influenced by ginsenosides. In clinical applications, its efficacy has been demonstrated in several clinical trials. As an advantage to anti-inflammatory, ginsenosides can improve glomerular endothelial barrier function; alleviate the apoptosis of renal cells induced by ischemia and reperfusion to protect the kidney function in AKI; inhibit the excessive accumulation of ECM, including collagen and fibronectin as well as fibrotic markers, especially TGF-β1 in renal tubular cells; inhibit apoptosis of glomerular mesangial cells; decrease the damage to podocyte cells, including apoptotic and necrotic changes. Ginsenosides exert their kidney-protected effects in the kidney (**Figure 3**). Collectively, ginsenosides are capable of being a remedy for kidney protection.

Effective therapeutics is currently available for this situation in several clinical trials. However, most of these studies reported contradictory or conflicting findings because of low methodological quality. For this reason, strong evidence is still needed to solve this issue, such as RCTs of rigorous trial design methods and a large, multicenter sample. Moreover, abundant studies have repetitively revealed the therapeutic potential of ginsenosides in kidney-associated human diseases. Meanwhile, new guidelines about Panax ginseng C.A.Mey. usage are imperative to guarantee safety and effectiveness, which is vital for standardized operation practice and experimentally controlled methods. This review provides the first systematic summary of studies examining the role of kidney protection, which will be helpful in the development of kidney-related diseases therapy in the future and gain more reliable and reproducible data.

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

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frontiers in Pharmacology

REVIEW
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Lychee Seed as a Potential Hypoglycemic Agent, and Exploration of its Underlying Mechanisms

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Food is people's primal want. A reasonable diet and healthy food not only provide nutrients for human growth but also contribute to disease prevention and treatment, while following an unhealthy diet can lead to an increased risk of many diseases, especially metabolic disorders, such as diabetes. Nature is enriched with different food sources, and it seems that purely natural products are more in line with the current concept of health, which enhance the formation of the notion that "Food/Diet Supplements from Natural Sources as a Medicine." As a delicious fruit, the medicinal values such as anticancer, antibacterial, antioxidation, and antiglycating properties of lychee have been found. Lychee (*Litchi* in Chinese) is a subtropical fruit plant belonging to the family Sapindaceae. It has been widely cultivated in warm climates worldwide, particularly in China, for thousands of years. In recent years, various phytochemical components such as quercetin, procyanidin A2, and (2R)-naringenin-7-O-(3-O-αL-rhamnopyranosyl-β-D-glucopyranoside) have been identified in a lychee seed, which may lend a lychee seed as a relatively safe and inexpensive adjuvant treatment for diabetes and diabetic complications. In fact, accumulating evidence has shown that lychee seed, lychee seed extracts, and related compounds have promising antihyperglycemic activities, including improving insulin resistance, anti-inflammatory effect, lipid regulation, neuroprotection, antineurotoxic effect, and renoprotection effect. In this review, we summarized publications on antiglycemic effects and mechanisms of lychee seed, lychee seed extracts, and related compounds, which included their efficacies as a cure for diabetes and diabetic complications in cells, animals, and humans, attempting to obtain a robust evidence basis for the clinical application and value of lychee seed.

Keywords: lychee seed, diet supplements, diabetes, pharmacological mechanisms, effect

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INTRODUCTION

Diabetes is a severe, long-term (or chronic) disease in the world, defined as a blood glucose profile higher than normal, due to a disturbed insulin secretion or a disturbed insulin effect or usually both (Petersmann et al., 2019). Based on the most recent data issued by the International Diabetes Federation (IDF) (Saeedi et al., 2019), the number of adults aged 20–79 years globally with diabetes has reached nearly 463.0 million in 2019. It is estimated that the number will rise to 578.4 million by 2030, and 700.2 million by 2045, which means that the global diabetes epidemic markedly increases at an incredible speed among populations. Obviously, it has become a significant global public health

TABLE 1 | The antihyperglycemic activity and the mechanisms of lychee seed in clinical trials, *in vitro*, and *in vivo* studies.

Activities	Pharmacological effects	Model	Material	Dose/ concentration (route of administration)	Duration	Species/exposure	Reference
Anti-diabetes	Decreased FPG	In vivo	Extract tablets of lychee seed	30 g/d (p.o., *n* = 45)	12 weeks	Patients with T2D	Zhang and Teng (1985)
	Lowered blood glucose and ameliorated symptoms	In vivo	Extract tablets of lychee seed water	3.6–5.4 g/d (p.o., *n* = 30)	12 weeks	Patients with T2D	Shen (1991)
	Lowered blood glucose	In vivo	Lychee seed ethanol extract	160 mg/kg/d (i.g., *n* = 10)	2 weeks	STZ-diabetic Wistar rats (220 ± 20 g)	Ren (2011)
	Lowered blood glucose	In vivo	Lychee seed extract fluid	0.1 ml/d (i.g., *n* = 20)	15 days	Alloxan induced diabetic mice (30–40 g)	Guo et al. (2013)
	Lowered blood glucose	In vivo	Lychee seed concentrated decoction	20 g/kg/d (i.g., *n* = 10)	10 days	Alloxan-diabetic Kunming mice (18–22 g)	Guo et al. (1999)
	Lowered blood glucose	In vivo	Total saponins of lychee seed	500 mg/kg/d (i.g.,*n* = 8)	2 weeks	Alloxan-diabetic Kunming male mice (18–20 g)	Yuan et al. (2006)
	Lowered blood glucose	In vivo	The alcohol extracted fract from lychee seed	300 mg/kg/d (i.g., *n* = 8)	2 weeks	High-fat/high-sucrose diet- diabetic SD rats (220 ± 20 g)	Jiang (2011)
	Lowered blood glucose	In vivo	The alcohol extracted fract from lychee seed	300 mg/kg/d (i.g., *n* = 8)	2 weeks	STZ-diabetic SD rats (220 ± 20 g)	Jiang et al. (2011)
	Lowered blood glucose	In vivo	Dry extract of lychee seed	2.6 mg/kg/d (i.g., *n* = 11)	30 days	Alloxan-diabetic Wistar rats (200 g)	Shen et al. (1986)
	Lowered blood glucose	In vivo	Lychee seed extract fluid	0.4 ml/d (containing 0.04 g crude drug,i.g., *n* = 14)	7 days	Alloxan-diabetic Kunming male mice (22–31 g)	Liang et al. (2009)
	Lowered blood glucose	In vivo	Lychee seed extract fluid	0.2 ml/d (i.g., *n* = 9)	7 days	Alloxan-diabetic Kunming mice (23–28 g)	Li et al. (2008)
	Lowered FBG and 2 h BG after OGTT; Improved IGT; Lowered FSG	In vivo	Saponin of lychee seed	0.2 g/kg/d (i.g., *n* = 12)	7 days	DX-induced insulin resistant SD rats (150–180 g)	Guo et al. (2003b)
	Displayed α-glucosidase inhibitory activity	In vitro	Total flavonoids of lychee seed	1 mg/ml	None	α -glucosidase inhibitory assay	Ren et al. (2017)
	Lowered FBG	In vivo	Lychee seed extract	0.3 ml (containing 0.015 g litchi seed extract, i.g.,n = 5)	12 weeks	db/db male mouse	Zhang et al. (2013)
	Lowered FBS	In vivo	Lychee seed extract fluid	0.4 ml/d (i.g., *n* = 12)	7 days	Alloxan-diabetic Kunming mice (22.5–25.5 g)	Chen et al. (2008)
	Displayed α-glucosidase inhibitory activity	In vitro	Lychee seed extract fluid	40 μL	None	α -glucosidase inhibitory assay	Zhou (2016)
	Displayed α-glucosidase inhibitory activity	In vitro	The crude extract, sugar-removed layer, pavetannin B2, procyanidin A2	IC_{50}: 0.691 μg/ml, 3.686 μg/ml, 0.04 μM, and 0.08 μM	None	α -glucosidase inhibitory assay	Choi et al. (2017)
	Decreased FPG	In vivo	Water extract of lychee seed	0.1 ml/kg/d (i.g., *n* = 10)	10 days	Alloxan-diabetic Kunming mice	Kuang et al. (1997)
	Displayed α-glucosidase inhibitory activity	In vitro	Polysaccharide	IC_{50}: 0.056 mg/ml	None	α -glucosidase inhibitory assay	Zhang et al. (2020)
	Displayed α-glucosidase inhibitory activity	In vitro	(2R)-Naringenin-7-O-(3-O-α-L-rhamnopyranosyl-β-D-glucopyranoside), (2S)-Pinocembrin-7-O-(6-O-α-Lrhamnopyranosyl-β-D-glucopyranoside)	1 mg/ml	None	α -glucosidase inhibitory assay	Ren et al. (2011)
	Reduced FBG and 1-h postprandial blood glucose	In vivo	Polysaccharides	400 mg/kg (i.g., *n* = 8)	30 days	Alloxan-diabetic ICR male mice (22–24 g)	Yuan (2010)
	Decreased FPG	In vivo	Lychee seed decoction	30 g/kg/d (i.g., *n* = 4)	2 weeks	Alloxan-diabetic SD male rats	Wu et al. (1991)
	Decreased FPG	In vivo			3 weeks		

(Continued on following page)

TABLE 1 | (*Continued*) The antihyperglycemic activity and the mechanisms of lychee seed in clinical trials, *in vitro*, and *in vivo* studies.

Activities	Pharmacological effects	Model	Material	Dose/ concentration (route of administration)	Duration	Species/exposure	Reference
			Total saponin extract from lychee seed	500 mg/kg/d (i.g., $n = 10$)		Alloxan-diabetic Kunming male mice (18–20 g)	Lou et al. (2007)
	Decreased FPG	In vivo	Lychee seed extract fluid	0.1 ml/d (i.g.,n = 8)	4 days	Alloxan-diabetic mice (20–24 g)	Chen et al. (2006)
	Displayed α-glucosidase inhibitory activity	In vitro	Semen lychee effective fractions	50 g/L	None	α -glucosidase inhibitory assay	Zhong et al. (2015)
	Inhibited the activities of both yeast and mammalian α-glucosidase	In vitro	Polysaccharide	IC_{50}: 75.24 μM, 66.97 μM	None	Yeast (*Saccharomyces cerevisiae*) and mammalian (rat-intestinal acetone powder) α-glucosidase	Wu et al. (2020)
Improving insulin resistance	Reduced the levels of TNF-α, hyper-leptinemia, and FFA	In vivo	Lychee seed extract fluid	3.8 g/kg/d (i.g., $n = 16$)	31 days	STZ-diabetic SD rats (150–180 g)	Guo et al. (2004)
	Decreased the mRNA expression of RETN, PTP1B, and GRP78	In vitro	Semen lychee effective constituents	0.2 mg/ml	48 h	DX-induced insulin resistant 3T3–L1 cells	Liao et al. (2014)
	Inhibited the mRNA expression of GRP78 and CHOP	In vivo	Lychee semen effective constituents	0.47 g/kg/d (i.g., $n = 8$)	4 weeks	High-fat feeding combined with STZ-diabetic SD rats (180–200 g)	Li et al. (2015)
	Improved IR	In vivo	Lychee seed water extractant	3.8 g/kg/d (i.g., $n = 16$)	31 days	High caloric diet combined with STZ-diabetic SD rats (150–180 g)	Guo et al. (2003a)
	Reduced IRI; increased ISI	In vivo	Lychee seed extracts	30 mg/d (i.g., $n = 6$)	6 weeks	STZ/high-fat diet induced SD rats (100–130 g)	Man et al. (2016)
	Changed microRNAs expression	In vivo	Lychee seed extracts	0.015 g/d (i.g., $n = 5$)	12 weeks	db/db male mouse	Zhang et al. (2013)
Antioxidant effect	Increased the activity of SOD; decreased content of MDA	In vivo	Lychee seed extract fluid	3.8 g/kg/d (i.g., $n = 16$)	31 days	STZ-diabetic SD rats (150–180 g)	Guo et al. (2004)
	Improved activity of SOD; decreased the content of MDA	In vivo	Saponin of lychee seed	0.2 g/kg/d (i.g., $n = 12$)	7 days	DX-induced insulin resistant SD rats (150–180 g)	Guo et al. (2003b)
	Scavenge free radicals	In vitro	Total flavonoids of lychee seed	IC_{50}: 0.00016 mg/ml	None	DPPH radical scavenging assay	Ren et al. (2017)
	Increased the activity of SOD; decreased content of MDA	In vivo	Lychee seed water and alcoholic extracts	62.50 g/kg/d(i.g., $n = 12$)	8 days	Alloxan-diabetic NIH mice(18–22 g)	Pan et al. (1999)
	Increased the level of GSH-PX; reduced the content of oxygen free radical	In vivo	Lychee seed extract fluid	0.1 ml/d (i.g., $n = 8$)	4 days	Alloxan-diabetic mice (20–24 g)	Chen et al. (2006)
	Accelerated the clearance of O_2-	In vivo	Lychee seed extract fluid	0.5 ml/d (containing 0.012 g crude drug, i.g., $n = 10$)	7 days	Alloxan-diabetic Kunming male mice (25–30 g)	Li et al. (2006)
Anti-inflammatory effect	Downregulation expression of TGF-β1,MCP-1 and MIF	In vivo	Lychee semen effective constituents	6 g/kg/d (i.g., $n = 10$)	6 weeks	High-sugar/high-fat feeding SD male rats (200–220 g)	Qi (2017)
	Increased mRNA levels of NF-κB	In vivo	Lychee seed extracts	30 mg/d (i.g., $n = 6$)	6 weeks	(STZ)/high-fat diet induced SD rats (100–130 g)	Man et al. (2016)
	Inhibited the expression of MCP-1 and ICAM-1 protein in kidney tissue; reduced IL-1β and IL-6 in serum	In vivo	Saponin of lychee seed	2.5 mg/kg/d (i.g., $n = 6$)	8 weeks	STZ-diabetic Kunming male mice (18–22 g)	Qin (2017)
Lipid regulation	Lowered concentrations of TC and TG	In vivo	Lychee seed extract fluid	3.8 g/kg/d (i.g., $n = 16$)	31 days	STZ-diabetic SD rats (150–180 g)	Guo et al. (2004)
	Reduced serum TG level	In vivo	Lychee semen effective constituents	0.47 g/kg/d (i.g., $n = 8$)	4 weeks	High-fat feeding combined with STZ-	Li et al. (2015)

(Continued on following page)

TABLE 1 | (*Continued*) The antihyperglycemic activity and the mechanisms of lychee seed in clinical trials, *in vitro*, and *in vivo* studies.

Activities	Pharmacological effects	Model	Material	Dose/ concentration (route of administration)	Duration	Species/exposure	Reference
						diabetic SD rats (180–200 g)	
	Reduced the content of TC, TG, and LDL-C	In vivo	Saponin of lychee seed	0.2 g/kg/d (i.g., *n* = 12)	7 days	DX-induced insulin resistant SD rats (150–180 g)	Guo et al. (2003b)
	Reduced the content of TC and TG; increased the content of HDL-C	In vivo	Lychee seed water and alcoholic extracts	62.50 g/kg/d (i.g., *n* = 12)	8 days	Alloxan-diabetic NIH mice (18–22 g)	Pan et al. (1999)
	Reduced the content of TC, TG; increased the content of HD L-C and ratio of HDL-C/TC	In vivo	Lychee seed water extractant	3.8 g/kg/d (i.g., *n* = 16)	31 days	High caloric diet combined with STZ-diabetic SD rats (150–180 g)	Guo et al. (2003a)
	Prevented the decrease of AMPK alpha 2 and p-AMPK levels; inhibited the synthesis of fatty acid, protein, and lipid metabolism	In vitro	Lychee semen active ingredients (LSE70 and LSE50)	2.5 μg/ml	24 h	HepG2 cell insulin resistance model	Man et al. (2019)
	Decreased TG, T-CHO, and LDH; increased the ratio of HDL-C to LDL-C	In vivo	Lychee seed extracts	30 mg/d (i.g., *n* = 6)	6 weeks	STZ/high-fat diet induced SD rats (100–130 g)	Man et al. (2016)
	Increased the content of HDL-C	In vivo	Lychee seed extract fluid	0.1 ml/d (i.g., *n* = 8)	4 days	Alloxan-diabetic mice (20–24 g)	Chen et al. (2006)
Kidney protection effect	Restrained the expression of HMC cell protein	In vitro	Saponin of lychee seed	20 mg/ml	48 h	HMC	Zhang (2016)
	Inhibited the proliferation of HBZY-1; reduced the protein level of TGF-β1, FN, and Col Ⅳ	In vitro	Total flavonoids of lychee	40 μg/ml	48 h	HBZY-1 induced by high glucose combined with TNF-α	Liu (2016)
	Inhibited the expression of MCP-1 and ICAM-1 protein; reduced the content of IL-1β and IL-6	In vivo	Saponin of lychee seed	2.5 mg/kg/d (i.g., *n* = 6)	8 weeks	STZ-diabetic Kunming male mice (18–22 g)	Qin (2017)
	Decreased the protein expression of FN and Col Ⅳ	In vitro	Total flavonoids of lychee	40 μg/ml	48 h	HBZY-1 induced by high glucose combined with TNF-α	Liu et al. (2016)
Neuroprotection and cognitive function improvement	Decreased Aβ and Tau deposition	In vivo	Lychee seed extract	2.78 g/kg/d (i.g., *n* = 12)	4 weeks	STZ-diabetic SD rats (180–220 g)	Zeng et al. (2016)
	Improved the transmit function of cholinergic nerve system in the cerebrum of mice	In vivo	Lychee seed extract fluid	0.5 ml/d (containing 0.012 g crude drug, i.g., *n* = 10)	7 days	Alloxan-diabetic Kunming male mice (25–30 g)	Li et al. (2006)
	Inhibited Tau hyperphosphorylation through improving IR *via* the IRS-1/PI3K/Akt/GSK-3β pathway	In vitro	Catechin, procyanidin A1, and procyanidin A2	10 μM	24 h	DX-induced HepG2 and HT22 cells	Xiong et al. (2020)
	Decreased Aβ, AGEs, and Tau protein	In vivo	Lychee seed extract	0.7 g/kg/d (i.g., *n* = 10)	28 days	High-fat/high-sugar/ high protein feeding combined with STZ-diabetic rats	Tang et al. (2018)

Abbreviation: fasting plasma glucose (FPG); type 2 diabetes (T2D); streptozotocin (STZ); Sprague-Dawley (SD); fasting blood glucose (FBG); 2-h blood glucose (2 h BG); oral glucose tolerance test (OGTT); impaired glucose tolerance (IGT); serum contents of fasting glucose (FSG); Dexamethasone (Dx); Institute of Cancer Research (ICR); fasting blood sugar (FBS); tumor necrosis factor-α (TNF-α); free fatty acids (FFAs); insulin resistance (IR); insulin resistance index (IRI); insulin sensitivity index (ISI); National Institutes of Health (NIH); superoxide dismutase (SOD); malondialdehyde (MDA); 1,1-diphenyl-2-picrylhydrazyl (DPPH); glutathione peroxidase (GSH-Px); transforming growth factor-β1 (TGF-β1); monocyte chemotactic protein 1(MCP-1); macrophage migration-inhibitory factor (MIF); nuclear factor-κB (NF-κB); intercellular cell adhesion molecule-1(ICAM-1); interleukin-1β (IL-1β); interleukin-6 (IL-6); total cholesterol (TC); triglyceride (TG); low-density lipoprotein cholesterol (LDL-C); high-density lipoprotein cholesterol (HDL-C); Hepatocellular carcinoma cell (HepG2 cell); total cholesterol (T-CHO); lactate dehydrogenase (LDH); human glomerular mesangial cells (HMC); rat glomerular mesangial cells (HBZY-1); fibronectin(FN); collagen IV (Col IV); amyloid beta (Aβ); advanced glycation end products (AGEs).

problem. Individuals with diabetes are more prone to develop complications such as retinopathy, nephropathy, coronary artery disease, peripheral arterial disease, and stroke, contributing to higher mortality rates (Ford, 2011; Tandon et al., 2012). Thus, the prevention and treatment of diabetes hold considerable importance. Currently, the main treatments for diabetes include insulin injection, oral diabetes medications, and pancreatic islet transplantation (Ryan et al., 2005; Doyle-Delgado et al., 2020). However, the available treatments only delay the progress of the disease rather than curing it, leading to the lengthy and costly therapy, and comprise side effects, which impart a heavy economic and psychological burden on patients.

Food is the first necessity of people. Poor diet is associated with a higher risk of many diseases (GBD 2017 Diet Collaborators, 2019), especially diabetes (Srour et al., 2020), while some healthy food is reported to improve glycemic control (Reynolds et al., 2020). Studies supported a positive association between dietary intake of momordica charantia and blood sugar reduction (Kibiti and Afolayan, 2015). Buckwheat also had effects on reducing serum glucose concentrations in diabetic rats (Kawa et al., 2003). Consequently, the notion that "Food/Diet Supplements from Natural Sources as a Medicine" has become popular and appealing among diabetic patients. In China, herbs in nature with homology of medicine and food have been widely studied. Lychee, a fruit tree belonging to family Sapindaceae, originating from China, is widely cultivated in warm climates in many regions around the world and is botanically related to Litchi chinensis Sonn (Jiang, 2003; Yang et al., 2006; Huang et al., 2014). Due to the high nutrients and savory flavor as well as the attractive red, lychee is widely favored by humans. Lychee seed, the dried mature seed of lychee, an ancient traditional drug–food homologous herbal medicine, was used to smooth Qi, dispel cold, alleviate polydipsia, and relieve pain in China (Kilari and Putta, 2016). Accumulating research studies recently focused on the antidiabetic activity of lychee seed, although the underlying mechanisms of action have not been studied thoroughly. A summary of antidiabetic studies of lychee seed will be helpful for offering a reference basis for deeper investigations and clinical use of this natural herb medicine (**Table 1**). We examined the electronic resources with the PubMed, EMBASE, Web of Science, and China National Knowledge Infrastructure based on the information limited to English and Chinese literatures up till Jun 2021.

BOTANICAL DESCRIPTIONS OF LYCHEE

Lychee (*Litchi chinensis* Sonn.) is a subtropical medium evergreen dome-shaped tree with a glossy grayish stem belonging to the family Sapindaceae. It generally grows to less than 10 m in height, rarely up to 15 m or more. The pinnate leaves which consist of 4–8 pairs of elliptic or lanceolate, long acuminate, glabrous leaflets are leathery, 5–7 cm long, and 2–4 cm broad. The yellowish-white flower is small in size with a tetramerous calyx. Terminal inflorescence is about 5–30 cm long with multibranched panicles and slender pedicels. The fruit is ellipsoidal or nearly round in shape, estimated at 2.5 cm in diameter and clothed with a coarse thicker ring or pericarp with strawberry to red in color at maturity. Inside the pericarp is lychee aril that is milky-white and semitransparent with a sweet, juicy, and delicious taste. A seed with a smooth and glossy surface is brown or reddish brown in color and elliptic to ovate in shape covered by a fleshy aril. The size of the seed varies greatly between 1 and 2 cm in length, as shown in **Figure 1**.

THE HISTORY OF LYCHEE SEED

Lychee seed, the dry mature seed of *Litchi chinensis* Sonn, is known to have a remarkable medicinal value in ancient China. The oldest available Chinese written source which described the application of lychee seed is Ben Cao Yan Yi traced back to the Song dynasty (AD 1116). In ancient Chinese medical practices, lychee seed was always used for hernia, orchitis, ulcers, and intestinal troubles. Diabetes-related symptoms were known as "Xiaoke" (emaciation and thirst) in the ancient Chinese medical literature (Tong et al., 2012). In Compendium of Materia Medica (Ben Cao Gang Mu) written by Li Shizhen (from 1518 to 1593 AD) during the Ming dynasty, the lychee seed was warm in nature, sweet in taste, and could act as a beneficial agent in thirst-quenching. Numerous Chinese patent medicines such as Jinlida granule (Tian et al., 2018) and Jiangtangtongmai tablets (Su et al., 2017) approved by the Chinese Food and Drug Administration, containing lychee seed, were clinically used for the treatment of diabetes.

POTENTIAL BIOACTIVE COMPOUNDS OF LYCHEE SEED RESPONSIBLE FOR HYPOGLYCEMIC ACTIVITIES

Lychee seed is thought to improve glycemic control *via* various bioactive compounds with great pharmaceutical and biomedical potential. The flavanones, flavonols, proanthocyanidins, and dihydrochalcone fractions of lychee seed are the most investigated for their hypoglycemic activities. With the continuous optimization of the lychee seed extraction process, a number of monomers have been successfully identified and isolated. Here, several single isolated compounds including (2R)-naringenin-7-O-(3-O-αL-rhamnopyranosyl-β-D-glucopyranoside) (Ren et al., 2011), (2S)-Pinocembrin-7-O-(6-O-a-L-rhamnopyranosyl-β-D-glucopyranoside) (Ren et al., 2011), quercetin (Ren et al., 2013), procyanidin A1 (Xiong et al., 2020), procyanidin A2 (Choi et al., 2017), and phlorhizin (Ren et al., 2013) exhibiting potential beneficial effects on regulating glycemia are prominently described in **Table 2**.

PHARMACOLOGY

Improving Insulin Resistance

Insulin has a pivotal function in ensuring the homeostasis of energy metabolism through a coordination of the storage and

FIGURE 1 | Lychee fruit, lychee aril, and lychee seed (color version of these figures is available at https://699pic.com/tupian-500450779.html?bindPhone=1, https://item.m.jd.com/product/53077629044.html?).

TABLE 2 | Antihyperglycemic compounds isolated from lychee seed.

Compound	Molecular formula	Reference
Flavanones		
2R)-Naringenin-7-O-(3-O-α-L-rhamnopyranosyl-β-D-glucopyranoside)	$C_{27}H_{32}O_{14}$	Ren et al. (2011)
(2S)-Pinocembrin-7-O-(6-O-a-L-rhamnopyranosyl-β-D-glucopyranoside)	$C_{27}H_{32}O_{13}$	Ren et al. (2011)
Flavonols		
Quercetin	$C_{15}H_{10}O_7$	Ren et al. (2013)
Proanthocyanidins		
Procyanidin A1	$C_{31}H_{28}O_{12}$	Xiong et al. (2020)
Procyanidin A2	$C_{30}H_{24}O_{12}$	Choi et al. (2017), Xiong et al. (2020)
Dihydrochalcones		
Phlorhizin	$C_{21}H_{24}O_{10}$	Ren et al. (2013)

utilization of fuel molecules in insulin-targeted organs (Castan-Laurell et al., 2012). Insulin resistance (IR) is a pathological condition defined by the inability of insulin to stimulate glucose disposal and is considered as a key player in the development of type 2 diabetes mellitus (Brown and Walker, 2016). Although the precise pathophysiology of IR in diabetes has not yet been delineated, inflammatory response, oxidative stress, insulin receptor mutations, endoplasmic reticulum stress, and mitochondrial dysfunction are currently regarded as the possible underlying mechanisms (Yaribeygi et al., 2019). Consequently, numerous genes such as INS, AKT1, IL-6, TP53, TNF, VEGFA, MAPK3, EGFR, EGF, and SRC have been revealed to be associated with the development of IR (Gao et al., 2020). The relatively prominent signaling pathways involved in the formation of IR are the pathways of insulin resistance, adipocytokine, insulin, PI3K-Akt, ERK, AMPK, and HIF-1 (Ozaki et al., 2016; Huang et al., 2018; Gao et al., 2020). In the glucose tolerance test, intragastric administration of a lychee seed water extractant remarkably decreased hyperinsulinemia and potentiated insulin sensitivity (Guo et al., 2003a). Another study indicated that lychee seed extracts could increase the quality of life of streptozotocin (STZ) combined with a high-fat diet–induced type 2 diabetes rats. Compared to the control group, the insulin resistance index in the lychee seed extract group was dramatically reduced, which in turn increased the insulin sensitivity index progressively (Man et al., 2016). The PI3K/AKT/mTOR signaling pathway makes essential contribution to the occurrence of IR. Activation of the PI3K/AKT/mTOR signaling pathway could improve insulin-induced glucose (Yin et al., 2017; Han et al., 2020). Lychee seed extracts significantly improved IR in a type 2 diabetes mouse model by elevating the expression levels of PI3K, AKT, and mTOR to trigger the PI3K/AKT/mTOR signaling pathway (Man et al., 2017). Recently, growing evidence has shown that microRNAs as crucial regulators of gene expression perform a critical role in the development of IR (Honardoost et al., 2014; Wen et al., 2014; Xihua et al., 2019). One study showed that the microRNA expression changed significantly in db/db mouse administered extract of lychee seed (0.015 g/d, i.g.) (Zhang et al., 2013). In addition, abundant studies have demonstrated that endoplasmic reticulum stress–induced pancreatic β-cell destruction is one of the vital mechanisms of progression for both insulin-dependent diabetes and non–insulin-dependent diabetes (Cnop et al., 2017). Endoplasmic reticulum stress can not only directly damage the insulin signaling pathway but also further promote IR in a variety of ways (Ozcan and Tabas, 2012; Dong et al., 2017). Experiments *in vitro* have confirmed that lychee semen effective constituents can significantly reduce the mRNA expression of glucose regulatory protein 78 (Grp78) (Liao et al., 2014; Li et al., 2015) which contributes to endoplasmic reticulum stress and the activation of unfolded protein response (UPR). Elevated pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and leptin levels have been demonstrated to be closely associated with IR (Ayina et al., 2016; Alzamil, 2020). In addition, plasma

free fatty acid (FFA) is viewed as a potential factor to IR and disrupts insulin secretion (Bergman and Ader, 2000). Lychee seed extracts could improve insulin sensitivity by reducing the levels of TNF-α, hyper-leptinemia, and FFA in diabetic rats (Guo et al., 2004).

Antioxidant Effect

Oxidative stress is induced by an imbalance between the production of free radicals and the antioxidant mechanisms, which is a well-known contributor to the pathogenesis and progression of diabetes *via* several molecular mechanisms, such as β-cell dysfunction and defects of the normal insulin signaling pathways (Yaribeygi et al., 2020). In addition, the excessive production of reactive oxygen species (ROS) inside the cell occupies a pivotal role in the onset of oxidative stress (Zhang et al., 2016). The body produces excess ROS, which is known to enhance nuclear factor (NF)-κB activity (Zheng et al., 2015), β-cell maturation, and apoptosis increase. In a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, the total flavonoids of lychee seed showed a potent antioxidant activity (Ren et al., 2017). Moreover, lychee seed extracts could significantly accelerate the clearance of O_2^- in the cerebrum of mice with diabetes induced by alloxan (Li et al., 2006). Malondialdehyde (MDA), an oxidative stress marker, is produced when ROS within cells oxidize unsaturated fatty acids (Kwiecien et al., 2014). Several animal studies revealed that lychee seed extracts remarkably improved the activity of superoxide dismutases (SODs) which was the central antioxidant defense system against O_2^- (Fukai and Ushio-Fukai, 2011) and decreased MDA in animal models of diabetic rats (Pan et al., 1999; Guo et al., 2003b; Guo et al., 2004).

Anti-Inflammatory Effect

The relationship between inflammation and diabetes has received extensive attention. It is believed that diabetes is a chronic inflammatory state (Wellen and Hotamisligil, 2005). Indeed, recent studies have emphasized and found substantial evidence that many inflammatory cytokines such as transforming growth factor beta 1 (TGF-β1) (Herder et al., 1984-2002; Olivieri et al., 2010; Shi et al., 2018), monocyte chemotactic peptide 1 (MCP-1) (Reddy et al., 2017), and macrophage migration-inhibitory factor (MIF) (Sánchez-Zamora and Rodriguez-Sosa, 2014; Abu El-Asrar et al., 2019) are reported to be responsible for the pathogenesis of the development of diabetes or diabetes complications. The currently available medical therapy mainly targets the underlying etiology. Hence, inhibition of excessive inflammatory responses might provide a potentially promising candidate for future therapeutics of diabetes. Lychee seed extracts could alleviate the inflammation reaction in rats with impaired glucose tolerance, which was associated with the downregulation expression of TGF-β1, MCP-1, and MIF (Qi, 2017). NF-κB is central to inflammatory responses and is tightly linked to various inflammatory diseases. Lychee seed extracts directly affected the mRNA levels of NF-κB, which prevented diabetes (Man et al., 2016). An experiment in diabetic nephropathy mice models has revealed that the saponin of lychee seed could delay the diabetic kidney inflammation development through inhibiting the expression of MCP-1 and intercellular cell adhesion molecule-1 (ICAM-1) protein in the kidney tissue, reducing the content of pro-inflammatory cytokines including interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the serum (Qin, 2017).

Lipid Regulation

Glucose and lipid metabolism are intrinsically related to one another in many aspects. Diabetic dyslipidemia is common in individuals with diabetes (Athyros et al., 2018). The pathophysiological mechanism of diabetic dyslipidemia is highly complex and multifactorial, yet accepted as a preponderant contributor in the occurrence of diabetic dyslipidemia is IR with an attendant increase in free fatty acid flux into the liver (Mooradian, 2009). The most predominant clinical presentation of the interaction is marked by elevated triglycerides (TGs), decreased high-density lipoprotein cholesterol (HDL-C), and predominance of small-dense low-density lipoprotein (LDL) (Parhofer, 2015). The saponin of lychee seed affected the lipid metabolism in dexamethasone (DX)-induced insulin-resistant rats by lowering the content of total cholesterol (TC), TG, and low-density lipoprotein cholesterol (LDL-C) (Guo et al., 2003b). Simultaneously, the potential lipid-modifying effect of lychee seed extracts was also demonstrated by the other two animal studies (Pan et al., 1999; Li et al., 2015).

Kidney Protection Effect

Diabetic kidney disease (DKD), a severe microvascular complication of diabetes, is the primary cause of end-stage renal failure and the single strongest predictor of mortality in diabetic patients (Reidy et al., 2014; Thomas et al., 2016). Strict glycemic management dramatically reduces DKD morbidity, which suggests that metabolic disorders resulting from hyperglycemia, including changes in energy utilization and mitochondrial damage, exert a critical role in the disease progression (Reidy et al., 2014). Presently, multifactorial management of DKD primarily includes diet therapy, glucose-lowering therapy, lipid control, and preserving renal function (Selby and Taal, 2020). Despite various therapeutic strategies, the morbidity and mortality of DKD remain high throughout the world. Traditional Chinese herbal medicine can possess antidiabetic effects and improve renal function on DKD obviously. Research showed that saponin of lychee seed could reduce the blood glucose and ameliorate pathological damage and kidney lesions of diabetic nephropathy model rats through repressing the expression of inflammatory factors and attenuating inflammatory responses in kidney tissue (Qin, 2017). Out of many cytokines implicated in fibrosis, transforming growth factor-β1 (TGF-β1), fibronectin (FN), and collagen IV (Col IV) promoting extracellular matrix (ECM) accumulation are the most notorious (Downer et al., 1988; Chen et al., 2017). In the rat mesangial cells induced by high glucose and tumor necrosis factor-α (TNF-α), the total flavonoids of lychee seed distinctly decreased the protein expression of TGF-β1, FN, and Col IV, which indicated the total flavone might improve the diabetic nephropathy fibrosis process (Liu, 2016; Liu et al., 2016). In addition, the saponin of the lychee seed was

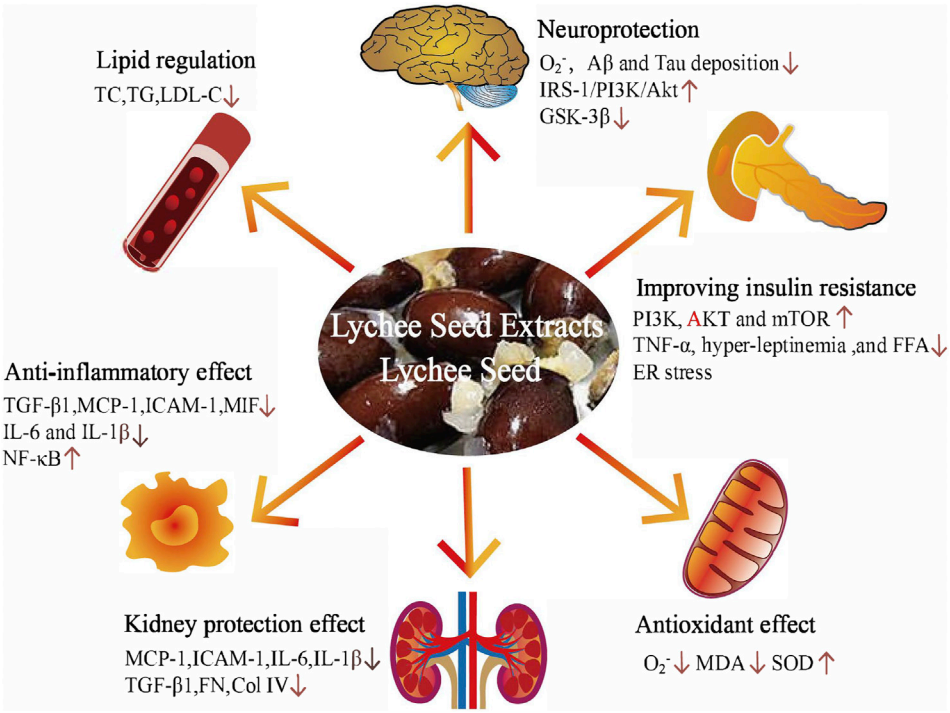

FIGURE 2 | The underlying mechanism of hypoglycemic activity of lychee seed and lychee seed extracts. Amyloid beta (Aβ); tumor necrosis factor-α (TNF-α); free fatty acids (FFAs); endoplasmic reticulum (ER); malondialdehyde (MDA); superoxide dismutase (SOD); monocyte chemotactic protein 1 (MCP-1); intercellular cell adhesion molecule-1 (ICAM-1); interleukin-6 (IL-6); interleukin-1 (IL-1β); transforming growth factor-β1 (TGF-β1); fibronectin (FN); collagen IV (Col IV); macrophage migration-inhibitory factor (MIF); nuclear factor-κB (NF-κB); cholesterol (TC); triglyceride (TG); and low-density lipoprotein cholesterol (LDL-C).

confirmed to obviously reduce the content of IL-6 and IL-1β secreted by human glomerular mesangial cells (HMC) and decrease the secretion of ECM to slow down the sclerosis process of glomerulus (Zhang, 2016).

Neuroprotection and Cognitive Function Improvement

Cognitive dysfunction is considered as a serious and common comorbidity or even a complication of diabetes (Biessels and Despa, 2018). They share common biological mechanisms including deficits in insulin signaling, neuroinflammatory pathways, mitochondrial (Mt) metabolism, the sirtuin-peroxisome proliferator-activated receptor-gamma coactivator 1α (SIRT-PGC-1α) axis, and Tau signaling (Zilliox et al., 2016). An animal test showed that the lychee seed extract fluid could protect the nervous system by significantly improving the transmit function of the cholinergic nervous system in the cerebrum of mice with diabetes induced by alloxan and accelerating the clearance of O_2^- (Li et al., 2006). In another research, compared with the model group, amyloid beta (Aβ) and Tau deposition of the experimental rats in the medium- and high-dose lychee seed extract administration groups [1.39 and 2.78 g/(kg.d)] were significantly decreased (Zeng et al., 2016). Similarly, investigators have found that lychee seed extracts consisting of numerous ingredients such as adenosine, 5-hydroxymethyluridine, and 4-p-coumaroylquinic acid dramatically protected against neuronal damage and prevented the decline in the cognitive function through lowering serum glucose, ameliorating IR, and suppressing the aggregation of Aβ, Tau protein, and advanced glycation end products (AGEs) in the hippocampus of type 2 diabetes rats (Tang et al., 2018), while further study demonstrated that polyphenols derived from lychee seed inhibited hyperphosphorylated Tau through improving IR *via* upregulating IRS-1/PI3K/Akt and downregulating GSK-3β (Xiong et al., 2020).

Based on studies on diabetes and diabetic complication intervention with lychee seed *in vivo* and *in vitro*, the underlying hypoglycemic mechanisms of lychee seed are summarized in **Figure 2**.

CONCLUSION AND PERSPECTIVE

The rising prevalence and financial burden of diabetes and its complications have made it one of the greatest health threats facing the 21st century. Although significant advances have been made toward a long-term therapeutic approach to treat diabetes, it is tough to control the blood glucose level precisely, and the use of oral hypoglycemic agents comes with many limitations, including side effects (gastrointestinal intolerance and myocardial events) (Nissen and Wolski, 2007; McCreight et al., 2016). Lychee seed as a natural source showed antidiabetic effects from lowering blood glucose to alleviating

diabetic complications. Its beneficial effects have also been validated by several clinical observations (Zhang and Teng, 1985; Shen, 1991). Through the literature review, the underlying mechanisms, improving insulin resistance, antioxidant effect, anti-inflammatory effects, lipid regulation, kidney protection effect, and neuroprotection and cognitive function improvement of lychee seed in treating diabetes are also worth investigating. For further research of lychee seed within this field, several issues should be considered. An *in vitro* research showed that saponin of lychee seed had no effect on glycometabolism in an insulin resistance model of pepatocellular carcinoma (HepG2) cells (Qin, 2017), which may be related to the site of drug action. The impact of saponin of lychee seed on improving IR may not be effected in hepatocytes but in other peripheral tissues such as muscle and fat. Thus, the corresponding site of action of lychee seed needs to be explicitly investigated. Most of the elucidation of the antidiabetic mechanisms scratches only at the surface, and researchers need to probe deeper into analyzing the detailed molecular mechanisms of the effects of lychee seed intervention. Consequently, comprehensive and much more robust evidence is desperately needed. As outlined in the above review, although some clinical studies show positive results in the treatment of diabetes, large, double-blind, randomized, placebo-controlled, multicenter clinical trials are needed.

In conclusion, lychee seed might be developed as a multi-target agent and prescribed as a useful adjuvant to the current treatment for diabetes and especially diabetic complications. Despite the enormous therapeutic potential, further comprehensive investigation from bench to clinical reasearch is warranted.

AUTHOR CONTRIBUTIONS

FL designed the study and is the corresponding author. YZ, DJ, and XA drafted the manuscript and figure. LD and YD drafted the table. All authors approved the final version of the manuscript.

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Phytopharmacological Evaluation of Different Solvent Extract/Fractions From *Sphaeranthus indicus* L. Flowers: From Traditional Therapies to Bioactive Compounds

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Sphaeranthus indicus L. is a medicinal herb having widespread traditional uses for treating common ailments. The present research work aims to explore the in-depth phytochemical composition and *in vitro* reactivity of six different polarity solvents (methanol, *n*-hexane, benzene, chloroform, ethyl acetate, and *n*-butanol) extracts/fractions of *S. indicus* flowers. The phytochemical composition was accomplished by determining total bioactive contents, HPLC-PDA polyphenolic quantification, and UHPLC-MS secondary metabolomics. The reactivity of the phenolic compounds was tested through the following biochemical assays: antioxidant (DPPH, ABTS, FRAP, CUPRAC, phosphomolybdenum, and metal chelation) and enzyme inhibition (AChE, BChE, α-glucosidase, α-amylase, urease, and tyrosinase) assays were performed. The methanol extract showed the highest values for phenolic (94.07 mg GAE/g extract) and flavonoid (78.7 mg QE/g extract) contents and was also the most active for α-glucosidase inhibition as well as radical scavenging and reducing power potential. HPLC-PDA analysis quantified rutin, naringenin, chlorogenic acid, 3-hydroxybenzoic acid, gallic acid, and epicatechin in a significant amount. UHPLC-MS analysis of methanol and ethyl acetate extracts revealed the presence of well-known phytocompounds; most of these were phenolic, flavonoid, and glycoside derivatives. The ethyl acetate fraction exhibited the highest inhibition against tyrosinase and urease, while the *n*-hexane fraction was most active for α-amylase. Moreover, principal component analysis

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CUPRAC, cupric reducing antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, Ethylenediaminetetraacetic acid; FRAP, ferric reducing antioxidant power; MTT, 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; PCA, principal component analysis; UHPLC-MS, ultra-high-performance liquid chromatography–mass spectrometry

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highlighted the positive correlation between bioactive compounds and the tested extracts. Overall, *S. indicus* flower extracts were found to contain important phytochemicals, hence could be further explored to discover novel bioactive compounds that could be a valid starting point for future pharmaceutical and nutraceuticals applications.

Keywords: *Sphaeranthus indicus*, phytochemicals, antioxidant, enzyme inhibition, UHPLC-MS, HPLC-PDA

1 INTRODUCTION

Plants are essential for human life for survival, shelter, food, and medicine. Great interest has developed for an investigation into medicinal plants as a novel source of enzymes inhibitors, natural antioxidant compounds, and treating a number of common ailments (Phumthum et al., 2018). Since times immemorial, as recorded for Mesopotamia, plants have been a major source to alleviate illness and suffering. Much awareness has improved since then. Botanists have advocated the traditional use of plants as a whole or as ingredients, which have been mainly based on their safety, availability, and affordability (Abioye et al., 2019). Among the naturopathies, these are the reasons which have increased the interest for elucidation of biological potential as well as chemical constituents of plants (Mamadalieva et al., 2019).

The knowledge of organic chemistry and growth in the pharmaceutical field have led toward the discovery of synthetic medicine but have also impacted the side effects and the high cost as a negative image of these discovered drugs. This ultimately has attracted the health-promoting effects of the natural products to be used as medicinal products (Cvetanović et al., 2018). Many plants having culinary uses have been detailed scientifically against worldwide common diseases like diabetes mellitus (DM), Alzheimer's, and cancer, owing to their medicinal properties. Nowadays, it is the need for such drugs that are safer to use in long-term therapy for these diseases without severe side effects (Mocan et al., 2016). Researchers have focused on developing a scientific rationale among the medicinal or dietary effects and chemical constituents of plants having traditional medicinal uses. There is a particular emphasis on such plants exhibiting antioxidant activities, as these plants are reported to have a lower risk of degenerative diseases, including cardiovascular disorders and cancer (Pérez-Jiménez et al., 2008). Researchers are exploiting an easy and cost-effective tactic toward the selection of medicinal plants by studying their traditional folklore uses among different ethnic groups, which is then followed up by their *in-vitro* and *in-vivo* tests or biological validation, leading toward the discovery of novel bioactive molecules. These biologically active molecules are further studied and modified to be used as therapeutic moieties (McRae et al., 2007).

Oxidative stress is caused by the generation of highly reactive oxygen species, which plays a key role in the pathogenesis of many physiological illnesses, including cell injury, cancer, and hepatic, cardiac, neurological, and renal problems (Oladejo et al., 2019). As a result of increased susceptibility of humans to various forms of lethal diseases, there has been a globally emerging trend toward the use of medicinal and nutritional plants as therapeutic antioxidants. In fact, an inverse relationship has been demonstrated between the dietary intake of antioxidant-rich medicinal plants and the prevalence of human diseases (Mahomoodally et al., 2021).

Sphaeranthus indicus L. (Asteraceae) is a strongly scented, branched, and annual erect, with branched tapering roots, medicinal herb widely distributed throughout the continents of Australia and Asia (Vikani et al., 2008). This plant has traditional uses against several ailments, for example, dried and powdered leaves of *S. indicus* are useful in the treatment of chronic skin diseases, urethral discharges, and jaundice (Saidapur, 1978). The extract of this plant is diuretic, styptic, and is believed to be beneficial against gastric and liver disorders (Chadha, 1976). Seeds and roots are used as anthelmintic and stomachic (Said and Kenawy, 1956). This plant is also believed to be used for skin disorders as a blood purifier (Kirtikar and Basu, 1918). The extract of this plant is also reported to inhibit the activity of the hyaluronidase enzyme and also possesses antibacterial activity (Lichtenbelt et al., 1998). The phytochemical analysis conducted on this plant has revealed the presence of flavonoids, carbohydrates, mucilage, alkaloids, and gums (Kokate, 1986). Another study has shown that it contains sesquiterpenes possessing anti-inflammatory and immune-stimulating activities (Sadaf et al., 2006). *S. indicus* has also been studied for its antimicrobial and antioxidant activities (Tandon and Gupta, 2020). Previously conducted review articles have highlighted the importance of this plant to have been used in various diseases, including epilepsy, mental illness, hemicrania, jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, and skin diseases, and have also discussed the important classes of phytochemicals present in this plant (Galani et al., 2010a; Makhija et al., 2011).

Keeping in view the abovementioned traditional uses, this study was conducted to evaluate the reactivity of different solvent extracts/fractions of *S. indicus* flowers. The detailed phytochemical profiling was established *via* determining the total phenolic contents (TPC) and total flavonoid contents (TFC), high-performance liquid chromatography–photo diode array (HPLC-PDA) polyphenolic quantification, and ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS) secondary metabolites composition. Similarly, the antioxidant activity of the tested extracts was tested using radical scavenging [2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)], reducing power [ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC)], total antioxidant capacity (phosphomolybdenum assay), and metal chelation activities. Similarly, the key enzyme inhibition potential against the clinically relevant enzymes included in

the most common pathologies, i.e., neurological problems [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)], diabetes (amylase and glucosidase), *Helicobacter pylori*–related infections (urease), and skin disorders (tyrosinase) was also investigated. Furthermore, in order to observe any co-relationships among the tested extracts and biochemical assays activities, exploratory multivariate statistical analyses, i.e., principal component analysis (PCA) and hierarchical clustered analysis (HCA), were also performed. To the best of the literature studied, this study could be the foremost attempt to explore the possible positive effects of this medicinal plant.

2 MATERIALS AND METHODS

2.1 Plant Collection, Extraction, and Fractionation

Shade dried flowers of *S. indicus* were obtained from the herbal clinic of the University College of Conventional Medicine (UCCM), Department of Eastern Medicine and Surgery, The Islamia University of Bahawalpur, Bahawalpur (Voucher Number 67/LS-16/X/17). The flowers of *S. indicus* were washed with cold water and placed for air drying, and finally ground into a fine powder using a kitchen mill grinder. The powdered drug was passed through a 50-mesh sieve (297 μm) and soaked in methanol for 24 h. After that, the soaked contents were pressed through a muslin cloth, filtered through Whatman filter paper #42 (pore size 2.5 μm). The solvent was evaporated through a rotary evaporator (Heidolph Co. Ltd., Japan). The dried methanol extract was further dispersed in distilled water and was sequentially fractionated with *n*-hexane, benzene, chloroform, ethyl acetate, and *n*-butanol solvents. All the resultant fractions were dried using the rotary evaporator.

2.2 Phytochemical Composition

2.2.1 Total Bioactive Contents

The total bioactive contents of all the extracts/fractions were determined by evaluating the TPC and TFC. The TPC was determined by the Folin–Ciocalteu method as reported previously, and the results were expressed as milligram gallic acid equivalent per gram of extract (mg of GAE/g extract). Similarly, the aluminum chloride colorimetric method, as reported earlier, was utilized to determine the TFC (Wolfe et al., 2003). The results of TFC were reported as milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

2.2.2 HPLC-PDA Polyphenolic Quantification

A list of 22 different polyphenolic standards was tested to be quantified in all the samples using HPLC-PDA analysis as reported previously. The analysis was performed on a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 PDA detector, and Empower v.2 Software (Waters Spa, Milford, MA, United States) was used for the acquisition of data (Locatelli et al., 2017).

2.2.3 UHPLC-MS Analysis

UHPLC-MS analysis of methanol and ethyl acetate extracts was performed (negative ionization mode) on the Agilent 1290 Infinity LC system coupled with Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source, as reported earlier (Saleem et al., 2019b). The METLIN database was used for the tentative identification of different secondary metabolites in the tested samples.

2.3 Biological Assays

2.3.1 Antioxidant Activity

Antioxidant potential of all the extract/fractions was evaluated by free radical scavenging (DPPH, ABTS), reducing antioxidant power (FRAP, CUPRAC), phosphomolybdenum, and metal chelation assays using the standard procedures, as has been reported previously (Ahmad et al., 2019). All the antioxidant assay results (except metal chelation) were expressed as milligrams of Trolox (water-soluble vitamin E analog) equivalent per gram of dry extract (mg TE/g extract), while ethylenediaminetetraacetic acid equivalent (mg EDTAE/g extract) was used for metal chelation assay. Chemical antioxidant assays, like the DPPH assay, are of no pharmacological relevance because they are chemical tests, and there is no evidence for health therapeutic benefits based on such chemical assays.

2.3.2 Enzyme Inhibition Assays

The inhibition potential of the methanolic extract and all other fractions was tested against α-amylase, α-glucosidase, urease, cholinesterases (AChE and BChE), and tyrosinase enzymes using standard *in-vitro* assays as reported previously (Grochowski et al., 2017; Ahmad et al., 2019). The results of the enzyme inhibition assays were presented as percentage inhibition against all the enzymes except for α-amylase, which was recorded as millimoles (mmol) of acarbose equivalents per gram of extract (ACAE/g). The percentage enzyme inhibition for all the tested enzymes except α-amylase was measured using the following equation:

$$Inhibiton\ (\%) = \left(\frac{Abs.\,of\ control - Abs.of\ sample}{Abs.\,of\ control}\right) \times 100$$

2.4 Statistical Analyses

All the assays were performed in triplicate and presented as average, with standard deviation. Standard curve, correlation coefficient (R^2), statistical comparison of means using one-way analysis of variance, all regarded $p < 0.05$ as significant. PCA and HCA were achieved to gain insights into the variability between extraction solvents in terms of the evaluated bioactivities. SPSS and R software v. 3.6.2 with FactoMineR was used for this purpose.

3 RESULTS AND DISCUSSION

3.1 Total Bioactive Contents and Phytochemical Composition

Phytochemicals, specifically phenols and flavonoids, are usually considered as most bioactive secondary metabolites in plants, which are also the defensive compounds that are produced as a

TABLE 1 | Total bioactive contents of *S. indicus* extracts/fractions.

Samples	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
Methanol	94.07 ± 0.28	78.70 ± 0.67
n-hexane	55.85 ± 0.25	40.02 ± 0.35
Benzene	55.97 ± 0.07	51.79 ± 0.26
Chloroform	73.36 ± 0.20	57.71 ± 0.18
Ethyl acetate	77.33 ± 0.25	72.36 ± 0.59
n-butanol	28.27 ± 0.20	37.22 ± 0.53

Data from the three repetitions are mean ± S.D. GAE: gallic acid equivalent; QE: Quercetin equivalent.

response to environmental stress (Rahman et al., 2018). In this study, different solvent extract/fractions of *S. indicus* were assessed for their TPC and TFC, and the obtained results are mentioned in **Table 1**. The highest value of TPC was found for methanolic extract (94.07 mg GAE/g), while the *n*-butanol fraction showed the lowest phenolic contents. Similarly, for flavonoid contents determination, a similar pattern to that of the TPC was noted, and the methanol extract showed maximum TFC value, i.e., 78.70 mg QE/g extract.

Similarly, to gain a more in-depth insight into the phytochemical composition of the studied plant, a list of 22 important standard phenolic phytochemicals were tested for their quantification in all the extracts/fractions of *S. indicus*; however, all the studied extracts were found to be quantified for nine of these compounds. The results of these quantified phenolics are presented in **Table 2**, and their respective HPLC-PDA chromatograms are shown in **Figure 1**. From the results, it could be seen that *S. indicus* methanol extract contained a higher amount of phenolics in comparison with the other extracts, with the highest amounts of rutin (6.45 μg/g extract) and naringenin (3.82 μg/g extract), while 3-hydroxybenzoic (0.54 μg/g extract) and sinapinic acid (0.36 μg/g extract) were quantified in lesser amounts, and epicatechin was detected as below the limit of detection (BLD). Likewise, the *n*-butanol extract contained gallic acid (1.61 μg/g extract), epicatechin (1.43 μg/g extract), and 3-OH-4-MeO benzaldehyde (0.21 μg/g extract). Interestingly, none of the tested phenolic standards were present in the *n*-hexane extract, and the benzene extract was found to contain only *t*-ferulic acid (BLD), which may be due to the nonpolar nature of these extracts. The ethyl acetate extract was found to contain two compounds, including chlorogenic acid (2.58 μg/g extract) and gallic acid (0.35 μg/g extract). Similarly, epicatechin (0.99 μg/g extract), 3-hydroxybenzoic acid (1.65 μg/g extract), and naringenin (0.66 μg/g extract) were quantified in the chloroform extract. Overall, this phenolic profiling confirms the presence of important secondary metabolites, so these plant extracts/fractions can be explored further for the isolation of bioactive molecules having potential important activities.

Furthermore, to have detailed individual secondary metabolites profiling, UHPLC-MS analysis was performed on the methanol and ethyl acetate extract/fraction (both of these extracts were found to have higher phenolic and flavonoid contents). Standard total ion chromatograms of the methanol and ethyl acetate extract/fraction with mass spectrometric peaks are shown in **Figures 2A,B**, respectively. Whereas the list of individual secondary metabolites as tentatively identified in the methanol and ethyl acetate extract/fraction is presented in **Tables 3**, **4**, respectively. As indicated in **Table 3**, the methanolic extract revealed the tentative presence of 22 different secondary metabolites, and most of these were phenolic and flavonoid derivatives, including quinic acid, benzoic acids, 7,8-dihydroxycoumarin, 3′-(6″-galloylglucosyl)-phloroacetophenone, robinetin 3-rutinoside, agecorynin C, absindiol, and moreollin. Similarly, the ethyl acetate fraction showed the tentative identification of 10 different secondary metabolites, including 10-acetoxyligustroside, 2,4,6-trihydroxybenzoic acid, syringin, and robinetin 3-rutinoside belonging to phenols and flavonoids classes. As far as our literature search, this is the first report on such detailed phytochemical profiling of this plant.

3.2 Antioxidant Activity

Free radicals are the agents that cause gene mutation and the transformation of molecules in the body. These reactions can be controlled by antioxidant defensive mechanisms in living organisms. A number of natural extracts and products are used as food supplements and as topical applications for their antioxidant activity. This requires further investigation of such extracts and products for their source and the compounds responsible for antioxidant activities (Dai and Mumper, 2010).

In the current research, the antioxidant activity of *S. indicus* extract/fractions was determined *via* six different assays (DPPH, ABTS, CUPRAC, FRAP, phosphomolybdenum, and metal chelation activity), and the results are presented in **Table 5**.

TABLE 2 | HPLC-PDA polyphenolic quantification of *S. indicus* extracts/fractions.

Samples	Polyphenolics quantified								
	Gallic acid	Chlorogenic acid	Epicatechin	3-hydroxybenzoic acid	3-OH-4-MeO benzaldehyde	Rutin	Sinapinic acid	*t*-ferulic acid	Naringenin
Methanol	nd	nd	BLD	0.54 ± 0.04	nd	6.45 ± 0.68	0.36 ± 0.04	nd	3.82 ± 1.02
n-hexane	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzene	nd	nd	nd	nd	nd	nd	nd	BLD	nd
Chloroform	nd	nd	0.99 ± 0.88	1.65 ± 0.87	nd	nd	nd	nd	0.66 ± 0.07
Ethyl acetate	0.35 ± 0.04	2.58 ± 0.99	nd	nd	nd	nd	nd	nd	nd
n-Butanol	1.61 ± 0.11	nd	1.43 ± 0.53	nd	0.21 ± 0.02	nd	nd	nd	nd

All values expressed are means ± S.D. of three parallel measurements; nd: not detected; BLD: below limit of detection.

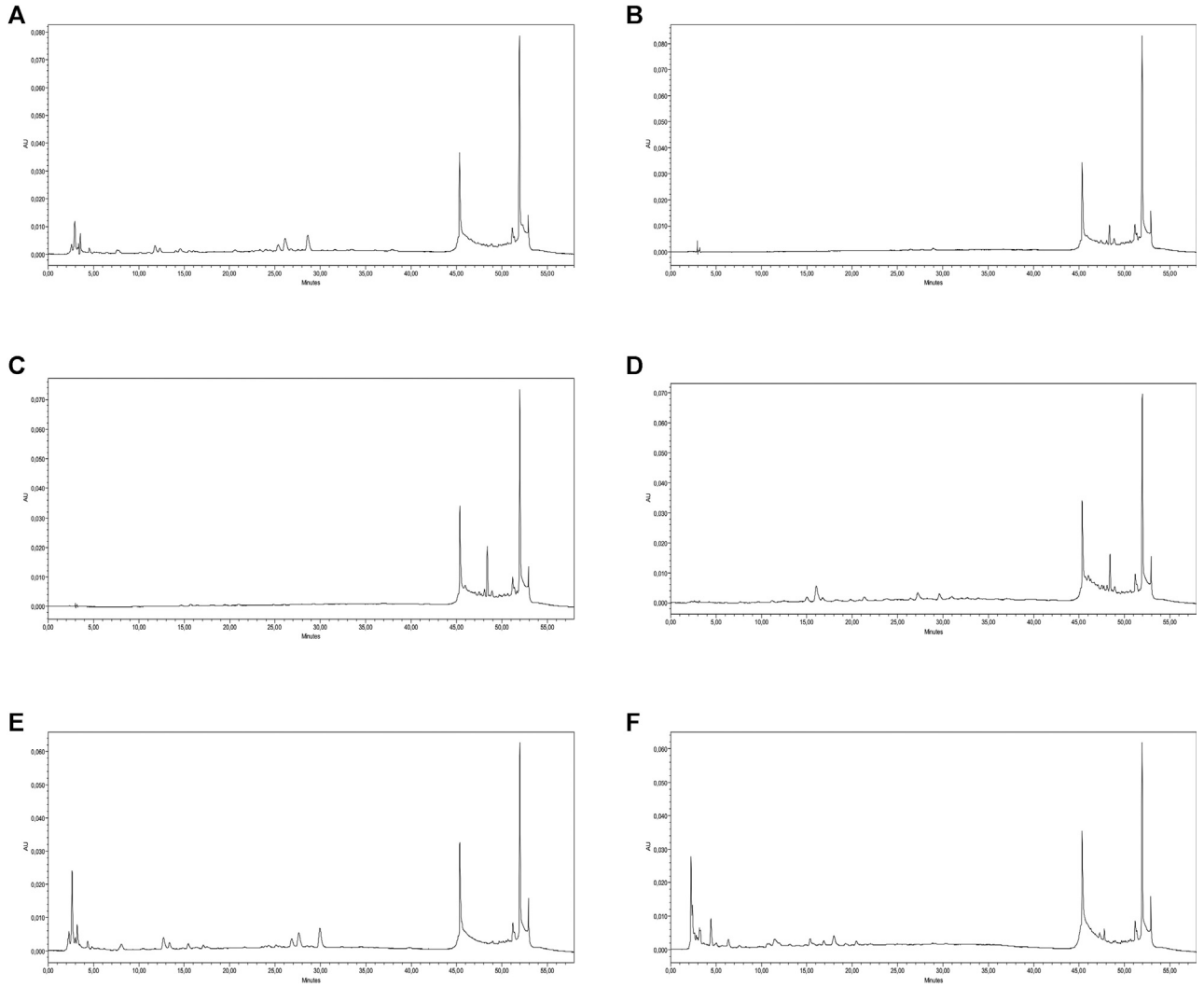

FIGURE 1 | HPLC-PDA chromatograms. **(A)** methanol, **(B)** *n*-hexane, **(C)** benzene, **(D)** chloroform, **(E)** ethyl acetate, and **(F)** *n*-butanol) of polyphenolics quantified in the tested extracts.

Overall, it was noted that the methanolic extract having the highest bioactive contents showed maximum values for radical scavenging and reducing power assays. This can be correlated to the presence of high amounts of phenols and flavonoids in this extract, as a positive relationship between bioactive components with reducing power and free radical scavenging as reported previously (Khan et al., 2019). While, for phosphomolybdenum assay, the benzene fraction showed the highest value, i.e., 5.33 mg TE/g, and the *n*-hexane fraction exerted the highest value for metal chelation activity (12.64 mg EDTA/g). Our results are in line with previous studies highlighting the considerable antioxidant activity of this plant (Shirwaikar et al., 2006; Tiwari and Khosa, 2009; Galani et al., 2010a; Kavitha and Satish, 2015; Vijayalakshmi and Rao, 2019). A flavonoid 5-hydroxy-7-methoxy-6-C-glycosylflavone with remarkable antioxidant potential has also been previously isolated from this plant (Mishra et al., 2007). Likewise, Tiwari and Khosa, (2009) reported the *in-vivo* antioxidant potential of methanolic extract of *S. indicus*, by increasing the levels of superoxide dismutase, catalase, and glutathione peroxides involved in the mechanism of reducing malondialdehyde levels in rats (Tiwari and Khosa, 2009). Similarly, many sesquiterpenes (including 2-hydroxycostic acid, eudesmenolide, and sphaeranthanolide) having antioxidant potential have also been reported in *S. indicus* (Galani et al., 2010b). HPLC polyphenolic quantification and UHPLC-MS analysis in the current study also show the presence of important phytocompounds like rutin and naringenin; both of these flavonoids have reported antioxidant potential (Yang et al., 2008; Cavia-Saiz et al., 2010). Likewise, some of the other compounds identified by phytochemical profiling, including quinic acid, ferulic acid, syringin, and coumarin derivative, have also been reported to have strong antioxidant properties (Hung et al., 2006; Srinivasan et al., 2007; Kostova et al., 2011; Ahmad et al.,

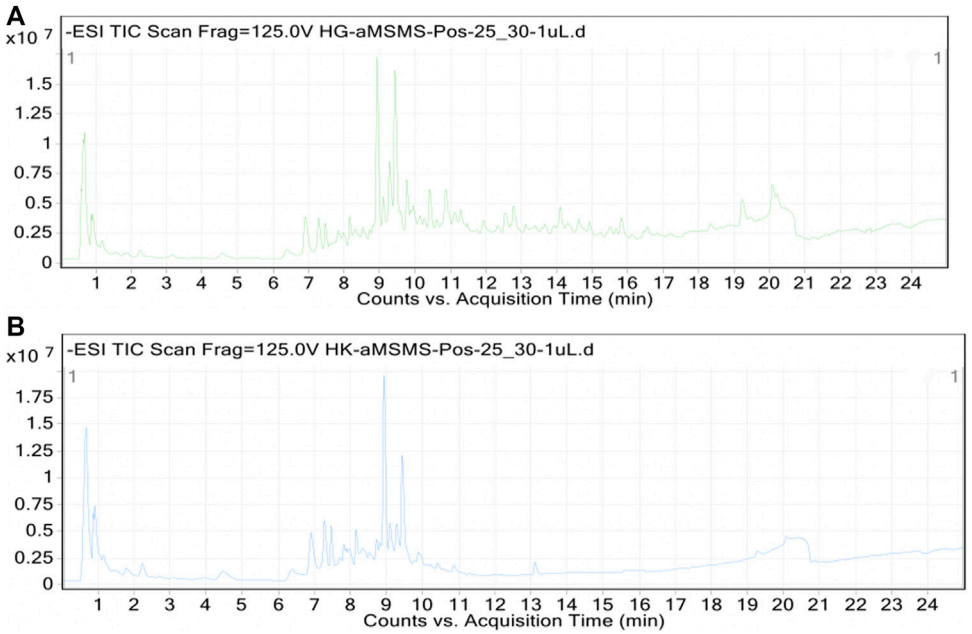

FIGURE 2 | Total ion chromatograms (TICs) of *S. indicus* methanol **(A)** and ethyl acetate **(B)** extracts.

TABLE 3 | UHPLC-MS analysis of *S. indicus* methanol extract (negative ionization mode).

Serial No.	Possible compound	Compound class	Mol. Formula	Mol. Mass	TR (min)	B. Peak (*m/z*)
1	Quinic acid	Phenol	$C_7H_{12}O_6$	192.06	0.665	191.05
2	2,3′,4,6-Tetrahydroxy-benzophenone	Xanthones	$C_{13}H_{10}O_5$	246.05	0.692	245.04
3	2,4,6-Trihydroxybenzoic acid	Phenol	$C_7H_6O_5$	170.022	1.557	169.01
4	3,4-Dihydroxybenzoic acid	Phenol	$C_7H_6O_4$	154.027	3.179	153.01
5	Scopolin	Phenolic	$C_{16}H_{18}O_9$	354.1	4.582	353.08
6	Vanilloloside	Glycoside	$C_{14}H_{20}O_8$	316.117	7.228	315.1
7	7,8-Dihydroxycoumarin	Flavonoid	$C_9H_6O_4$	178.027	7.454	177.01
8	Syringin	Phenol	$C_{17}H_{24}O_9$	372.14	7.819	371.13
9	3′-(6″-Galloylglucosyl)-phloroacetophenone	Flavonoid	$C_{21}H_{22}O_{13}$	482.11	7.902	481.1
10	4-*p*-Coumaroylquinic acid	Phenol	$C_{16}H_{18}O_8$	338.102	8.005	337.09
11	Thevetin B	Glycoside	$C_{42}H_{66}O_{18}$	858.43	8.159	857.42
12	Robinetin 3-rutinoside	Flavonoid	$C_{27}H_{30}O_{16}$	610.15	8.722	609.15
13	Formononetin 7-(6″-methylmalonylglucoside)	Flavonoid	$C_{26}H_{26}O_{12}$	530.14	9.778	529.14
14	Ferulic acid	Phenol	$C_{10}H_{10}O_4$	194.059	9.833	193.051
15	Agecorynin C	Flavonoid	$C_{22}H_{24}O_9$	432.14	9.96	431.13
16	Tetracaffeoylquinic acid	Phenol	$C_{43}H_{36}O_{18}$	840.19	10.882	839.18
17	5,8,12-trihydroxy-9-octadecenoic acid	Fatty acid	$C_{18}H_{34}O_5$	330.24	10.977	329.23
18	Coriandrone D	Phenol	$C_{18}H_{24}O_8$	352.15	11.537	351.15
19	9,16-dihydroxy-palmitic acid	Fatty acid	$C_{16}H_{32}O_4$	288.23	11.756	287.22
20	Cinnamodial	Sesquiterpene	$C_{17}H_{24}O_5$	308.163	11.951	307.155
21	Absindiol	Phenol	$C_{15}H_{22}O_4$	266.15	11.955	265.144
22	Moreollin	Flavonoid	$C_{35}H_{42}O_8$	590.29	14.635	589.28

TR: retention time; B. peak: base peak.

2015). These compounds might be responsible for the antioxidant and reducing activities exhibit by the methanolic extract of *S. indicus*. Benzene and *n*-hexane fractions showed the highest value for phosphomolybdenum and metal chelation assays, respectively. This revealed no linear correlation with the bioactive constituents of the said fractions. Literature supports the theory that this trend might be possible due to the presence of some non-phenolic compounds in these fractions (Saleem et al., 2019a; Khurshid et al., 2019; Zengin et al., 2019).

3.3 Enzymatic Assays

An increasing trend toward the utilization of natural products for treating some common diseases like diabetes, gastritis,

TABLE 4 | UHPLC-MS analysis of *S. indicus* ethyl acetate fraction (negative ionization mode).

Sr. no	Possible compound	Compound class	Mol. Formula	Mol. Mass	TR (min)	B. Peak (*m/z*)
1	Lippioside I	Phenol	$C_{25}H_{30}O_{13}$	538.16	0.653	537.16
2	Theobromine	Alkaloid	$C_7H_8N_4O_2$	180.07	0.67	179.06
3	10-Acetoxyligustroside	Phenol	$C_{27}H_{34}O_{14}$	582.19	0.872	581.19
4	2,4,6-Trihydroxybenzoic acid	Phenol	$C_7H_6O_5$	170.022	1.543	169.015
5	Scopolin	Phenolic Glycoside	$C_{16}H_{18}O_9$	354.1	4.498	353.088
6	Vanilloloside	Glycoside	$C_{14}H_{20}O_8$	316.12	7.223	315.11
7	Syringin	Phenol	$C_{17}H_{24}O_9$	372.14	7.816	371.135
8	Thevetin B	Glycoside	$C_{42}H_{66}O_{18}$	858.43	8.157	857.42
9	Robinetin 3-rutinoside	Flavonoid	$C_{27}H_{30}O_{16}$	610.15	8.721	609.15
10	Tetracaffeoylquinic acid	Phenol	$C_{43}H_{36}O_{18}$	840.19	10.876	839.18

TR: retention time; B. peak: base peak.

TABLE 5 | Antioxidant activities of *S. indicus* extracts/fractions.

Samples	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC mg TE/g	FRAP (mg TE/g)	Phosphomolybdenum (mg TE/g extract)	Metal chelating (mg EDTAE/g extract)
Methanol	235.66 ± 2.71	206.81 ± 4.41	458.2 ± 6.31	241.28 ± 1.93	2.66 ± 0.23	4.63 ± 1.35
n-Hexane	14.74 ± 3.62	Na	120.68 ± 6.77	55.79 ± 0.04	4.74 ± 0.39	12.64 ± 0.89
Benzene	30.94 ± 0.85	Na	115.33 ± 1.63	71.98 ± 0.10	5.33 ± 0.17	7.65 ± 0.41
Chloroform	132.62 ± 2.39	116.61 ± 3.44	253.72 ± 2.46	125.89 ± 1.64	4.94 ± 0.22	9.20 ± 0.86
Ethyl acetate	110.73 ± 3.65	69.90 ± 4.58	187.56 ± 0.16	129.55 ± 5.29	0.81 ± 0.05	3.59 ± 0.56
n-Butanol	123.49 ± 2.39	130.83 ± 4.54	185.25 ± 2.57	137.27 ± 2.32	1.05 ± 0.12	4.52 ± 0.15

[a]*TE: trolox equivalent; EDTAE: EDTA equivalent. All values expressed are means ± S.D. of three parallel measurements; na: not active.*

TABLE 6 | Enzyme inhibition studies of *S. indicus* extracts/fractions.

Samples	AChE	BChE	α-glucosidase	α-amylase	Urease	Tyrosinase
Methanol	82.78 ± 0.57	47.68 ± 0.58	87.16 ± 0.17	0.67 ± 0.01	75.63 ± 0.74	83.18 ± 2.16
n-Hexane	71.46 ± 0.72	83.74 ± 0.57	12.62 ± 0.12	0.80 ± 0.03	13.46 ± 0.45	51.37 ± 1.05
Benzene	68.53 ± 0.74	78.46 ± 0.85	23.57 ± 0.53	0.71 ± 0.03	29.87 ± 0.53	63.41 ± 3.17
Chloroform	81.72 ± 0.65	81.65 ± 0.76	37.85 ± 0.21	0.76 ± 0.01	61.42 ± 0.68	79.86 ± 1.23
Ethyl acetate	42.56 ± 0.57	82.57 ± 0.68	42.79 ± 0.27	0.48 ± 0.01	82.35 ± 0.79	86.13 ± 2.81
n-Butanol	39.24 ± 0.26	34.23 ± 0.62	17.53 ± 0.43	0.44 ± 0.02	37.71 ± 0.46	31.48 ± 1.01
Acarbose	nt	nt	95.83 ± 0.16	nt	nt	nt
Eserine	91.27 ± 1.17	82.82 ± 1.09	nt	nt	nt	nt
Thiourea	nt	nt	nt	nt	98.21 ± 0.18	nt
Arbutin	nt	Nt	nt	nt	nt	84.6 ± 1.9
Kojic acid	nt	Nt	nt	nt	nt	87.24 ± 2.43

Concentration of standards were set at 1 mM; Values expressed are means ± S.D. of the three parallel measurements; nt: not tested. Eserine was used as the standard for AChE and BChE, acarbose for α-glucosidase and α-amylase; thiourea for urease; arbutin and kojic acid for tyrosinase enzymes.

gastroduodenal ulcers, Alzheimer's disease, and hyperpigmentation has been observed (Zengin et al., 2016; Khurshid et al., 2019). In this study, the methanolic extract and various fractions of *S. indicus* were evaluated against α-amylase, α-glucosidase, urease, AChE, BChE, and tyrosinase enzymes. The results of the enzyme inhibition assays are presented in **Table 6**.

DM is considered to be a global epidemic. It is considered to be at the 8th position for deaths worldwide. An estimated 425 million adults had diabetes worldwide in 2017, and this number is predicted to rise to 629 million by 2045 (Gomes et al., 2019). Oligosaccharides and disaccharides in the small intestine are converted into glucose by the α-glucosidase enzyme. Inhibition of this enzyme reduces the rate of carbohydrate digestion and due to which its absorption is also delayed in the digestive tract. Inhibitors of α-glucosidase have the potential to prevent the development of type-2 DM (Liu et al., 2011). As presented in **Table 6**, a considerable α-glucosidase inhibition was shown by the methanolic extract (87.16%), and the *n*-hexane fraction was most active against α-amylase (0.80 ± 0.03 mmol ACAE/g extract), whilst all the other fractions showed the least inhibition. The higher α-glucosidase inhibition by the methanol extract can be due to the higher number of flavonoid compounds in this extract, as previously, this class of phytochemicals have been reported for α-glucosidase potential (Liu et al., 2014).

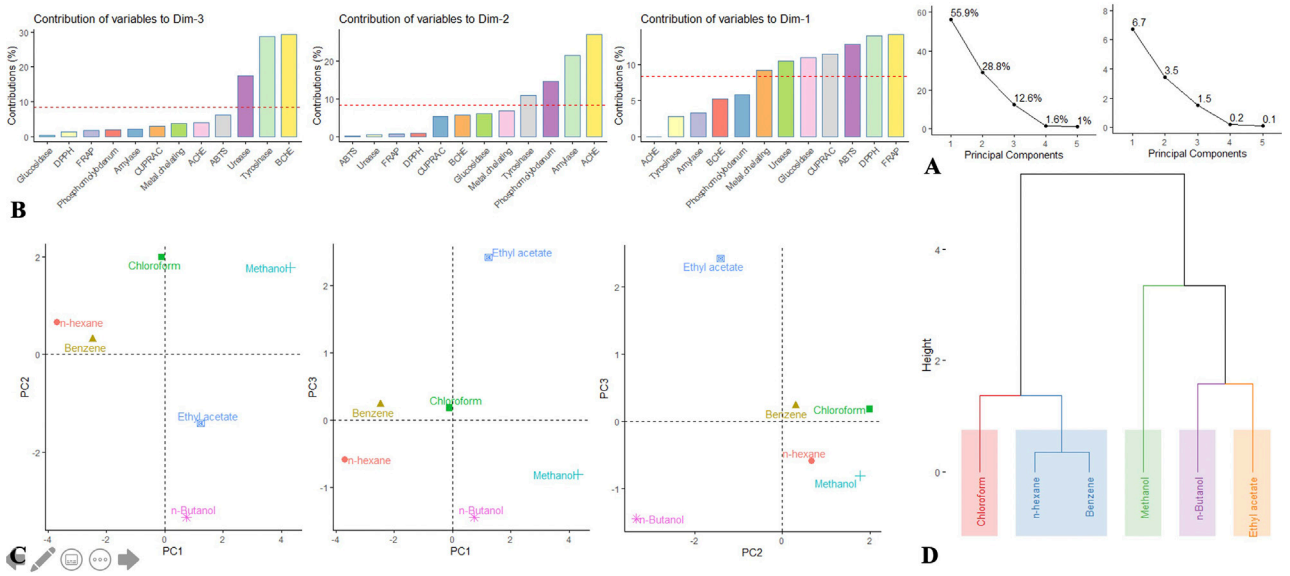

FIGURE 3 | (A–C) Principal component analysis. **(A)** Eigenvalue and percentage of explained variance by each dimension. **(B)** Contribution of bioactivities to principal components. **(C)** Distribution of the samples on the factorial plane PC1 vs PC2, PC1 vs PC3, and PC2 vs PC3. **(D)** Dendrogram of HCA.

Urease is an enzyme that hydrolysis urea and produces ammonia and carbon dioxide. Gastritis and gastroduodenal ulcer can be caused by *H. pylori*, and urease enzymes aid the growth of these bacteria in the acidic environment of the stomach. Urease produces a cloud of ammonia that is used by the bacterium for its protection (Liaqat et al., 2017); thus the inhibition of this enzyme is important to control *H. pylori*–related infections. In this study, the maximum urease inhibition was shown by the ethyl acetate fraction (82.35%). This phenomenon might be due to the presence of chlorogenic acid in the ethyl acetate fraction as quantified by the HPLC-PDA analysis, which has been previously reported for urease inhibition activity (Xiao et al., 2012).

Similarly, neurodegenerative disorders are a significant health alarm in several developed countries where the elderly population face abnormal emotional changes (Rajakumar et al., 2017). AChE inhibitors are used for the symptomatic treatment of Alzheimer's disease, for myasthenia gravis, and also in other dementias (Pohanka, 2014). On the other hand, BChE is synthesized in the liver and is reported to have more activity in the human blood than that of AChE (Eddleston et al., 2008). Efforts are in progress to use BChE inhibitors for prophylactic treatment of nerve agent poisoning (Chilukuri et al., 2005). In this study, the methanolic extract showed maximum inhibition for AChE (82.78%), while the *n*-hexane fraction presented the highest BChE (83.74%) inhibition. Likewise, ethyl acetate fraction also showed considerable inhibition of BChE (82.57%).

Tyrosinase enzyme is catalyzed in three steps during melanin biosynthesis. So, tyrosinase inhibitory effects have significance in the cosmetic industry to alter the tone of the skin (Shimizu et al., 2000). Overproduction and accumulation of melanin pigment in the skin can lead to several disorders, including neurodegenerative disorders (Khurshid et al., 2019). The ethyl acetate fraction showed maximum tyrosinase inhibition, i.e., 86.13%, while the methanolic extract showed 83.18% inhibition. The methanolic extract was found to be rich in several phenolic and flavonoid compounds that might be responsible for inhibiting mushroom tyrosinase enzyme, as previously reported studies have indicated the anti-tyrosinase capacity of different phenolics and flavonoids (Bouzaiene et al., 2016; De Freitas et al., 2016; Cespedes et al., 2017; Choi et al., 2019).

3.4 Exploratory Multivariate Analyses

As has been seen *via* the univariate analysis, there is a high difference among the extraction solvents. To better describe and for greater understanding of the overall activity variations between these solvents, we undertook to perform an exploratory multivariate analysis, i.e., PCA and HCA. Firstly, the data matrix was submitted to PCA with the intention of reducing the dimensionality of the data set by compressing it onto a smaller set of the principal component (PC). The obtained results are given in **Figures 3A–C**; based on Kaiser Criterion, only the first three components that contained most of the information were selected. PC1 resumed 55.9% of the total information and was largely determined by FRAP, DPPH, ABTS, CUPRAC, and to a limited extent glucosidase (**Figure 3B**). PC2 captured 28.8% of the information and was linked to AChE, amylase, and to a lesser extent to phosphomolybdenum. PC3 synthetized 12.6% of the information and was bound to BChE, tyrosinase, and urease. In essence, PC1, PC2, and PC3 combined explained almost 97% of the variation in the data set, and the resulting scatter plots PC1 *vs* PC2, PC1 *vs* PC3, and PC2 *vs* PC3 are depicted in **Figure 3C**. By observing the three scatter plots, a great variability among the extraction solvents can be noted, albeit benzene and *n*-hexane seemed to be close enough. To better reflect the potential clusters,

a complementary analysis, i.e., HCA was done. Results depicted in the figure revealed five distinct clusters (**Figure 3D**).

Both exploratory multivariate analyses allowed to highlight the differential impact of extraction solvents on the biological activities of *S. indicus*. Thus, depending on the solvent employed for recovering the bioactive molecules, extracts obtained from the same material of a plant may differ with respect to their biological activities. Moreover, considering the difference in chemical structure and physicochemical properties of the bioactive molecules, it is impossible to propose a universal standard solvent. Therefore, the selection of the extraction solvent must take into account, the type of molecules expected to be extracted from the plant material, as well as the activities being assessed. In addition, it must be able to preserve the quality of the chemical structure of the desired molecules, as reported previously (Harborne, 1998).

4 CONCLUSION

This research work has highlighted the positive effects by determining the detailed phytochemical and biological composition of different polarity solvent/fractions obtained from *S. indicus* flowers. Phytochemical profiling as achieved by HPLC-PDA and UHPLC-MS analyses has revealed the identification of important secondary metabolites belonging to phenolic, flavonoid, and glycoside classes. The most polar solvent extract/fractions were found to contain the higher bioactive contents. All the tested extracts/fractions showed varying antioxidant and enzyme inhibition potential. Moreover, statistical analyses confirm the relationship among contents and the observed biological activities. Overall, the results obtained by this comprehensive report provide a framework for the utilization of *S. indicus* flower extract/fractions as a natural source for bioactive compounds. However, further work regarding isolation and characterization studies are recommended.

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/s.

AUTHOR CONTRIBUTIONS

Conceptualization, Writing - original draft: HIA; Methodology: MFN; UK; Conceptualization, Data curation, Writing - original draft: HS; Formal analysis, Editing: ML; SAZA; MA; Conceptualization, Project supervision: HMSK; NA; Writing - review and editing: MS; AA.

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REVIEW
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Advances in the Extraction, Purification, Structural Characteristics and Biological Activities of *Eleutherococcus senticosus* Polysaccharides: A Promising Medicinal and Edible Resource With Development Value

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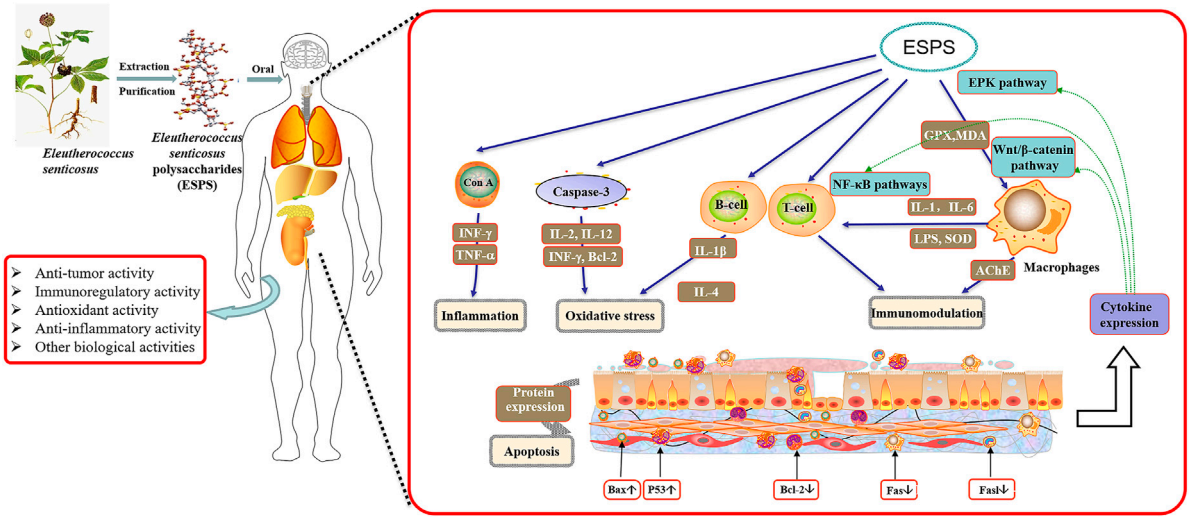
In recent years, natural polysaccharides have received growing attention and interest in view of their values in food, medical, cosmetics and other fields. *Eleutherococcus senticosus* (*E. senticosus*) is a medicine and food homologous plant that possess anti-tumor, anti-inflammatory, central nervous system and cardiovascular protection, anti-radiation, enhancement of human microcirculation, improvement of physical fatigue effects, mainly based on lignans, flavonoids and coumarin types. *E. senticosus* polysaccharides (ESPS), act as a kind of polysaccharide extracted and isolated from the root and rhizome of *E. senticosus*, have been found in many applications of medicine and food for their unique biological activity. Nevertheless, the existing studies are mostly concerned with small molecules of *E. senticosus*, less attention is paid to polysaccharides. Moreover, the types and structural characterization of ESPS reported in existing literature were also not summarized. In this paper, the research progress of ESPS is reviewed from the aspects of extraction, separation, structural characterization and biological activity, future perspectives from points of efficient extraction, resource utilization and quality control standards were also proposed, which provide reference for the further development and utilization of ESPS.

Keywords: extraction, purification, structure, bioactivities, Eleutherococcus senticosus polysaccharides (ESPS,)

HIGHLIGHTS

- The extraction and purification techniques of *Eleutherococcus senticosus* polysaccharides were summarized.
- Types and structural characterization of *Eleutherococcus senticosus* polysaccharides isolated and purified at present were preliminarily discussed.
- The modern pharmacological research and biological activity of *Eleutherococcus senticosus* polysaccharides were summarized, and its application prospect was proposed.



GRAPHICAL ABSTRACT |

INTRODUCTION

Eleutherococcus senticosus, commonly known as "Ci-wu-jia" or "*Acanthopanax senticosus*", is botanically from the dried root and rhizome or stem of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim [syn. *Acanthopanax senticosus* (Rupr. and Maxim.) Harms] belong to the Araliaceae. It is a medicine and food homologous plant that is widely distributed in northeast China, which contains multi-kinds of nutrients and components. Among variety of active ingredients, flavonoids (quercetin, hyperoside, quercetin, rutin, etc.), coumarins (isofraxidin, scopolamine, etc), lignans (ferulic acid glucoside, eleutheroside, carotin, syringin, etc.,) (Lee et al., 2012) played an important role in its activities. Moreover, some trace elements such as Ca, P, Mg, Fe, a variety of amino acids and polysaccharides also play a role in mediating the immune function of the body (Liu, 2010), antioxidant, anti-tumor, anti-inflammatory, central nervous system and cardiovascular protection, anti-radiation, enhancement of human microcirculation, improvement of physical fatigue, etc., (Luan, 2012; Pan, 2019a). As a medicinal and food homologous plant, various products such as oral liquid, tea, capsule preparation, wine of *E. senticosus* have been developed and commercialized, and favored by those who pay attention to health. The products of *E. senticosus* are listed in **Figure 1**.

In recent years, polysaccharides have demonstrated good therapeutic effects against tumors, hyperlipemia, diabetes and hypertensive diseases (Shen et al., 1991; Duan, 2015). The development and utilization of polysaccharides have also become a hot spot. *E. senticosus* polysaccharides (ESPS) are an important biologically active ingredient extracted from the roots and rhizomes of *E. senticosus*. The content of alkaline polysaccharides in *E. senticosus* is about 2–8%, and that of the water-soluble polysaccharides is 2.3–5.7% (Gao, 2019). Studies had shown that ESPS possessed immunoenhancement (Xu et al., 2005), anti-tumor (Hao and Nan, 2013), anti-virus (Zhou, 2018), anti-fatigue, anti-oxidation, anti-diabetes (Fu et al., 2012), hepatoprotective, and other biological activities (Chen and Huang, 2018; Ganesan and Xu, 2019; Zeng et al., 2019), which prompts the huge application and development potential of ESPS. In this paper, the extraction and purification process and structure characterization of ESPS were systematically introduced, and the studies on biological activities of ESPS were further reviewed in order to expand the application of ESPS.

EXTRACTION AND PURIFICATION

Extraction of Crude Polysaccharides

Hot Water Extraction

Polysaccharides are a category of substances with higher polarity, higher water soluble and insoluble in EtOH. Because of the nature of polysaccharides, hot water extraction in combination with EtOH-precipitated has been commonly used in laboratories and industrial production. EtOH-precipitated polysaccharides are suitable for almost all water-soluble polysaccharides. The principal principle is to separate the polysaccharides by reducing the dielectric constant of the aqueous solution to dehydrate the polysaccharides for precipitation. There are several ways to extract ESPS (**Table 1**). Feng et al. used a single factor test to optimize the extraction conditions of ESPS and obtained the optimal condition: pulverization degree above 80 mesh, solute extraction 50% EtOH, extraction pH = 6, soaking for 2 h, degreasing treatment with petroleum ether. Under these conditions, the extraction yield of crude ESPS could reach 3.27 ± 0.07% (Feng, 2016). An orthogonal experiment was developed from the experimental data in order to predict the ESPS yield by Cheng, the optimal condition was found to be: extraction temperature 80°C, extraction once, time of 150 min and solid-liquid ratio of 1:20 g/ml with a maximum pectin yield

TABLE 1 | Summary of the extraction process of ESPS.

Method	Pros	Cons	Optimization Factors	Optimization Results	ESPS Content	References
Hot water extraction	The production cycle is short, the experiment cost is low, experiment operation is simple and the degree of damage to the structure of the extract is small.	Part of the protein will be extracted and protein must be removed; low selectivity, low extraction rate.	Material/liquid, extraction time, times, temperature, alcohol precipitation temperature, time, pH	90°C, 150 min, material/liquid 1:18, extraction twice; alcohol precipitation condition -20°C, 36 h, ethanol extractant ratio 4:1, pH=7	263.3 mg/ml	Zhai (2007)
—	—	—	Extraction temperature, time, material/liquid l, times	80°C, 150 min, material/liquid 1:20, extract once extraction time> extraction temperature> extraction times> material/liquid	23.21 mg/g	Chen (2011a)
—	—	—	Material/liquid, extraction time, temperature, times	90°C, 2.5 h, material/liquid 1:25 (g/mL), extract 3 times.	23.95 mg/g	Chen (2015a)
—	—	—	*E. senticosus* pulverization degree, extraction solute, pH, soaking time degreasing solvent	degree of pulverization:>80 meshes, solute is extracted by 50% ethanol, pH = 6 (petroleum ether degreasing treatment soaking 2 h	3.27±0.07% (n = 3)	Feng (2016)
—	—	—	Extraction parts (rhizome, leaves, fruits) extraction time, temperature, alcohol concentration, material/liquid	95°C, 116 min, material/liquid 31:1 best extraction part fruit, alcohol concentration 85%,	13.21%	Shen (2017)
—	—	—	Extraction temperature, time, material / liquid	80°C, 1.5 h, material/liquid 1:21	10.14%	Yu (2019)
Ultrasonic extraction	More efficient, faster, high extraction yield, low energy consumption	Ultrasound will damage the structure of polysaccharides, resulting in difficulty in separation and difficult to apply to industrial production	Extraction temperature, material/liquid, time, ultrasonic power	58°C, 73 min, material/liquid 1:25; ultrasonic power 85 W	1.532±0.037% (n = 3)	Chen et al. (2018)
			Ultrasonic power, material/liquid , extraction temperature, time	57°C, 42 min, material/liquid 1:40 g/mL ultrasonic power 90 W	3.86%	Chen (2015b)
Microwave extraction	Strong penetration, uniform and fast heating, high selectivity.	Polysaccharides often contain some pigments, so they need to be decolorized.	—	It was the first time to extract ESPS using microwave technology and determine its content.	5.01%	Li and Liu (2003)
—	—	—	Extraction time, microwave power, material/liquid	22.5 min, material/liquid 1:25 22.5 min, material/liquid 1:25	1.52% ± 0.09% (n = 3)	Zhang and Guo (2015)
Alkaline extraction	Higher total glucose content	The yield of polysaccharide was slightly lower, and it will damage the structure of acid polysaccharide and neutral polysaccharide	Alkali concentration, material/alkali ratio, alkali extraction time	Alkali extraction time>material-alkali ratio>alkali concentration 10 h, material/alkali 1:16. alkali concentration 4%,	5.70%	Wang (2006)
—	—	—	Graded alcohol precipitation, step-by-step alcohol precipitation	Used step-by-step alcohol precipitation method, more and higher content of polysaccharides can be obtained	6.01%	Li (2019)

(23.3 mg g^{-1}) (Chen, 2011a). Based on the single factor test, some researches adopted the Box-Behnken center combination design test and response surface method (RSM) to compare rhizomes, fruits, and leaves, and finally determined that the fruit was the best extraction part. This technique gives a maximal yield of 13.21% for material-liquid ratio of 31:1 in 116 min, extraction temperature 95°C (Shen, 2017). Some scholars also investigated the drying method and found that the freeze-drying method was better than the spray-drying method (Zhang and Sun, 2005).

However, the hot water extraction method will introduce some impurities like protein, which needs to be removed. Sevag reagent, trifluoro trichloroethane, trichloroacetic acid, anion and cation exchange resin and the enzymatic hydrolysis are the commonly used mediums. In ESPS literature, protein is mostly removed by the Sevag method (Jiao, 2008; Li, 2019). Wang et al. compared the deproteinization effects of Sevag, trichloroacetic acid (TCA)-Sevag, chitosan coagulation and enzyme on ESPS. The results showed that the protein removal rate: enzyme 93.1% = chitosan coagulation 93.1% > TCA-Sevag 83% > Sevag 28%, polysaccharide loss rate: chitosan coagulation 47% > TCA-Sevag 43% > Sevag 18% > enzyme 7%. Results indicated that enzyme deproteinization has the best effect (Wang, 2009).

FIGURE 1 | *Eleutherococcus senticosus*. Salad from leaves **(A)**; tea **(B)**; pulp **(C)**; wine **(D)**; tablets **(E)**; cortex **(F)**.

Ultrasound-Assisted Extraction (UAE)

The ultrasound-assisted extraction (UAE) method were employed for increasing the movement frequency and speed of macromolecules, strengthening the solvent penetration ability, and shortening the extraction period of polysaccharides by ultrasonic waves (Cui, 2012). In some experiments, the RSM was used to optimize the extraction of ESPS by ultrasound-assisted extraction (UAE), by which the optimum levels of the parameters were obtained as follows: extraction temperature 58°C, extraction time 73 min, liquid/solid ratio 25:1, ultrasonic power 85 W (Chen et al., 2018). However, the extraction time should not be too long because ultrasound has a mechanical shearing effect, which may damage the structure of ESPS. Moreover, the UAE method is difficult to be widely applied in the field of industrial production in view of the limitations of the instruments.

Microwave Extraction

Water molecules in the cell are rapidly heated and pressurized under microwave due to its strong penetrating power, which increased the extraction rate by breakage of cell wall and release of intracellular polysaccharides into the extract.

Li et al. removed fat-soluble impurities and interfering components gradually with petroleum ether, ether and EtOH and then ectracted *E. senticosus* crude polysaccharide by microwave technology (Li and Liu, 2003). Zhang et al. used the RSM based on Box-Behnken design to optimize the extraction of ESPS from three aspects of the microwave power, microwave processing time and material-to-liquid ratio parameters. The results showed that microwave extraction 22.5 min, microwave power 450 W, material-liquid ratio 1:25 ($g{\cdot}ml^{-1}$) achieved the best performance (Zhang and Guo, 2015). Furthermore, microwave extraction of ESPS often requires depigmentation. Ion exchange, adsorption, oxidation and metal complexation are common methods for subsequent depigmentation.

Alkali-Extraction

Polysaccharides could be divided into acidic, alkaline and neutral polysaccharides, which can be extracted by adjusting the acidity and alkalinity like some small molecule compounds. For example, Jiang et al. extracted lemon polysaccharides under alkaline conditions with an extraction rate of 8.81% (Jiang et al., 2017), Li et al. used alkaline leaching method to optimize the extraction of acidic Tuckahoe polysaccharides, the extraction rate was increased to 78.5% (li, 2018). Similarly, corn silk polysaccharide was comparatively extracted by acid extraction and water under the same experimental conditions. The extraction rate 33.36% obtained by acid was higher than the water extraction (7.44%) (Li and Zhou, 2020). Thus, polysaccharides could also be extracted by the acid and alkaline leaching method due to their hydrolysis. However, little related literature on extracting ESPS were obtained in view of the fact that the acid-base method may destroy the structure of the active polysaccharides.

Li et al. compared the extraction of ESPS with water extraction and alkali-extraction. The yield of crude polysaccharides from water extraction and EtOH precipitation was 20.8%, and total carbohydrate content in which was 5.19%. Alternatively, the value

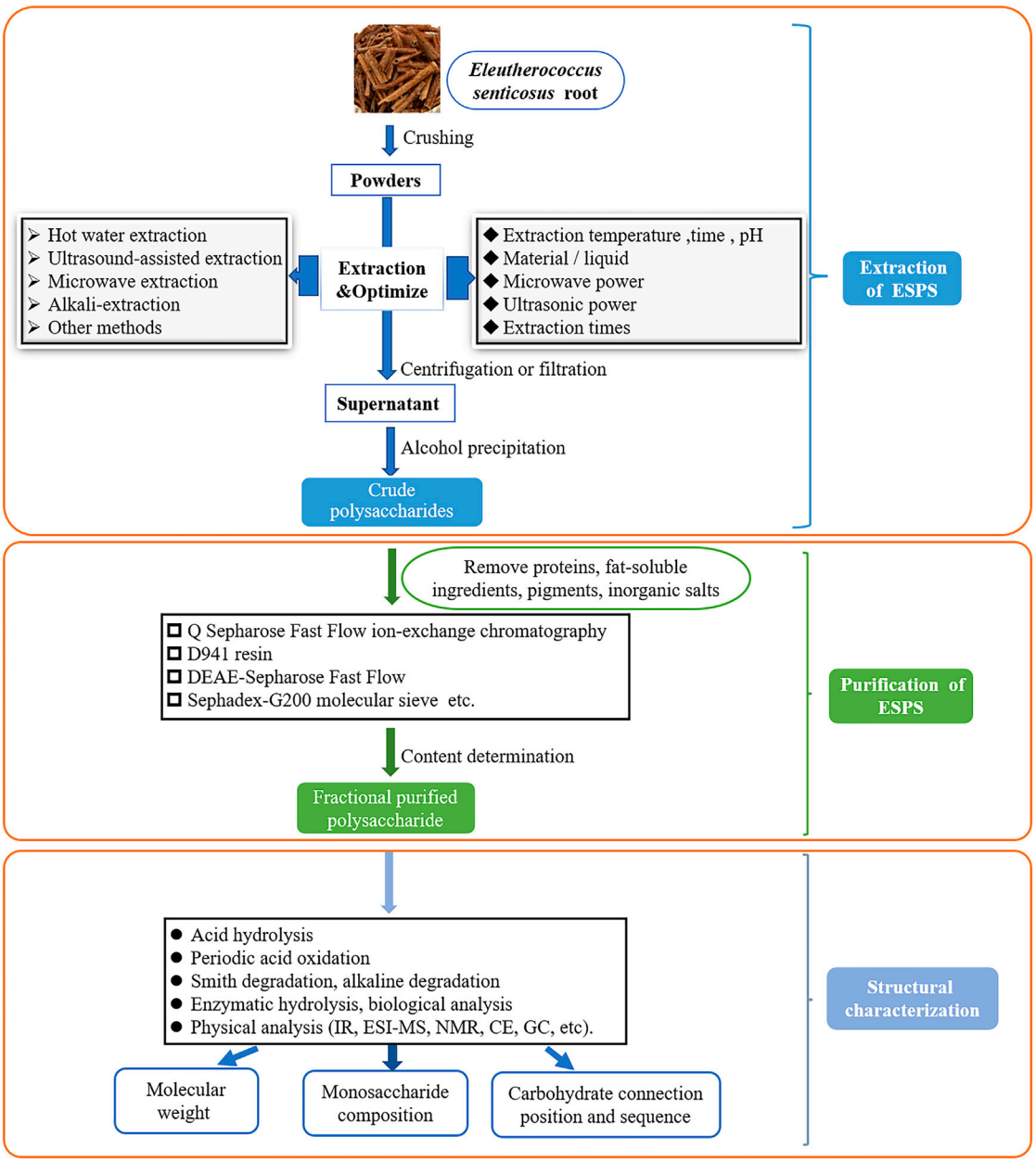


FIGURE 2 | Schematic diagram of extraction, purification and structural characterization of ESPS.

obtained from alkali-extraction and EtOH precipitation were 18.31 and 6.01%, respectively (Li, 2019).

Other Methods

Aside from the common technologies mentioned above, enzymatic hydrolysis, supercritical fluid extraction (SFE) (Wei, 2018), enzyme-microwave-ultrasonic-assisted extraction (Yin et al., 2018) were also employed in the extraction of plant polysaccharides. However, these methods have not been applied to the ESPS extraction at present, which needs further research. Moreover, the crude polysaccharides extracted by above methods need to remove proteins, fat-soluble ingredients, pigments, inorganic salts and other small molecule impurities before subsequent tests. **Figure 2** summarized the diagram of extraction, purification and structural characterization of ESPS.

Purification of Eleutherococcus Senticosus Polysaccharides

Polysaccharides in medicinal materials often coexist with other active ingredients. The separation and purification of homogeneous polysaccharide from crude polysaccharides was usually achieved by EtOH precipitation according to the solubility differences in EtOH. Normally, EtOH precipitation

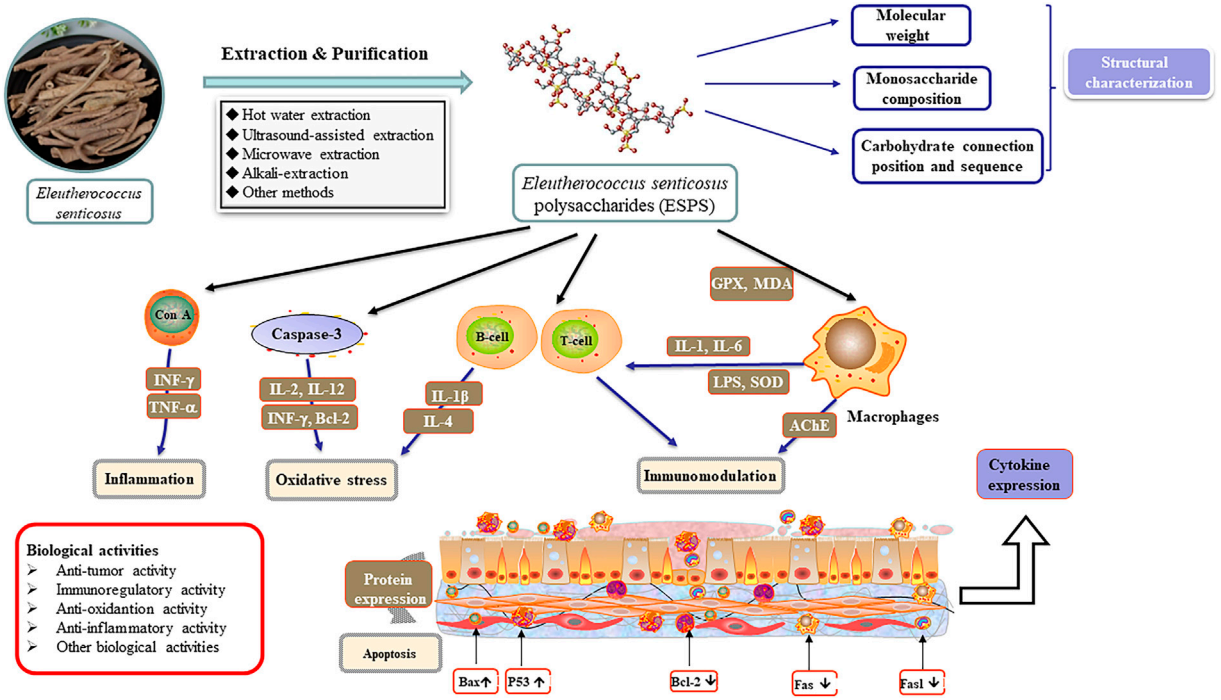


FIGURE 3 | Schematic diagram of biological activity mechanism of ESPS.

could be divided into step-alcohol-sinking and graded-alcohol-sinking, respectively. Related experiments showed that the purification performance of ESPS by step-alcohol-sinking performed more perfect in view of the higher content (Li, 2019). Besides, the concentration of alcohol in the graded-alcohol-sinking method would also affect the biological activity of ESPS. Li et al. compared the immune enhancement effects of ESPS obtained from 20, 40, and 60% alcohol precipitations. After comparison, ESPS obtained from 40% EtOH-precipitated exhibited better resistance to the splenic lymphocyte proliferation induced by ConA and LPS in a dose-dependent manner (Li, 2020).

In recent years, new materials and technologies have been increasingly applied in the separation and purification of polysaccharides accompanied by the rapid development of the science and technology. Among which, ion exchange chromatography has been used for the preparation of various types of components of traditional Chinese medicine in view of their advantages of good separation effect, fast separation speed, wide application range, and simple operation. Usually, diethylaminoethyl (DEAE)-cellulose is first used as an exchange agent to purify the polysaccharide to be acidic or neutral. The separated one was further purified by Sephadex gel according to the difference in molecular size. Based on this principle, scholars have developed different classification and types of fillers to achieve the systematic separation of polysaccharides with different properties. For example, Wang et al. employed Q Sepharose Fast Flow ion-exchange chromatography column to purify three kinds of ESPS,NASC-1, ASH-1 and ASA-1 that extracted by cold alkali, hot alkali and acid solutions, respectively (Wang, 2006). Bai et al. classified the ESPS from the rhizomes of *E. senticosus* into neutral ASPN and acidic ASPA-1 and ASPA-2 by DEAE-cellulose column. Subsequently, Sepharose CL-6B was further used to separate ASPA-1 into two uniform grades, namely ASPA-1-A and ASPA-1-B (Bai, 2015). Similarly, Zhai et al. adopted D941 resin, DEAE-Sepharose Fast Flow to chromatograph the crude polysaccharide and then used Sephadex-G200 molecular sieve to obtain the purified neutral and acidic polysaccharide As-1 and As-2, respectively (Zhai, 2007).

Content Determination

Content determination is an important procedure to control and evaluate the quality of polysaccharide products. Phenol-sulfuric acid and anthrone-sulfuric acid methods were the most commonly used ones. The principle is that polysaccharides are hydrolyzed into monosaccharides and further dehydrated to furfural or carboxymethyl furfural due to the concentrated sulfuric acid. Subsequently, phenol or anthraquinone can undergo condensation reaction with furfural derivatives to produce orange-yellow and blue-green compounds. Within 100–200 mg, the color depth increases with the increase of polysaccharide content, which can be determined by colorimetry. The compound produced by the phenol-sulfuric acid method has a maximum absorption peak at a wavelength of 490 nm, so the absorbance of the sample is measured at λ = 490 nm (Bai, 2015).

A study compared the phenol-sulfuric acid and the anthrone-sulfuric acid methods for the determination of ESPS content and found that the results of the two methods were similar, but the

TABLE 2 | Structural characterization of polysaccharides isolated from the different parts of *Eleutherococcus senticosus*.

No	Compound Name	Medical Parts	Monosaccharide Composition[a]	Main chain composition/ structural domain	Branch composition	M.W. (KDa)	Reference
1	PES-A	Root	Glc: Gal: Ara =3.3:2:1	—	—	7	Xu (1983)
—	PES-B	Root	—	—	—	76	Xu (1983)
—	PES-W	Root	—	—	—	—	Xu (1983)
2	As-II	Root	Glc	—	—	150	wang (1986)
—	As-III	Root	Ara: Xyl:4-O-methyl-D-GlcA =1:11:1	—	—	30	wang (1986)
3	AS-2	Root	Ara: Xyl: Rha: Gal: Glc =1.6:1.2:1.8:1.0:3.6	β-(1→3)-Glc, β-(1→4)-Glc	(1→4)-Rha, (1→6)-Gal, (1→3)-Gal, (1→6)-Glc	78	Zhang et al. (1993)
4	ASP	Root	—	—	—	—	Han et al. (2003)
5	ASC-1	Root	—	—	—	—	Wang et al. (2006)
—	ASC-2	Root	Rha: Ara: Xyl: Glc: Gal	—	—	—	Wang et al. (2006)
—	ASC-2C	Root	—	—	—	17	Wang et al. (2006)
—	ASC-2S	Root	—	—	—	11	Wang et al. (2006)
6	ASC-1P2B	Root	Xyl	—	—	—	Wang et al. (2006)
7	ASH-1	Root	—	—	—	—	Wang et al. (2006)
—	ASH-1P3	Root	Rha: Ara: Xyl: Man: Glc: Gal	—	—	—	Wang et al. (2006)
8	ASA-1	Root	—	—	—	12	Wang et al. (2006)
—	ASA-2	Root	—	—	—	—	Wang et al. (2006)
9	ASA-1P3A	Root	Glc: Xyl: Gal= 22.4:5.2:1	(1→6)-Glc	(1→4)-Glc, (1→3)/ (1→4)-Xyl,	38.3	Wang et al. (2006)
—	ASA-1P3B	Root	Glc: Xyl: Gal =7.6:19.4:1	(1→2)-Glc , (1→4)-Xyl	(1→3)-Xyl, (1→2), (1→4), (1→6)-Glc	7.2	Wang et al. (2006)
10	ASP-2-1	Leaf	Rha: Xyl: Glc: Man: Ara: Gal: GlcA =7.45: 18.63:25.15:0.93:8.35:2.79:5.69	—	—	14.6	Chen (2011b)
11	ESPS	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara: Fuc=1.5:13.6:0.6:14.9:17.1:24.2:11.6: 15.4:1.1	—	—	17	Bai (2015)
—	—	—	GlcA: Rha: Xyl: Fru: Glc=1.10:6.38:2.30: 2.83:12.34	—	—	—	Zhang and Sun, (2015)
—	—	—	Ara: Glu: Gal: Xyl: GalA =51.4:24.5:10.2: 5.7:4.9	—	—	74,38,45,23	Wang et al. (2016)
—	—	—	Ara: Xyl: Gluc: Man=7.1:22.3:7.6:1.0	—	—	—	Meng (2018)
12	ASPA	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara: Fuc= 0.3:13.5:1.9:27:3.6:22.3:15.1: 15.7:0.6	RG-1, AG-II, XGA	—	—	Bai (2015)
13	ASPN	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara: Fuc =4.3:4.5:1:1.7:25.7:32.9:7.1:22.6:0.2	—	—	—	Bai (2015)
14	ASPA-1	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara = 0.2:13:0.8:35.9:0.6:17:25.3:7.2	RG-1, XGA	—	—	Bai (2015)
—	ASPA-2	Root	Man: Rha: GlcA: GalA: Glc: Gal: Ara = 0.4: 11.4:1.8:15.7:2.2:44.7:23.8	RG-1	—	400	Bai (2015)
15	ASPA-1-A	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara: Fuc=0.4:14.8:1.1:26.9:0.6:20.4:27.1: 8.3:0.4	RG-1, XGA	—	48,40,37	Bai (2015)
—	ASPA-1-B	Root	Man: Rha: GlcA: GalA: Glc: Gal: Xyl: Ara: Fuc=0.4:11.7:0.9:46.2:1:14.1:20.4: 5.1:0.2	RG-1, XGA	—	11,10	Bai (2015)
16	ABPS-21	Stem	Gal: Glc: Rha: GalA =3.0:2.0:2.0:1.0 →4)-D-Galp-(1→4)-D-Glcp-(1→4)-D-	α-L-Rhap-(1→4)-β-D-Glcp-(1→,	106	—	Hu et al. (2015)
—	—	—	—	Galp-(1→4)-D-Galp-(1→2)-α-L-Rhap-(1→,	α-D-GalpA-(1→O-6 1,4,6-linked β-D-Glcp	—	—
—	—	—	—	—	α-L-Rhap-(1→4)-β-D-Glcp-(1→,	—	—

(Continued on following page)

TABLE 2 | (*Continued*) **S**tructural characterization of polysaccharides isolated from the different parts of *Eleutherococcus senticosus*.

No	Compound Name	Medical Parts	Monosaccharide Composition[a]	Main chain composition/ structural domain	Branch composition	M.W. (KDa)	Reference
—	—	—	—	—	α-D-GalpA-(1→ O-6 1,4,6-linked β-D-Galp	—	—
—	ABPS-21P	Stem	Gal: Glc: Rha =3.0:1.0:1.0	—	—	8.25	Hu et al. (2015)
17	ANP	Buds	Ara: Man: Glc: Gal=1.0:2.6:2.5:1.4	—	—	10.7	Lee et al. (2015)
—	AAP	Buds	Ara: Gal: 4-O-methyl-D-GlcA = 5:10:1	AG-II	—	84	Lee et al. (2015)
18	CASPs	Root	Ara: Man: Rha: Gal: Glc =1:1.1:3:4.7:5	—	—	70,38,120,19	Xie et al. (2015)

[a]*Ara, arabinose; Fuc, fucose; Fru, fructose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose; AG, arabinogalactans; RG, rhamnogalacturonan; XGA, xylogalacturonan.*

phenol-sulfuric acid method was more convenient. Using phenol-sulfuric acid method, the content of ESPS after freeze-drying was 51.9–56.5%, while that obtained by spray-drying was 50.5–53.1% (Zhang and Sun, 2005).

Existing data from literatures reveal that the variation explained by the content of ESPS ranged from 1.52 to 13.21% due to the limitation of the extracted part and process of *E. senticosus*. How to further increase the content of ESPS is still the direction we need to work hard on.

Structural Characterization of Polysaccharides

The polysaccharide belongs to a kind of high molecular polymer with a relative molecular weight (Mw) ranging from tens of thousands to tens of millions connected by glycosidic bonds. The structure identification of polysaccharides could be accomplished by the determination of the purity, monosaccharide composition, Mw, position and sequence of carbohydrate connection, glycoside bond configuration and oxygen ring. Several technologies including acid hydrolysis, methylation, periodic acid oxidation, Smith degradation, alkaline degradation, enzymatic hydrolysis, biological analysis, physical analysis (including IR, MS, ESI-MS, MALDI-MS, NMR, CE, GC, etc.,) would be employed.

The early research on structure of ESPS was limited to incomplete and inaccurate characterization by equipment and technology. Mw and monosaccharide composition were the only detected items for ESPS. The Mw of polysaccharides was obtained by measuring the standard dextran with known Mw. Based on this approach, PES-A and PES-B with 7 and 76 kDa together with immunologically active polysaccharides As-II and As-II with 150 and 30 kDa were purified from the root of *E. senticosus* (Xu, 1983). In the 21st century, the structure research and chemical modification technology of ESPS become more mature, high performance gel permeation chromatography (HPGPC) analysis technology with more accurate and fast superiority is developed to determine the Mw distribution and uniformity of polysaccharides based on the molecular sieve principle. Accordingly, several ESPS with Mw ranging from 7 to 400 kDa were reported (Wang, 2006). Their structural information is provided in **Table 2**. It is worth noting that the separation range of the HPGPC column possess the significant influence on the determination results. Bai et al. compared the Mw of the same ESPS samples separated by two specifications of columns from TSK-gel company. Results showed that Mw of ESPS-1-A and ASPA-1-B were 48 and 11 kDa by G-3000PWXL, while the value were 37 and 10 kDa by G-4000PWXL, respectively (Bai, 2015). As ASPS-1-A possessed larger Mw, more significant influence on it was present. This reminds us that the choice of chromatographic column is very important.

The determination of monosaccharide composition of polysaccharides has been improved and perfected. In the initial stages, thin layer chromatography (TLC) method was used by comparing the specific Rf value with each monosaccharide standard. While TLC can only provide the type of monosaccharides like ASC-2 and ASH-1P3 (Wang, 2006), their proportion could not be given as TLC is an unquantifiable method. Instead, gas chromatography (GC) could realize the measurement of above two respects based on the derivatization method. After GC analysis, the proportion of monosaccharides of PES-A (Xu, 1983), ASA-1P3A and ASA-1P3B had been supplied, which also provides key information for the subsequent estimate of ESPS types. Besides, the results in **Table 2** showed that GalA and Gal possessed relatively high proportion in ESPS.

In recent years, more in-depth research into chain sequencing and structural domain of carbohydrate is developed based on the requirement of pharmacology, toxicology and structural modification. According to the sequence, mode, and monosaccharide types of glycosylation in the main chain, the structure of ESPS could be divided into three types, namely RG-I, XGA and AG-II. 1) RG-I: Most of ESPS belong to the polysaccharides with RG-I structure due to high content of Rha and GalA connected through 1→4 linkage. Besides, the Rha/GalA ratios in different samples ranged from 0.25 to 0.73 indicated that they may be inducted as pectin. 2) XGA: XGA domain represent for some polysaccharides with higher Xyl content. Accordingly, the Xyl/GalA ratios 1.0 and 0.44 obtained from ASPA-1-A and ASPS-1-B suggest that XGA domain existed in both components. Moreover, several XGA domains also coexist with RG-I domain in other ESPS samples (Bai, 2015). 3) AG-II: Three kinds of ESPS named ASPA (Bai, 2015), ANP (Lee et al., 2015), and AAP (Lee et al., 2015)were found as AG-II pectin due to major connection of Ara and Gal through 1→3 linkage. However, the structure of ESPS was irregularly in branch composition.

BIOLOGICAL ACTIVITIES

Anti-tumor Activity

Anti-lung Cancer Activity

Lung cancer remains the leading cause of cancer deaths all over the word. It is generally divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively. However, SCLC was harder to cure due to its high invasiveness, low tumor differentiation, high deterioration as well as poor results of chemotherapy and surgery. Some studies had confirmed that ESPS could inhibit the proliferation of human SCLC cell line H446 using MTT, FCM and Hoechst33258 staining methods (Zhao, 2008a), and promote the apoptosis of H446 cells by up-regulating the expression of Bax and p53 genes and down-regulating the expression of Bcl-2 genes (Zhao, 2008b), and lead to G2/M phase arrest to interfere with H446 cell cycle progression by activating the ERK1/2 pathway (Zhao, 2010). Besides, the intervention effect of ESPS on the incidence of Lewis LC was also observed. *In vivo*, inhibition of tumor growth by ESPS was also manifested by decreasing the levels of PAI-1 and μPA in plasma, tumor and lung tissue. Moreover, combined administration of cyclophosphamide (CTX) and ESPS exhibited better effect than a single group of drugs (Zhang, 2001). Similarly, Sun et al. explored transwell and wound healing assay to evaluate the anti-tumor activity of ESPS on NCI-H520 cells after 24 and 48 h, decreased proliferation as well as repressed invasion and migration associated with Wnt/β-catenin pathway mediated-EMT provided new theoretical knowledge for NSCLC (Sun et al., 2019). In addition, Aidi injection is a proprietary Chinese medicine extracted from *E. senticosus* to treat stage IIIB/IV non-small cell lung cancer, and this therapeutic effect seems to be related to ESPS (Wang et al., 2018).

Anti-liver Cancer Activity

In recent years, some studies have found that ESPS also showed remarkable biological activity against liver cancer. In 1997, Zhang et al. found that ESPS had a significant inhibitory effect on hepatocellular carcinoma induced by chemical carcinogens (3'-me-DAB) (Zhang et al., 1997). *In vitro* experiments showed that ESPS could induce apoptosis and G0/G1 phase cell cycle growth arrest in HepG2 cells by targeting Wnt/β-catenin pathway (Wang et al., 2016). Anti-hepatic carcinoma assay experiment of ASC-1 and ESPS on mouse H22 transplanted tumors also showed a preventive effect. Compared with the white water group, the tumor suppression rate of ASC-1 reached 39.13% (Wang, 2006), and the tumor quality of the ESPS group was significantly lower than that of the control group (Meng, 2018). It can be inferred that ESPS indirectly suppressed hepatic carcinoma by enhancing the immunity of the body.

Other Anti-cancer Activity

Malignant tumors remain a serious disease that threatens human health. Currently, previously discovered ESPS have shown effects on cervical cancer, sarcoma, and laryngeal cancer of varying degrees along with the advantages of safety, efficacy and low toxicity. It can be used as antineoplastic and anti-tumor adjuvant drugs. As early as 1993, Cao et al. found that intragastric administration of ESPS at 10 mg/kg promoted the proliferation of mouse spleen cells and enhanced the activity of LAK cells, which provides an ideal biological response adjustment for LAK/IL-2 tumor cell therapy agents (Cao and Du, 1993). Meng et al. founded that different doses of ESPS (200, 100 and 50 mg/kg) could significantly inhibit the growth of sarcoma S180 and cervical cancer U14 on tumor-bearing mice and prolonged their survival time. Among which, 100 mg/ml dose possessed the best activity (Meng, 2018). Simultaneously, Tong et al. confirmed that ESPS has a direct anti-tumor effect on sarcoma S180 cells, whose inhibition rate exceeds 70% and half effective inhibitory concentration is 0.38 g/L (Tong, 1994). Besides, studies have also shown that ESPS can inhibit the proliferation of human cervical cancer HeLa cells via promoting the expression of Bax protein. At the same time, it may down-regulate the expression of survivin protein (Jin, 2014; Jin, 2016), thereby promoting the apoptosis of HeLa cells. ESPS inhibited the proliferation of CD133-positive stem cells in laryngeal cancer Hep-2, which may be related to the influence of ESPS on the expression levels of PD-L1 and Bcl-2 to promote stem cell apoptosis (Chen et al., 2019). This important discovery lays the foundation for the targeted therapy of laryngeal cancer stem cells.

Immunoregulatory Activity

Immunomodulation is considered to be one of the important physiological functions for the body to identify and remove antigenic foreign bodies as well as maintain physiological dynamic balance and relative stability. Abnormalities in the immune system could cause a variety of diseases such as infection, inflammation and cancer (Yu et al., 2018). In recent years, ESPS has been proved to have a certain regulatory effect on the immune system of mice. On the one hand, it can directly activate the immune function of T, B lymphocytes and macrophages by regulating immune organs; on the other hand, it can also play its biological activity by regulating the level of cytokines. Many studies have shown that intraperitoneal injection or intragastric administration of ESPS could increase the weight of thymus and spleen and improve immune function in mice (Chen, 1984; Xie, 1989a; Xu, 1990). Moreover, this regulatory effect may be positively correlated with the dose. Sun et al. found that the immune organ index, peripheral blood leukocyte count and macrophage phagocytosis of CTX-induced immunocompromised mice were increased by intragastric administration of different doses of ESPS aqueous solution (6 mg/10 g, 12 mg/10 g and 24 mg/10 g), and the efficacy of high dose group was significantly (Sun et al., 2018). Equally, Luo et al. also found that high-dose ESPS could significantly promote the proliferation of spleen lymphocytes and increase the ratio of $CD3^+CD4^+/CD3^+CD8^+$ (Luo, 2013a). In addition, ESPS can also improve the innate immune function of the body by reducing the levels of cytokines such as IFN-γ, IL-2, IL-6 and TNF-α (Diao, 2008a; Han et al., 2014; Zhang, 2015). *In vitro*, ESPS could significantly promote lymphocyte proliferation induced by concavalinA and LPS, and this effect is not related to endotoxin (Chen et al., 2011). These studies provide abundant evidence for ESPS as a natural immunostimulant agent. However, the present research on the immune activity of ESPS remains on the animal

level, and whether it can be used in clinic needs more in-depth research.

Antioxidant Activity

As we all know, the production of free radicals has the beneficial effects of regulating cell growth and inhibiting viruses and bacteria (Diao, 2008c). Nevertheless, excessive free radicals will lead to excessive reactive oxygen species (ROS) formation and break the redox homeostasis. This is closely related to some human chronic diseases, such as cancer, arteriosclerosis and aging (Diao et al., 2010b). ESPS have been proved to have significant scavenging effects on a variety of free radicals, such as hydroxyl radicals, superoxide anions and hydrogen peroxide. *In vitro*, Chen and Zhao reported that ESPS had obvious scavenging activity evaluated by ferric reducing antioxidant power assay (FRAP), Fenton's reaction, pyrogallol's auto oxidation and DPPH radical scavenging experiments in a concentration-dependent manner (Jiao, 2008) (Meng et al., 2005; Diao, 2008d; Chen, 2011b). Xia et al. compared the antioxidant capacity of two water-soluble polysaccharides (ASP-B2 and ASP-B3) extracted from the leaves of *E. senticosus*. It is worth noting that the clearance rates of ASP-B2 and ASP-B3 for DPPH reached 91.75 ± 0.59% and 91.58 ± 1.58% at 2.0 mg/ml, respectively (Diao, 2008b). Likewise, ESPS extracted from fruits also showed antioxidant activity. Among the concentration range from 0.05 to 0.8 mg/ml, the scavenging rates of ESPS to DPPH and ABTS increased in a concentration-dependent manner, and the highest scavenging rates were 65.21 ± 1.85% and 58.02 ± 1.25%, respectively (Diao, 2009b).

Meantime, researchers also focused on the antioxidant activity of ESPS *in vivo*. It is reported that the activities of superoxide dismutase (SOD) and GSH-Px increased in a dose-dependent manner after 15 days of intragastric administration of ESPS (50, 100 and 200 mg/kg/d) in rats with cerebral ischemia-reperfusion. As SOD is the most important antioxidant enzyme for scavenging free radicals *in vivo*, the result suggests that ESPS may improve cerebral ischemia-reperfusion injury by improving the antioxidant capacity of brain tissue (Xie et al., 2015). Similarly, Long et al. found that ESPS could also improve the growth performance of chicks by increasing the activities of SOD and GSH-Px (Long et al., 2021). Besides that, some domestic scholars have also found that ESPS can significantly protect rat hippocampal neurons from oxidative stress damage induced by H_2O_2 (Diao et al., 2010b; Liu et al., 2013). Above studies indicated that ESPS seem to be used as a potential natural antioxidant. However, there is a big gap between the clinical application and fundamental research, which need further explore in the future.

Anti-inflammatory Activity

Inflammation is a response to pathogens and tissue damage. This process is mainly caused by a series of pathological reactions of the injured site by stimulating immune cells to release inflammatory mediators. However, long-term inflammation is the main cause of aging and serious diseases, such as inflammatory bowel disease, cardiovascular disease (CVD), hepatitis and cancer (Diao, 2009d). In the past decades, the natural polysaccharides extracted from *E.* senticosus showed significant anti-inflammatory activity. Many studies have shown that ESPS could regulate the level of inflammatory mediators *in vivo* and reduce the infiltration of inflammatory cells in multiple organs. It is reported that ESPS pretreatment reduced the secretion and expression of inflammatory cytokines such as IL-2, IL-4 and INF-γ, which has a certain protective effect on immune liver injury (Zhu et al., 1982; Xie, 1989c; Luo, 2013b; Han, 2013; Zhang, 2018; Zhai, 2020). Similarly, ESPS could also inhibit the activation of NF-κβ in mouse liver tissue and endotoxic shock induced by LPS/D-GalN (Diao, 2009c). Xie et al. also reported that intragastric administration of ESPS could reduce the contents of IL-1β and TNF-α in brain tissue and exhibit a certain protective effect on cerebral ischemia-reperfusion injury (Zhang et al., 2019). In addition, Han and Fan et al. have verified that ESPS could improve a variety of inflammatory bowel diseases induced by LPS, whose effect is mainly mediated by NF-κB/MLCK, HIF-1α/COX-2 and TLR4/NF-κB signal pathways (Yang et al., 1983; Yang et al., 1985; Poolsup et al., 2004; Luo, 2008; Mo, 2012a; Mo, 2012b; Han et al., 2016a; Han et al., 2016b; Lu, 2016; Yang, 2016; Han et al., 2017; Pan, 2019b; Zhang et al., 2020a). Clinically, Poolsup et al. conducted a statistical analysis of 433 patients with upper respiratory tract infection and found that *E. senticosus* was more effective than placebo. Whether this effect is related to ESPS needs to be further explored (Poolsup et al., 2004).

Other Biological Activities

Apart from to above biological activities, ESPS also possess the activities of analgesia, anti-radiation, anti-fatigue as well as compatibility with other drugs to treat diabetes and leukemia. As early as the end of the 20th century, ESPS was reported to increase the ability to produce IFNs in S801 and S7811 leukemia cell lines (Diao, 2008b; Diao, 2008c; Diao, 2008d; Diao, 2009a; Diao, 2009b). Similarly, the proliferation of leukemia cells K562 and Pmur388 original from human and mice were also inhibited by ESPS (Diao, 2009c; Jin, 2016). Moreover, Mo et al. reported that ESPS also had anti-radiation effect (Diao et al., 2010a; Diao et al., 2010b). ESPS has also shown a protective effect on the gastrointestinal tract, reproductive system, and peripheral blood cells of rats irradiated by 60Co gamma rays (Diao et al., 2010c). It's worth noting that magical effect of ESPS as an adjuvant therapy for diabetes was discovered by the rapidly reduced blood glucose after combined use of ESPS with Metformin, whose efficacy on the level of blood lipids (TC and TG), thiobarbituric acid reactive substances (TBARS), AST, ALT, ALP, total bilirubin, creatinine and urea was better than that of Metformin (Fu et al., 2012).

Besides, ESPS can also improve exercise-induced energy metabolism, increase serum creatinine level and reduce protein decomposition, which exhibits a significant anti-fatigue potential (Han, 2007; Bang et al., 2015). Others used metabonomics methods to investigate the mechanism of ESPS on Alzheimer's disease and CVD rats by detecting the endogenous substances, 20 potential biomarkers such as 6-dimethylaminopurine and L-acetylcarnitine were screened out (Lu, 2015). **Table 3** and

TABLE 3 | Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
Anti-lung cancer	ESPS	Root	Female C57/ BLmice	*In vivo*	i.p.	0.12 ml/piece	—	μPA ↓	Intervene of lung cancer	PAI-1 ↓	Zhang (2001)
	ESPS	Root	H446 cells	*In vitro*	—	240,480,960 μg/ml	48 h	—	Cell proliferation ↓	P53, Bax↑, Bcl-2 expression ↓ Apoptosis	Zhao (2018)
	ESPS	Root	H446 cells	*In vitro*	—	240,480,960 μg/ml	48 h	TNF-α, IL-1	Cell proliferation ↓	P53,Bax↑,Bcl-2↓p-38 expression EPK pathway, Apoptosis	Zhao (2018)
	ESPS	Root	H446 cells	*In vitro*	—	240,480,960 mg/l	48 h	—	G2/M arrest ↓	EPK MAP kinase pathways	Zhao (2010)
	ESPS	Root	NSCLC NCI-H520	*In vitro*	—	10,20,40,80,160, 320 mg/ml	12,24,48 h	MMP2, MMP9, FN1, wnt3a	Proliferation, metastasis ↓	Wnt/β-catenin pathways	Sun et al. (2019)
	ASC-1	Root	A-549 cells	*In vitro*	—	ASC-1 35.7 μM	11 days	—	Cell viability ↓	—	Wang, (2006)
	ASA-1	Root	—	—	—	ASA-1 125 μM	—	—	—	—	—
	ASH-1	Root	—	—	—	ASH-1 163.5 μM	—	—	—	—	—
Anti-liver cancer	ESPS	Root	Mice, HEPA cells	*In vivo*	s.c.	0.34 g/kg /d	10 days	—	The life of mice ↑	—	Chen (1984)
	ASC-1	Root	Male Kunming mice,	*In vivo*	i.g.	2 mg/piece	11 days	ASC-1 prevent seed tumors	—	—	Wang, (2006)
	ASA-1	Root	—	H22 cells	i.p.	0.04 mg/piece	—	—	ASA-1no anti-tumor effect	—	—
	ESPS	Root	Kunming mice,	*In vivo*	i.g.	50,100,200 mg/kg/d	10 days	TNF-α,TNF-γ,	Survival days ↑,regulate cytokines	—	Meng, (2018)
	—	—	H22cells	—	—	—	—	IL-2, IL-12	—	—	—
	PAS	Root	C57BL/6 mice	*In vivo*	i.p.	10 mg/kg /d	14 days	IL-2R, LAK	LAK activity ↑, splenocyte ↑	—	Cao and Du, (1993)
	ESPS	Root	Male Wister rat	*In vivo*	i.g.	20%	70 days	γ-GT ↑	Inhibit tumor	—	Zhang et al. (1997)
	ESPS	Root	HepG2 cells	*In vitro*	—	0,10,20,40,80 mg/L	48 h	—	Apoptosis↑, G0/G1 phase arrest in	Wnt/β-catenin pathway	Wang et al. (2016)
Anti-other tumors	ESPS	Root	Kunming mice,	*In vivo*	i.g.	50,100,200 mg/kg/d	10 days	IL-2,IL-12,INF-γ	Cell growth ↓	Apoptosis	Meng et al. (2018)
	ESPS	Root	Kunming mice,	*In vivo*	i.g.	50,100,200 mg/kg/d	10 days	TNF-α,TNF-γ,	Survival days ↑, regulate cytokines	Immunomodulation	Meng, (2018)
	—	—	U14 cells	—	—	—	—	IL-2, IL-12	—	—	—
	ESPS	Root	Hela cells	*In vitro*	—	1,2,4,8,16 mg/ml	24,48,72 h	—	Cell proliferation ↓	Bax expression ↑ , Apoptosis	Jin (2014)
	ESPS	Root	Hela cells	*In vitro*	—	1,2,4,8,16 mg/ml	24,48,72 h	—	Cell proliferation ↓	Survivin expression ↓, Apoptosis	Jin (2016)
	ESPS	Root	Kunming mice,	*In vivo*	i.g.	50,100,200 mg/kg /d	10 days	TNF-α,TNF-γ,	Survival days ↑, regulate cytokines	Immunomodulation	Meng, (2018)
	—	—	S180cells	—	—	—	—	IL-2, IL-12	—	—	—
	ESPS	Root	Mice S37,S180 cells	*In vivo*	s.c	0.34 g/kg/d	10 days	—	Excited reticuloendothelial system	Immunomodulation	Chen (1984)
	ESPS	Root	S180cells	*In vitro*	—	1,10,100,500,	24 h	—	Cell proliferation ↓	Apoptosis	Tong (1994)

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TABLE 3 | (*Continued*) Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
	—	—	—	—	—	1000 mg/l	—	—	—	—	—
	ESPS	Root	Hep 2 CD133	*In vitro*	—	100 mg/l	24,48,72 h	Bcl-2,PD-L1	CD133 stem cells ↓	Apoptosis	Chen et al. (2019)
Immuno-modulatory	PES-W	Root	Female C57BL mice	*In vivo*	i.p.	100 mg/kg /d	5 days	—	Immunity↑, hyperplasia of	Immunomodulation	Xu (1983)
	PES-A	—	—	—	—	—	—	spleen and thymus ↑	—	—	—
	PES-B	—	—	—	—	—	—	—	—	—	—
	PES-W	Root	Female C58BL mice	*In vivo*	i.p.	100 mg/kg/d	4 days	—	The phagocytic function of mice	Immunomodulation	Xu (1983)
	PES-A	—	—	—	—	—	—	—	Peritoneal macrophages ↑	—	—
	PES-B	—	—	—	—	—	—	—	—	—	—
	ESPS	Root	Mice	*In vivo*	i.p.	30 mg/kg/d	3 days	—	Sleep time↑, number of thymus ↓	Immunomodulation	Chen (1984)
	ESPS	Root	Mice	*In vivo*	i.p.	30 mg/kg /d	3 days	—	Sleep time↑, white blood cells↑	Immunomodulation	Chen (1984)
	ESPS	Root	Mice	*In vivo*	s.c.	0.067 g/kg/d	3 days	—	Formation of antibodies in the spleen ↑	Immunomodulation	Chen (1984)
	ASII	Root	Mice	*In vivo*	—	—	—	—	Strong phagocytosis ↑	Immunoregulation	Wang, (1986)
	ASIII	—	—	—	—	—	—	—	—	—	—
	ESPS	Root	Male BALB/c, C57BL/6 mice	*In vivo*	i.p.	100 mg/kg/d	10 days	ConA, LPS	Stimulate T-cell proliferation	—	Xie et al. (1989a)
Immuno-Modulatory	ESPS	Root	Male BALB/c, C57BL/7 mice	*In vivo*	i.p.	100 mg/kg/d	15 days	ConA, LPS	Number of antibody secreting cells↑ C57BL/7 mice delayed-type hypersensitivity↑	Immunomodulation	Xie (1989b)
	ESPS	Root	LACA mice	*In vivo*	i.p.	12.5,25,50,100 mg/kg	4/9 days	—	Humoral immune response↑	—	Xu, (1990)
	PES	Root	Female C57BL/JCR mice	*In vitro*	s.c.	100 mg/kg	4 days	LPS	PES possesses mitogenic activities	Immunomodulation	Shen et al. (1991)
	—	—		—	—	—	—	—	Enhanced LPS, lymphocyte	—	—
	—	—	—	—	—	—	—	—	Transformations ↓, stimulates B-cells	—	—
	ESPS	Root	Kunming mice	*In vivo*	i.g.	25,50,100 mg/kg	7 days	CD3+,CD4+,	The proliferation of splenic	Immunomodulation	Luo (2013a)
	—	—	—	—	—	—	—	CD8+	lymphocytes ↑,	—	—
	—	—	—	—	—	—	—	—	CD3+CD4+/ CD3+CD8+↑	Immunomodulation	—
	ESPS	Root	Kunming mice	*In vivo*	i.g.	25,50,100 mg/kg	7 days	CD4+/CD8+,	The body's cellular immune	Immunomodulation	Luo (2013b)
	—	—	—	—	—	—	—	IL-2	function ↑	—	
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	14 days	—	The phagocytic function of	Immunomodulation	Zhai (2020)
	—	—	—	—	—	—	—	—		—	—

(Continued on following page)

TABLE 3 | (*Continued*) Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
									immunosuppressed mice ↑		
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	7 days	—	The production of hemolysin HC50↑	Immunomodulation	Zhai (2020)
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	7 days	—	The weight of the thymus ↓	Immunomodulation	Zhang, (2015)
	ESPS	Root	BALB/c, DTH mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	7 days	CD4+/CD8+	Immunosuppressive effect	—	Zhang, (2015)
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	7 days	IL-2,IL-4,IFN-γ	Cytokine content ↓	Cytokine expression	Zhang, (2015)
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36,25,72.5,145 mg/kg	14 days	HC50	Sheep red blood cell production	Immune stress	Zhang, (2015)
	—	—	—	—	—	—	—	—	Hemolysin ↑	—	—
	ESPS	Root	Weaned piglets	*In vitro*	—	0,40,80,160, 320 μg/ml	21 days	NO, iNOS, NF-κB,	Cooperate with ConA to promote	Apoptosis	Han, (2013)
	—	—	—	—	—	—	—	Th1	T- cell proliferation	—	—
	ESPS	Root	Weaned piglets	*In vitro*	—	800 mg/kg	14d	IL-2,IL-6, TNF-α,	Modulate the release of	Cytokine expression	Han et al. (2014)
	—	—	—	—	—	—	—	α-AGP	Pro-inflammatory cytokines	—	—
	ESPS	Root	ICR mice	*In vivo*	i.g.	6,12,24 mg/10g/d	21 days	TNF-α, INF-γ	Regulate the serum hemolysin level,	Apoptosis	Sun et al. (2018)
	—	—	—	—	—	—	—	—	Cell apoptosis ↓ to enhance the	—	—
	—	—	—	—	—	—	—	—	Body's humoral immune function.	—	—
	PES	Root	Mice	*In vivo*	i.p.	125 mg/kg/d	5 days	BSAIgG↑	Enhance the defense.	Antioxidant index	Zhu et al. (1982)
Anti-inflammatory	ESPS	Root	Kunming mice	*In vivo*	i.g.	20 mg/kg/d	10 days	IL-1, IL-2,	Improve immune cytokines	Cytokine expression	Meng, (2018)
	—	—	—	—	—	—	—	IL-10, TNF-α	—	—	—
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36.25,72.5,145 mg/kg	7 days	IL-1, IL-1β, IL-2, IL-4	Number of enzymes ↓,protect liver	Antioxidant index	Zhang (2018)
	CASPs	Root	Wistar male rats	*In vivo*	i.g.	50,100,200 mg/kg	15d	IL-10↑, IL-1β, TNF-α↓	Inflammatory cytokines↓	—	Xie et al. (2015)
	ESPS	Root	Male BALB/c mice	*In vivo*	i.g.	14.5 mg/ml/d	7 days	IL-4, Cyclic GMP	Improve the body's immunity,	Glutathione metabolism, purine	Yang, (2016)
	—	—	—	—	—	—	—	—	Anti-inflammatory, Antioxidant,	Generation metabolism, cysteine ,	—
	—	—	—	—	—	—	—	—	and supply the body's energy	methionine metabolism pathways	—
	ASPS-1-1	Root	Male BALB/c mice	*In vivo*	i.g.	6.9 mg/ml	7 days	IL-1β, TNF-α, IL-2,	Liver cell damage ↓	—	Yang, (2016)
	ASPS-2-1	—	—	—	—	4.6 mg/ml	—	IL-4, IL-6	—	—	—
	ASPS-3-1	—	—	—	—	2.0 mg/ml	—	—	—	—	—
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36.25,72.5,145 mg/kg	7 days	IL-2, IL-4,	Secretion and expression of	Cytokine expression	Zhang (2018)

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TABLE 3 | (*Continued*) Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
	—	—	—	—	—	—	—	—	inflammatory cytokines ↓	—	—
	ESPS	Root	BALB/c mice	*In vivo*	i.g.	36.25,72.5,145 mg/kg	7 days	MDA, IL-1β, TNF-α,	The activity of inflammatory	Cytokine expression	Zhang et al. (2019)
	—	—	—	—	—	—	—	NO, GSH-Px, SOD,	Cytokines, adhesion factors↓,	—	—
	—	—	—	—	—	—	—	ICAM-1, iNOS,	The secretion and expression of	—	—
	—	—	—	—	—	—	—	NF-κB	Inflammatory cytokines↓	—	—
	ESPS	Root	KM mice	*In vivo*	i.g.	14.5 mg/ml/d	7 days	IL-2, IL-6	By enhancing the body's immune function, scavenging free radicals, liver cell apoptosis↓, and other mechanisms	Bile secretion, Cysteine metabolism, Ubiquinone and other terpenoid quinone biosynthesis, Citrate cycle regulating the body's cytokines Purine metabolism, Riboflavin metabolism, Primary bile acid biosynthesis, Biosynthesis of unsaturated fatty acids, Cysteine and methionine metabolism	Lu (2016)
Anti-leukemia	ESPS	Root	Mice, L615 cells	*In vivo*	i.p.	0.34 g/kg/d	5 d	—	No significant effect on survival time	Oxidative stress	Chen (1984)
	ESPS	Root	S801, S7811 cells	*In vitro*	—	10 μg/ml	single	—	Improve the ability of cells to produce interferon	Oxidative stress	Yang et al. (1983)
	ESPS	Root	S801,S7811 cells	*In vitro*	—	10 μg/ml	single	—	The life span of the transcribed dry money mRNA↑ or mRNA inactivation rate↓	Apoptosis	Yang et al. (1985)
	ESPS	Root	K562 cells	*In vitro*	—	1,10,100,500, 1000 mg/l	24 h	—	Cell proliferation↓	Apoptosis	Tong (1994)
	ESPS	Root	K562 cells	*In vitro*	—	0.405, 0.810, 1.620, 2.430, 3.240 mg/mL	24 h	—	K562 cell apoptosis ↑	Apoptosis	Luo (2008)
	ASC-1	Root	P-388 cells	*In vitro*	–	35.69 μM	single	—	Mouse white blood cell activity↓	—	Wang, (2006)
	ASH-1	—	—	—	—	164.23 μM	single	—	—	—	—
Anti-oxidation	ESPS	Root	·OH, O^{2-}	*In vitro*	–	3 mg/l	single	—	Elimate ·OH and O^{2-}	Oxidative stress	Meng et al. (2005)
	ESPS	Root	·OH, O^{2-}	*In vitro*	–	3 mg/l	single	—	Elimate ·OH and O^{2-}	Oxidative stress	Jiao, (2008)
	—	—	—	—	—	—	—	—	Inhibit lipid peroxidation of red blood	—	—
	—	—	—	—	—	—	—	—	cell membrane↓	—	—
	ASP-2-1	Leaf			—	0.2 mg/ml	single	—	Elimate ·OH, O^{2-}, DPPH·	Oxidative stress	

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TABLE 3 | (*Continued*) Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
			Mice spleen cells	*In vitro*							Chen (2011a)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 g/ml	7 days	SOD,NOS,MDA	Improve cell resistance to oxidative stress damage	Bax expression ↓,Bcl-2 expression ↑	Diao (2008a)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	—	Improve the anti-apoptotic ability	Bax expression ↓,Bcl-2 expression ↑	Diao (2008b)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	LDH,SOD,MDA	Improve the anti-apoptotic ability	P53 expression	Diao (2009a)
	ESPS	Root	SD rats hippocampal	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	NO, iNOS mRNA↓	The expression of iNOSmRNA↓	iNOSmRNA expression	Diao (2008c)
	ESPS	Root	SD rats hippocampal	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	—	Anti-apoptotic ability ↑	P53 expression ↓	Diao (2009b)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	SOD, CAT, GSH-Px, MDA	Activity of Antioxidant ↑	Antioxidant index	Diao (2008d)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	MDA, ROS, H_2O_2	The content of oxygen free radicals	Oxidative stress ↓	Diao (2009c)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	1.25, 2.5, 5, 10 μg/ml	7 days	SOD, GSH-Px, OGG1mRNA	OGG1mRNA expression ↑	OGG1mRNA expression	Diao (2009d)
	CASPs	Root	Wistar male rats	*In vitro*	i.g.	50, 100, 200 mg/kg	15 days	SOD, GSH-Px↑, MDA↓	Oxidative damage ↓	Oxidative stress	Xie et al. (2015)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	2.5,5,10 μg/ml	7 days	Fas, Fasl↓	Cell apoptosis↓	Fas,Fasl↓, Apoptosis	Diao et al. (2010a)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	2.5,5,10 μg/ml	7 days	—	Gene expression↓, the activity↑	C-fos,p53 expression	Diao et al. (2010b)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	2.5,5,10 μg/ml	7 days	NF-κB↓	Gene expression↓	NF-κB pathways	Diao et al. (2010c)
	ESPS	Root	SD rats hippocampal neurons	*In vitro*	—	2.5,5,10 μg/ml Caspase-3 mRNA	24 h	Caspase-3	Resist oxidative stress damage	Oxidative stress	Liu et al. (2013)
Other biological activities	ESPS	Root	Kunming mice	*In vivo*	—	300 mg/kg	14 days	DAO, occludin-1, zonula occludens-1	Improves intestinal integrity	TLR4/NF-κB signaling pathway	Han et al. (2016a)
	ESPS	Root	Kunming mice	*In vivo*	i.g.	300 mg/kg	14 days	Occludin-1, HSP70	Gene mRNA expression of epidermal growth factor and its receptor ↑	mRNA expression	Han et al. (2016b)
	ESPS	Root	Male Wistar rat	*In vivo*	i.g.	50,100,200 mg/kg	10 days	—	Protect the body weight of irradiated rats	—	Mo (2012a)
	ESPS	Root			i.g.	50,100,200 mg/kg	10 days	WBC, RBC, PLT		—	Mo (2012b)

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TABLE 3 | (*Continued*) Pharmacological activities and involved mechanism of ESPS.

Biological activity	Compound Name	Medical Parts	Model	Vivo/ Vitro	Administration Route[a]	Dose	Duration	Cytokine/ Enzyme /Protein	Results	Involved Mechanism	References
			Male Wistar rat	*In vivo*					Protect peripheral blood cells from radiation exposure		
	ESPS	Root	Adult flies	*In vivo*	i.g.	15,30 mg/ml	5 days	—	Protects the intestinal tract from DSS	EGFR, JNK, Notch pathways	Zhang et al. (2020a)
	ESPS	Root	BALB/C mice	*In vivo*	i.g.	300 mg/kg	7 days	MLCK, TJ, NF-κB, LPS	Associated with inhibition of the NF-κB/ MLCK pathway	NF-κB/MLCK pathway	Han et al. (2017)
	ESPS	Root	Kunming mice	*In vivo*	i.g.	50,100,200 mg/kg	7 days	IL-6, TNF-α, IL-1β	Improve immune organ index and regulate cytokine levels	—	Pan (2019b)
	ESPS	Root	Male SD rat	*In vivo*	i.g.	100 mg/kg	20 days	—	Neuroprotection, anti-apoptosis	Anti-apoptosis	Lu (2015)
	ESPS	Root	Male kunming mice	*In vivo*	i.g.	450,150 mg/kg/d	14 days	—	Ameliorate energy metabolism, creatinine, improve the level of serum the breakdown of protein↓ free radical content of the body	Energy metabolism	Han, (2007)
	ESPS	Root	Kunming mice	*In vivo*	i.g.	50,100,200 mg/kg/d	5 days	IL-6, TNF-α	Inhibit swelling, improve immune organs, immune cytokines	Immunomodulation	Meng, (2018)
	ESPS	Root	Female SD rat	*In vivo*	s.c.	100 mg/kg/d	2/5 days	sICAM-1, TGF-β1, LYM, RT, GLU, LDH	Improve pleural effusion	Immunomodulation	Meng, (2018)
	ESPS	Root	Kunming mice	*In vivo*	i.g.	50,100,200 mg/kg/d	7 days	IL-1β, TNF-α	Improve immune organs, immune cytokines	Immunomodulation	Meng, (2018)
	ASP	Root	Male Wistar rats	*In vivo*	i.g.	200 mg/kg	28 days	SOD, GPX, TC, TG, TBARS	Adjust the pathophysiological	Antioxidant index parameters of diabetic rats	Fu et al. (2012)
	ESPS	Root	Kunming mice	*In vivo*	i.g.	50,100,200 mg/kg	5 days	SOD, GSH-PX,	Improve the Antioxidant capacity CAT, MDA	Antioxidant index caused by D-Gal	Meng, (2018)
	ESPS	Root	Female SD rat	*In vivo*	i.p.	50,100,200 mg/kg/d	5 days	SOD, MDA, AChE, ChAT	Regulate enzyme activity in brain tissue and serum	Immunomodulation	Meng, (2018)
	ANP	Buds	RAW-Blue TM cells	*In vitro*	—	—	—	—	Promoted by the interaction through	—	Lee et al. (2015)
	AAP	—	(RAW 264.7)	—	—	—	—	—	the membrane receptors	—	—
	PEA	Root	—	*In vitro*	i.g.	1.75 g/kg	24 h	CRP	Suppress hypoglycemia and	Inflammation inflammation to relieve alcohol	Bang et al. (2015)

[a]*i. g. intragastric administration; i. v. intravenous injection; i. p. intraperitoneal injection; s. c. subcutaneous injection.*

Figure 3 summarize the biological activities and related mechanism of ESPS, respectively.

CONCLUSIONS AND FUTURE PERSPECTIVES

Consult the related literature and data about *E. senticosus*, we can find that the related studies mainly focused on small molecule components, which ignored macromolecular substances such as protein, polysaccharides, pectin, and cellulose. As the effective ingredient of *E. senticosus*, polysaccharide possess a wide range of pharmacological effects with the advantages of non-toxic, two-way immune regulation, and has great potential for clinical application. However, research on polysaccharides has increased resistance due to its high molecular weight, complex structure, low content, difficulty of separation and low purity. The research work of its future perspectives is mainly reflected in the following aspects.

1) From aspects of efficient extraction, the existing methods expose the disadvantages of low yield and long extraction time, and therefore cannot be industrialized in mass production. Instead, a wide range of green environmental technologies such assisted extraction with enzymes and chelating agents have not been studied and applied in the extraction of ESPS.
2) As for the resource utilization, the residues of herbs and fruits after extraction of small molecules are rich in a large number of macromolecular substances, such as pectin, polysaccharides and cellulose. Thus, recirculated extraction is conducive to the sustainable development and maximum utilization of traditional Chinese medicine. It has been reported that relative high yield of sugars and pectin was separately extracted from residues of *Ginseng* (Zhang et al., 2020b) and Sweet Potato Peels (Hamidon et al., 2020). Meanwhile, Chen et al. found that a large amount of ESPS could be extracted from petioles, leaves and fruits (Chen, 2009). However, no literature on the reuse and expansion of *E. senticosus* resource were found. Collectively, extraction of ESPS from the residue and other medical parts will provide us future research direction.
3) Recently, the rapid development and application of biological macromolecules in new drug delivery system and edible film provide people new directions and technologies for pharmaceutical and food preservation. If ESPS could pack or interact with other ingredients (flavonoids, saponins, protein) and then act as drug delivery system is relatively empty areas.
4) At present, several products derived from *E. senticosus* such as capsules, wine and tea have long been developed and marketed in view of their excellent health-care function and clinical therapeutic effect. However, ESPS has not been put into clinical use in view of the absence of quality control standards and reference substance. How to establish a comprehensive quality management system is a problem worth thinking about.

In conclusion, health has received more and more attention with the development of science and the improvement of living standard, and the exploitation of functional food has become a research hotspot and development trend. *E. senticosus,* a homology of medicine and food as the main raw materials, has displayed the most economic and developing value. Meanwhile, the emergence of ESPS can better inherit, innovate and develop traditional Chinese medicine culture and products. It is believed that ESPS will be widely applied soon in the fields of clinical and functional food with the deepening of research and the maturity of technology.

AUTHOR CONTRIBUTIONS

XL: Investigation, Resources, Writing-original draft. CC: Writing-original draft. JQ: Conceptualization, Writing-review and editing, Funding acquisition. AL: Writing-review and editing, Funding acquisition.

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GLOSSARY

AchE acetylcholinesterase
AG arabinogalactans
ALP Alkaline phosphatase
ALT alanine aminotransferase
AST aspartate aminotransferase
Ara arabinose
ChAT choline acetyl-transferase
ConA Concanavalin A
CVD cardiovascular disease
CTX cyclophosphamide
EGFR epidermal growth factor receptor
ERK regulated protein kinase
ESPS Eleutherococcus senticosus polysaccharides
Fuc fucose
Fru fructose
FT-IR Fourier transform infrared spectroscopy
Gal galactose
GC gas chromatography
Glc glucose
GalA galacturonic acid
GlcA glucuronic acid
GSH glutathione
GSH-Px glutathione peroxidase
HPLC high-performance liquid chromatography
i.g. intragastric administration
i.v. intravenous injection
i.p. intraperitoneal injection
IFN interferon
ICAM intercellular adhesion molecule
iNOS inducible nitric oxide synthase
IL interleukin
JNK c-jun N-terminal kinase
LAK lymphokine-activated killer cell
LC liquid chromatography
LC Lung cancer
LPS lipopolysaccharide
Man mannose
MAPK mitogen-activated protein kinase
MDA malonaldehyde
MMP mitochondrial membrane potential
MS mass spectrometry
NF-κB nuclear factor-kappa B
NSCLC non-small cell lung cancer
NMR nuclear magnetic resonance
NO nitric oxide
OGG1 8-hydroxyguanine DNA glycosidase
PLT platelets
Rha rhamnose
RBC red blood cells
RG rhamnogalacturonan
Rib ribose
SCLC small cell lung cancer
s.c. subcutaneous injection
SFE supercritical fluid extraction
SOD superoxide dismutase
TBARS thiobarbituric acid reactive substances
TCA trichloroacetic acid
TLR toll-like receptor
TC serum total cholesterol
TG triglyceride
TNF tumor necrosis factor
UAE ultrasound-assisted extraction
WBS white blood cells
XGA xylogalacturonan
Xyl xylose.

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Mercury Content in Dietary Supplements From Poland Containing Ingredients of Plant Origin: A Safety Assessment

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Mercury (Hg) is a fairly common environmental pollutant. Chronic exposure to this element may cause, inter alia, kidney damage, and disturbances in the functioning of the nervous system. Literature data indicate that food, including dietary supplements (DS), may sometimes be contaminated with Hg. Therefore, the aim of the study was to assess Hg content in DS containing ingredients of plant origin. The study covered 200 DS available for sale in Poland. Hg content was determined by using the AAS method with the amalgamation technique using the AMA-254 analyzer. The highest average Hg content was found in preparations used as adjuncts for lowering glucose levels (23.97 ± 38.56 μg/kg). The highest percentage of PTWI (1.143%) was found in DS aimed at improving vitality. Due to the fact that DS are commonly used, their quality should be constantly monitored.

Keywords: mercury, dietary supplements, herbs, food safety, PTWI

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INTRODUCTION

Dietary supplements (DS) constitute a large group of food products. They are used by patients for prophylactic purposes, to support therapy or to supplement the diet with missing nutrients, and are sometimes treated as drugs (due to their similarity in terms of pharmaceutical form). It is also commonly believed that DS have a natural composition, are not harmful, have no side effects, and cannot be overdosed. Since they are sold for example in the form of tablets, capsules, syrups, and lozenges, they tend to be mistaken for medical products. As a result, they are often used by people with weakened immunity, diseases of various systems, or confirmed vitamin and mineral deficiencies. Data show that the popularity of DS and their presence in the market are steadily increasing (The Law on Food and Nutrition Safety, 2006; Main Sanitary Inspectorate, 2021).

Among DS available in Poland, the largest market share is held by preparations with magnesium (7.56%); immunostimulants (6.58%); probiotics (6.13%); supplements for strengthening bones, muscles, and joints (4.75%); vitamins and minerals for adults (4.65%); beauty supplements (4.40%); and food supplements with substances which improve vision (4.08%) (Dietary supplements, 2017).

The danger of using DS is related to the registration procedures, which are extremely straightforward. DS are not subjected to detailed quantitative or qualitative tests, confirming their high quality and safety. Unlike in the case of drugs, during the registration process, it is not necessary to prove that a supplement actually contains the ingredients that it claims to contain or

present the results of research on their safety (Regulation of the Minister of Health, 2011; Main Sanitary Inspectorate, 2021).

Mercury (Hg) is a major food contaminant. This toxic element can be released from primary natural sources (e.g., volcanic activity), primary anthropogenic sources (e.g., mining or natural gas extraction), or secondary anthropogenic sources (industrial processes). It is widely distributed in the environment; therefore, the general population is unable to avoid exposure to it (Vianna et al., 2019). Hg occurs mainly in three forms: elemental, inorganic (e.g., as mercury (I) chloride, mercury (II) chloride, or mercury (II) sulfide), and organic (methylmercury, dimethylmercury, ethylmercury, or phenylmercury) (EPoCitF, 2012). The abovementioned compounds are characterized by different bioavailability and toxic effects. The latter group includes methylmercury, which is the most common form of Hg in the food chain. For most people, the main source of exposure is diet, particularly one rich in fish and seafood (Communication from the Co, 2005; Richardson et al., 2011).

Chronic exposure to Hg can result in a number of health consequences, including disorders of the nervous system and kidneys (Johnson-Arbor et al., 2021; Novo et al., 2021). Metallic Hg enters the body *via* inhalation. Inorganic Hg compounds can mainly result in gastrointestinal disturbances and damage to the renal tubules, as well as the formation of free radicals that destroy DNA. Among the organic forms, methylmercury is most dangerous because of its high toxicity to humans. Approximately 95% of it is absorbed from food. Erythrocytes are the main accumulation site of methylmercury. Unfortunately, Hg can also penetrate the placenta and fetus and even cross the blood–brain barrier and the blood–cerebrospinal fluid barrier. Other health consequences include muscle weakness, peripheral vision disorders, problems with coordination of movements, and speech, hearing, and walking impairment (Bernhoft, 2012).

Contamination with this element has been highlighted in many reports (Kabata-Pendias, 2007; CD, 2008; Rice et al., 2014). However, it should be emphasized that there is low public awareness of the health consequences of excessive consumption of DS. Traditional herbal remedies from Asia (EPoCitF, 2012) may pose the greatest threat. It was shown that 17% of traditional herbal preparations exceeded the safety limits for Hg (Martena et al., 2010).

In 2008, the European Union set (Commission Regulation, 2008) the maximum permissible level of Hg in DS at 0.1 mg/kg. However, the European Commission Regulation (Commission Regulation, 2018) does not include data on Hg content in DS, which may indicate that this source presents a lower health risk than other food products listed in the regulation, for example, tree nuts (standard: 0.02 mg/kg), edible herbs and flowers (0.03 mg/kg), wild mushrooms (0.5 mg/kg), oilseeds (0.02 mg/kg), teas, coffee beans, or herbal infusions (0.02 mg/kg).

Our previous studies of 30 plant-based DS revealed contamination with Hg. The highest average content was found in DS supporting immunity (9.62–17.1 μg/kg) and those for the urinary system (9.98–21.2 μg/kg) (Socha et al., 2013). Alarming data were published in 2018. Hg content of 4212.04 μg/kg and 1806.12 μg/kg was detected in DS containing the following ingredients of plant origin: bamboo shoots (85.72 mg/portion), horsetail (52.63 mg/portion), and algae *Chlorella pyrenoidosa* Chick (100% of algae, no specific data on the content) (Brodziak-Dopierała et al., 2018). The abovementioned data indicate that supplements containing herbs may still be a cause for concern and require strict control.

Therefore, the aim of our research was to evaluate the content of Hg in DS containing ingredients of plant origin. In addition, exposure indicators related to the regular use of Hg-contaminated DS were assessed, which made it possible to perform such a comprehensive assessment of exposure to the abovementioned element.

MATERIALS AND METHODS

Materials

In this research, 200 DS were included. All the analyzed products were available for sale in Poland. The study samples consisted of DS for the following: acne therapy support (n = 6), cholesterol control (n = 7), detoxification (n = 5), digestive tract support (n = 21), glucose level control (n = 5), for hair, skin and nails, called nutricosmetics (n = 17), immunity (n = 18), memory (n = 9), the nervous system (n = 4), sore throat (n = 11), the urinary tract (n = 10), for veins (n = 6), for vision and eye health (n = 5), vitality (n = 17), and supplements containing vitamins and minerals (n = 23) for weight loss (n = 25) and others (n = 11). All the studied DS contained ingredients of plant origin.

The DS were purchased in stationery and online drugstores belonging to nationwide pharmacy chains.

Methods

Preparation of DS for Analysis

Solid DS were homogenized in a vibrating mill (Testchem, Radlin, Poland), while liquid ones were mixed using a Vortex Mixer Benchmixer (Benchmark, Sayreville, NY, United States of America). The weighed samples (0.02 g or 50 μL, with an accuracy of 1 mg) were placed in a cuvette, and Hg content was determined.

Determination of Hg Content

The content of Hg was measured using atomic absorption spectrometry (AAS), using an Advanced Mercury Analizer (AMA)-254 (Leco Corp. Altec Ltd. Prague, Czech Republic), according to the methodology described previously (Bielecka et al., 2020). This method facilitates the separation of Hg from its compounds, both inorganic and organic, and transforming it into an atomic form.

The process of determining the content of Hg consisted of 3 steps. The first step was to dry the sample and then burn it in an oxygen stream; medicinal oxygen was used as the carrier gas. The second step was to pass the released Hg vapor through the catalytic column; the vapors were captured by the amalgamator. The third step was to release Hg from the amalgamator and measure its content using the AAS method at a wavelength of 254 nm. The method's limit of quantification was 0.003 ng Hg/g sample.

Quality Control of the Method

Quality control of the method was performed using certified reference material – *Mixed Polish Herbs* (INCT-MPH-2), obtained from the Institute of Nuclear Chemistry and Technology (Warsaw, Poland). The particular analytical steps were analogous to the procedure for determining Hg content in the samples. The recovery rate was 102%, and the precision rate was 2.1%.

Comparison to the Norm

The obtained results were compared to the applicable Commission Regulation (Commission Regulation, 2008), establishing the maximum levels of certain contaminants in foodstuffs, according to which the maximum level of Hg in DS is 0.1 mg/kg.

Assessment of Consumption Safety

The risk of the health consequences related to the consumption of Hg in DS was estimated by calculating selected exposure indicators, such as the estimated daily intake (EDI), the estimated weekly intake (EWI), the percentage of provisional tolerable weekly intake (% PTWI), and Hg consumption during 1 month and 1 year. The EDI [μg/day] was calculated using the following formula:

$$EDI = C \times Cons,$$

where C [μg/kg] is the concentration of Hg in the sample and Cons [kg] is the daily portion of the studied supplement, considering the weight of the portion and maximum daily dosage. The EWI [μg/week] was estimated by multiplying the EDI value by seven (which corresponds to 1 week). To determine the % PTWI [μg/kg/week], the following equation was used:

$$\%PTWI = \left[\left(\frac{EWI}{BW}\right)/4\right] * 100,$$

where BW is the average body weight of an adult in Poland (the weight of 70 kg was assumed). The obtained results were compared to the norm established by the European Food Safety Authority at 4 μg/BW/week (EPoCitF, 2012).

Moreover, the THQ index (target hazard quotient) was calculated for selected DS using the following formula:

$$THQ = \frac{Fr \; x \; D \; x \; Cons \; x \; C}{RfD \; x \; BW \; x \; T} \; x \; 10^{-3},$$

where Fr is the frequency of exposure [365 days/year], D is the time of exposure [70 years], Cons is the average DS consumption per day [g], C is the concentration of Hg in the DS [mg/kg], RfD is the oral reference dose [0.3 μg/kg body weight/day], BW is the body weight [kg], and T is the time of exposure [365 days/year x 70 years].

The interpretation of the THQ is as follows: if the THQ value is above 1, it may indicate a potential risk associated with the consumption of the heavy metal in question with the DS. On the other hand, when the value is below 1, it indicates a low non-carcinogenic risk.

The supplementation materials provide Hg content per portion (i.e., one capsule, one tablet, *etc.*), daily consumption (content in one portion multiplied by the number of portions recommended for consumption by the manufacturer), and weekly consumption – calculated analogously by multiplying the daily consumption for 7, monthly – for 30, and yearly – for 365.

Statistical Analysis of the Results

Data were analyzed using Statistica 13.3 (TIBCO Software Inc. Palo Alto, CA, United States). In order to assess the consistency of the data distribution with normal distribution, the Shapiro–Wilk, Kolmogorov–Smirnov, and Lilliefors tests were used. Due to the lack of normality in the data distribution, the Kruskal–Wallis analysis of variance (ANOVA) was performed to compare the content of Hg in individual categories and between pharmaceutical forms. The table lists the mean (X), standard deviation (SD), minimum (Min), and maximum (Max) levels to compare the results with the literature data, the median (Me), lower (Q1), and upper (Q3) quartile levels due to lack of normality of the data distribution. The level of statistical significance was set at $p < 0.05$.

RESULTS

Taking into account all studied DS (n = 200), the mean Hg content was 3.37 ± 7.65 μg/kg and the median content was 1.69 μg/kg, while the range of quartiles ranged from 1.10 to 2.86 μg/kg.

Table 1 shows the content of Hg in the tested DS, with division into categories. Detailed data on Hg content in individual DS are included in the Supplementary Materials section: **Supplementary Tables S1–S17**.

In this study, the highest median Hg content (5.94 μg/kg) was detected in the group of supplements responsible for controlling glucose levels, while the lowest was in the detoxifying supplements (0.64 μg/kg). The average concentrations of Hg ranged from 0.23 μg/kg in a supplement for the digestive tract to 91.40 μg/kg in a product designed to control blood glucose levels.

Among the supplements supporting acne treatment, the highest content of Hg (6.01 μg/kg) was found in a supplement containing the extract of *Viola tricolor* L. (wild pansy) (100 mg).

In the case of DS used to help in reducing cholesterol levels, the highest content of Hg (2.16 μg/kg) was found in a supplement containing red yeast rice extract with monacolin K (250 mg) and phytosterols (47.5 mg).

Considering the subgroup comprising detoxifying supplements (n = 5), the maximum Hg concentration (3.52 μg/kg) was detected in products containing silver birch extract (600 mg), common dandelion extract (350 mg), *Orthosiphon spicatus (*Thunb.*)* Backer, Bakh. f. Steenis not Benth. extract (350 mg), ginseng root extract (300 mg), white nettle extract (300 mg), broadleaf plantain extract (250 mg), *Pilosella officinarum* Vaill. extract (250 mg), maypop extract (200 mg), fennel extract (175 mg), olive extract (175 mg), green tea extract (100 mg), and lemon extract (100 mg).

TABLE 1 | Hg content in DS and health risks of their use.

Category of the supplements	n	Content of Hg [μg/kg]		Indicators		Intake of Hg [μg] min–max		PTWI min–max [%]
		X ± SD min-max	Me Q_1-Q_3	EDI [μg]	EWI [μg]	Monthly	Annual	
Acne	6	2.24 ± 1.95 0.71–6.01	1.83 1.06–2.17	0.001–0.003	0.004–0.021	0.015–0.088	0.186–1.069	0.001–0.007
Cholesterol control	7	1.29 ± 0.56 0.42–2.16	1.32 1.00–1.55	0.001–0.002	0.004–0.012	0.016–0.051	0.192–0.616	0.001–0.004
Detox	5	1.20 ± 1.30 0.49–3.52	0.64 0.65–0.68	0.001–0.017	0.004–0.117	0.017–0.502	0.206–6.109	0.001–0.042
Digestive tract	21	1.91 ± 1.66 0.23–7.10	1.42 0.91–2.14	0.000–0.014	0.001–0.097	0.002–0.418	0.029–5.082	<0.001–0.035
Glucose level	5	23.97 ± 38.56 0.80–91.40	5.94 0.99–20.72	0.001–0.119	0.004–0.830	0.018–3.558	0.223–43.283	0.002–0.296
Nutricosmetics	17	4.13 ± 5.32 0.56–22.00	2.48 1.20–3.72	0.000–0.068	0.003–0.474	0.013–2.030	0.162–24.693	0.001–0.169
Immunity	18	2.62 ± 2.73 0.34–11.88	1.90 0.90–2.71	0.001–0.047	0.005–0.327	0.022–1.399	0.263–17.025	0.002–0.117
Memory	9	1.45 ± 0.98 0.66–3.79	1.46 0.92–2.69	0.000–0.006	0.003–0.041	0.012–0.177	0.147–2.159	0.001–0.015
Nervous system	4	2.41 ± 1.64 0.80–4.64	2.09 1.49–3.01	0.002–0.009	0.011–0.065	0.046–0.277	0.564–3.371	0.004–0.023
Throat	11	1.54 ± 0.87 0.27–3.20	1.50 1.12–1.99	0.003–0.050	0.018–0.352	0.078–1.510	0.948–18.371	0.006–0.126
Urinary tract	10	4.49 ± 4.90 0.67–13.84	1.99 1.37–7.84	0.001–0.012	0.005–0.082	0.019–0.350	0.236–4.264	0.002–0.029
Veins	6	4.50 ± 2.49 1.27–8.57	3.76 3.66–5.44	0.001–0.011	0.005–0.076	0.021–0.324	0.252–3.947	0.002–0.027
Vision	5	6.06 ± 8.91 1.27–21.81	1.39 1.38–4.46	0.001–0.018	0.004–0.126	0.016–0.538	0.196–6.544	0.001–0.045
Vitality	17	2.06 ± 1.38 0.46–5.32	1.81 1.11–2.65	0.000–0.457	0.003–3.201	0.014–13.720	0.172–166.932	0.001–1.143
Vitamins and minerals	23	4.21 ± 8.61 0.57–42.96	1.86 1.18–3.35	0.001–0.036	0.006–0.252	0.026–1.079	0.312–13.124	0.002–0.090
Weight loss	25	3.08 ± 3.62 0.52–17.26	1.78 1.21–2.88	0.001–0.026	0.004–0.184	0.018–0.790	0.215–9.617	0.001–0.066
Other	11	1.56 ± 0.69 0.54–2.47	1.36 1.09–2.29	0.000–0.023	0.001–0.163	0.006–0.699	0.070–8.509	<0.001–0.058

X–average, SD–standard deviation, Me–median, Q1–quartile 1, Q3–quartile 3, EDI–estimated daily intake, EWI–estimated weekly intake, PTWI–provisional tolerable weekly intake.

Among the DS recommended to improve digestive tract function (n = 21), the greatest amount of Hg (7.10 μg/kg) was detected in a sample containing mint leaves.

The next studied subgroup included supplements for glucose level control (n = 5). In this subgroup, one sample with the highest Hg concentration, among whose ingredients were extracts of *Gymnema sylvestre* R. Br. and *Trigonella foenum graceum* L. (fenugreek), contained the highest level of detected Hg, which was nearly the maximum permissible amount of Hg (91.40 μg/kg).

In the subgroup of supplements with bioactive substances, which could play a role in improving the condition of hair, skin, and nails (n = 17), the highest level of Hg (22.00 μg/kg) was observed in a sample containing *Chlorella pyrenoidosa* Chick (200 mg).

Analyses of Hg content in the subgroup of supplements recommended to strengthen the immune system (n = 18) revealed the highest amount of the element (11.88 μg/kg) in a sample containing the extract of acerola.

Among the DS recommended for memory support (n = 9), the highest level of Hg (3.79 μg/kg) was recorded in supplements with extracts of *Panax ginseng* C.A. Meyer (71.43 mg), *Ilex paraguariensis* A. St.-Hill (150 mg), and *Bacopa monnieri* (L.) Wettst (36 mg).

In the case of supplements supporting the functioning of the nervous system (n = 4), the greatest Hg level (4.64 μg/kg) was found in a sample containing lemon balm leaf.

In our study, the highest Hg (3.20 μg/kg) concentration in the analyzed DS for patients with throat symptoms (n = 11) was observed in products containing extracts of *Salvia officinalis* L. (11.25 mg), *Althaea officinalis* L. (11.25 mg), *Tilia cordata* Mill (10 mg), *Matricaria recutita* L. (8 mg), propolis (5.25 mg), *Sambucus nigra* L. (3.75 mg), and *Thymus vulgaris* L. (6.25 mg).

Ten of the tested products were dedicated to supporting the urinary tract. In this subgroup, the highest content of Hg (13.84 μg/kg) was detected in DS based on cranberry fruit extract (360 mg).

The maximum Hg content in the next studied group, that is, supplements to improve the condition of veins (n = 6) was 8.57 μg/kg. These DS were based on *Vitis vinifera* L. leaf extract (84 mg), and grape seed extract (79 mg).

In the subgroup of supplements claiming to support vision (n = 5), the highest level of Hg (21.81 μg/kg) was found in supplements containing bilberry fruit extract (290 mg) and Aztec marigold flower extract (15 mg).

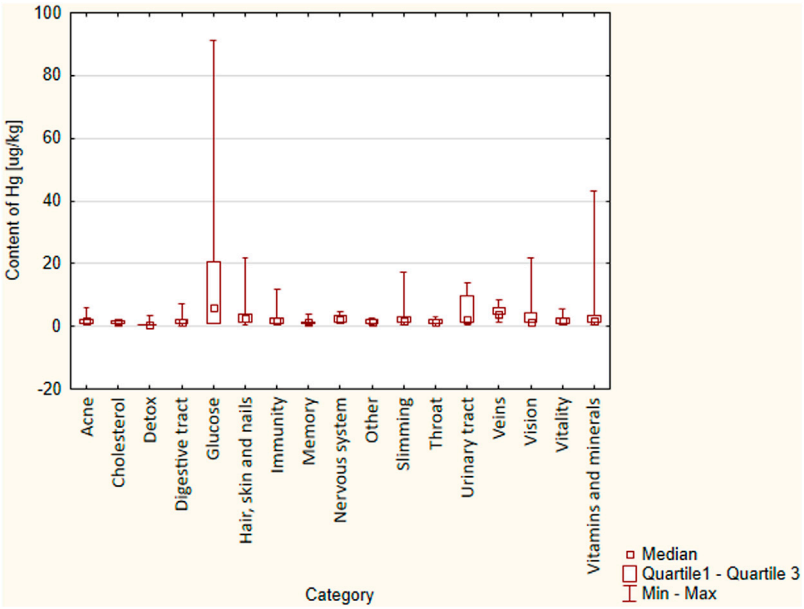

FIGURE 1 | Differences in Hg content between the category of DS (p = 0.068, results were not statistically significant).

Among the DS recommended to improve vitality (n = 17), the highest content of the tested toxic element (5.32 μg/kg) was found in a sample based on *Ginseng* extract C.A. Meyer (50 mg) and ginkgo extract (40 mg).

In the next studied subgroup—DS containing vitamins and minerals—the highest concentration of Hg (42.96 μg/kg) was detected in a supplement containing extract of *Withania somnifera* (L.) Dunal (80 mg).

The largest surveyed group (n = 25) included products designed to promote weight loss. The highest concentration of Hg (17.26 μg/kg) was observed in one sample containing the following substances: extract of *Camellia sinensis* (L.) Kuntze (105 mg), extract of *Zingiber officinale* Rosc (100 mg), extract of cayenne peper (70 mg), extract of green coffee (5 mg), extract of *Cinnamomum* Scheffer (5 mg), extract of *Paullinia cupana* Kunth (6.6 mg), extract of *Citrus sinensis* (L.) Osbeck, extract of *Citrus grandis* Osbeck, and extract of *Citrus aurantium vel. dulcis* L. (10 mg).

In our research, 11 products were not classified into any of the studied subgroups. The highest Hg content (2.47 μg/kg) was found in a product containing extracts of *Melissa officinalis* L. leaves (300 mg), *Humulus lupulus* L. (100 mg), and *Rhodiola rosea* L. (100 mg).

The study showed that the examined categories of DS did not differ significantly in terms of Hg content (p = 0.068) (**Figure 1**).

Moreover, it was assessed that the pharmaceutical form of the DS affected the content of the tested element–p = 0.045 (**Figure 2**). The preparations available in the form of sachets for infusion were characterized by the highest median: 2.66 μg/kg (Q1-Q3: 1.36–5.49 μg/kg).

Our analyses showed that the content of Hg in all tested DS was below 0.1 mg/kg. In the case of one DS, the content of this element was above 0.09 mg/kg, which is a value close to the maximum allowable concentration.

The calculated percentage of the PTWI for Hg due to intake of studied DS is included in **Table 1**. The lowest percentage of the PTWI was lower than 0.001%, while the highest percentage of the PTWI was calculated for one DS designed to boost vitality (1.143%) (Table S14). Generally, the values of this indicator in the vast majority of samples were lower than 1%, ranging from 0.001 to 0.030%.

In the case of the DS with the highest content per sample (category: glucose, 91.40 μg/kg) and the one with which the most Hg would be consumed during a single day (category: vitality, 22.00 μg/kg, it is recommended to consume 15 servings per day), the THQ index was calculated as follows: 4.10E-03 and 3.22E-03, respectively.

DISCUSSION

DS are used by consumers from different age groups. Due to their similarity to medicinal products, they are applied in the treatment of various diseases. For the abovementioned reason, they should be of high quality. Registration procedures and national regulations do not require qualitative research; therefore, this kind of research is of interest to various authors.

Our research has shown that the highest average Hg content was found in DS used for lowering glucose levels (23.97 ± 38.56 μg/kg). The supplement with the highest concentration of the element (91.40 μg/kg) contained two ingredients of plant origin, namely, extract of *Gymnema sylvestre* R. Br (185 mg) and extract of *Trigonella foenum graceum* L./fenugreek (90 mg). Moreover, they contain chromium and lipoic acid as well.

It can be assumed that high concentration of Hg in *Gymnea sylvestrae* R. Br. may stem from the fact that the plant is mainly grown in Asia. China is a country where Hg is the most prevalent toxic element (Huang et al., 2022); hence, plants are likely to absorb it.

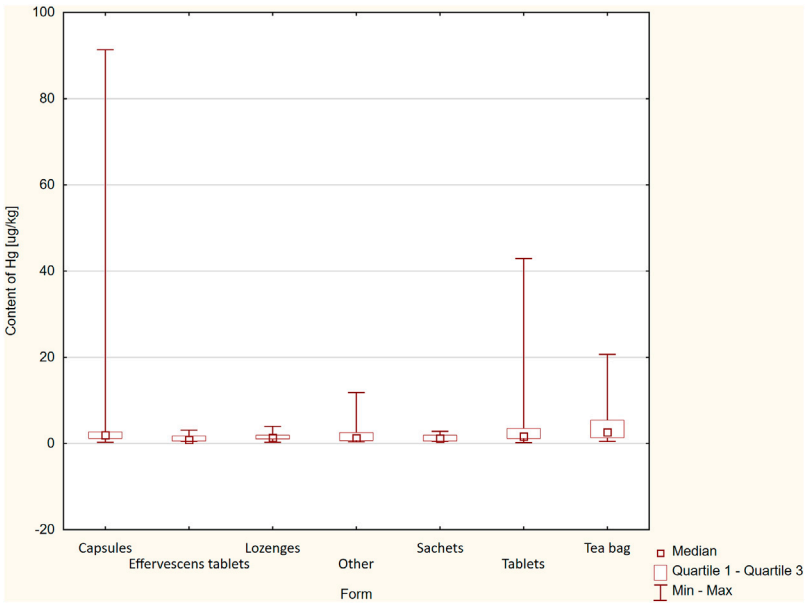

FIGURE 2 | Differences in Hg content between forms of DS (p = 0.045).

To our knowledge, the research conducted in this project involved more DS than in most previous publications to better assess consumer exposure. Research on Hg content in 24 DS containing ingredients of plant origin was carried out by Brodziak–Dopierała et al. (Brodziak-Dopierała et al., 2018). The authors measured the content of Hg using the same method that we did and found that the studied supplements contained 0.02 to 4,293.07 μg/kg of Hg. The average concentration was almost 58 times higher than that shown in our study (193.77 vs 3.37 μg/kg). The second highest result (1806.12 μg/kg) involved a DS containing *Chlorella pyrenoidosa* Chick algae. In our study, a *Chlorella*-based product with the recommended dose of as many as 15 tablets/day also proved to have one of the highest amounts of Hg (22.001 μg/kg). The result obtained by us, however, was as much as 82 times lower than the highest one in the abovementioned studies. Moreover, the authors showed that the preparations in tablets were characterized by a significantly higher mean Hg content compared to capsules (274.80 ± 917.64 μg/kg vs 5.95 ± 7.30 μg/kg). Our research looked at more pharmaceutical forms and revealed that the infusion bags had the highest median Hg content.

Chlorella has good Hg absorption properties. It prevents the reabsorption of Hg from the gastrointestinal tract; therefore, it can be used as an effective absorbent to remove Hg from the body (Yadav et al., 2020). In a study conducted by Caldas et al., Hg was detected in all samples, while none of the samples exceeded the acceptable limit (from <0.01 to 0.09 μg/g) (Caldas and Machado, 2004). On the other hand, in a study analyzing the Polish supplement market, a preparation based on *Chlorella* had one of the highest concentrations of Hg: 1810 μg/kg, which exceeded the acceptable standard (100 μg/kg) (Brodziak-Dopierała et al., 2018).

Other data, including Hg content in 24 DS, come from Mexico. The content of the discussed element ranged below 240–850 μg/kg. According to the authors, Hg at the detection limit level was present in only 5 DS (about 21% of samples). The highest content (850 μg/kg) was found in a DS containing Guaco stem (*Mikania guaco* Bonpl), red vine leaves (*Vitis viniferol* L.), horse plant (*Equisetum arvense* L.), and gorongoro bark (García-Rico et al., 2007). Hg content in DS can be explained by the presence of this ingredient in the form of cinnabar (HgS), especially in Chinese preparations, including those used for the treatment of throat diseases (Bin et al., 2001; Wu et al., 2002).

Studies assessing the quality of 49 pharmaceutical products from Korea containing raw materials of plant origin showed that the content of total Hg in these preparations was high. For example, the highest amount was found in a preparation containing royal jelly—as much as 159.89 μg/kg—which is about 1.76 times higher than the highest result obtained by us. The mean methylmercury content of the herbal preparations in this study was 31.18 μg/kg, while the preparations containing Spirulina had 0.62 μg/kg of Hg (Lee and Lee, 2013).

Another study of DS from Poland assessed the quality of 33 products containing macro and microelements (n = 7), vitamins (n = 5), and nutricosmetics (n = 6) and classified as "other" (n = 15). The average content of Hg was 5.5 μg/kg, with the highest in a preparation containing vitamin C and rutin (16.7 μg/kg) (Kowalski and Frankowski, 2015).

Another study of DS from Poland involved a quality assessment of 41 DS containing terrestrial plants and microalgae. The authors showed that 29.3% of the investigated DS were contaminated with Hg. The average content of Hg in the products containing ingredients of plant origin was 5 ± 8 μg/kg, while in those based on microalgae — 3 ± 6 μg/kg. The highest concentration (28 μg/kg) was found in tablets which contained *Rehnabbua glutinosa* (Gaertn.) Steud. radix and Wolfiporia (Ćwieląg-Drabek et al., 2020).

The prevalence of Hg contamination in Ayurvedic herbal DS was demonstrated by Mikulski et al. (Mikulski et al., 2017). The

presence of Hg was detected in as many as 38% of the tested preparations. It is very disturbing that the content of the toxic element ranged from 800 to 279, 000, 000 μg/kg. Brihat Vatchintamani Ras (139,500 μg/0.5 g pill) was characterized by the highest content of Hg per one pill.

In contrast, studies of 10 DS from Turkey, carried out using the ICP-OES method, showed no Hg in the tested preparations (Canbay and Doğantűrk, 2017).

The presence of Hg in DS containing ingredients of plant origin can be explained by the fact that plants are one of the best agents for removing Hg2 + impurities from soil. Bioabsorption is based on mechanisms such as chelation, ion exchange, and species of the structural polysaccharide cell wall network absorption by physical forces and ion entrapment in inter- and intra-fibrillar capillaries. For example, Hg is selectively accumulated by *Carica papaya* L. wood or *Ricinus communis* L. (Basha et al., 2009; Kumar et al., 2017). It should be emphasized that the absorption of Hg may be toxic not only to humans but also to plants themselves.

DS can be one of the sources of exposure to Hg. Other sources of exposure to organic forms of Hg include fossil fuel emissions, medical waste incineration, dental amalgams, and various other products, including skin creams, bactericidal soaps, teething powders, painkillers, thermometers, blood pressure gauges, barometers, bulbs, and batteries. Other sources of organic Hg include phenylmercury and ethylmercury compounds, which used to be components of latex paints before the 1990s, and thimerosal, which was used as a preservative in vaccines (Rice et al., 2014).

Chronic exposure to Hg may result in, inter alia, disruption of the endocrine system. Hg is mainly stored in the thyroid and pituitary gland. Previous research has shown that the concentration of the element in these organs ranged from 6.3 to 77 ng/g, while in another study, it amounted to 28 ng/g. These levels exert neurotoxic and cytotoxic effects (Rice et al., 2014). Exposure to Hg can cause changes in the nervous system, which is associated with a toxic increase in reactive oxygen species (Fernandes Azevedo et al., 2017). However, the THQ index calculated by us does not indicate an increased non-carcinogenic risk resulting from consuming the DS under investigation.

In another study, the DS which had the highest % PTWI (3.91%) contained two ingredients of plant origin: extract of millet (50 mg) and extract of wheat germ (50 mg) (Brodziak-Dopierała et al., 2018). The highest % PTWI calculated in our study amounted to 1.143% and was detected in a preparation containing *Guarana* Kunth seed extract.

Hg, along with several other elements (Cd and Pb), has been recognized as an impurity arising from the food chain (Tchounwou et al., 2012), which is also confirmed by our study. Among the many factors affecting the concentration of Hg in food are natural factors, including not only growing conditions (type of water and soil) and cultivation practices but also meteorological conditions (i.e. geological areas for Hg-rich formations and atmospheric deposition rate). The fact that supplement ingredients are often plant-based may account for Hg contamination. Plants quite easily absorb heavy metals from soil and water, and these can remain in the final product, even after processing. It should also be emphasized that the location of the source of raw materials for the production of supplements is of considerable importance and may directly affect the content of Hg in the final product (Bandara et al., 2020).

Summing up, although the content of Hg in the studied DS was lower than that reported in most of the literature and did not exceed the permissible maximum content prescribed by law, it should be emphasized that since Hg is a toxic element, any amount of it may be harmful to health. During the production of DS, strict procedures for obtaining raw materials from crops controlled in terms of environmental pollution, including soil, as well as procedures for cleaning plant materials and eliminating the risk of contamination of the final product at all stages should be implemented.

CONCLUSION

DS containing ingredients of plant origin are mostly safe in terms of Hg content, but it should be stressed that Hg is a highly toxic element, and its long-term use may pose a health hazard. This is especially dangerous in the case of chronically ill people who use several DS at the same time. Consumers and pharmacists should pay attention to the origin of DS and the recommended number of tablets taken during the day, as in some cases, higher doses can lead to increased exposure to Hg. In addition, there is a recognized need for DS to be tested for quality and safety before being placed on the market.

DATA AVAILABILITY STATEMENT

The results of the research carried out may be available from the authors. Requests to access the datasets should be directed to anna.puscion-jakubik@umb.edu.pl.

AUTHOR CONTRIBUTIONS

Conceptualization, KS, RM-Ż, and AP-J; methodology, AP-J and KS; software, AP-J, AM, DA, MI, MG, and JB; formal analysis, AP-J, KS, and RM-Ż; data curation, DA, MI, AM, MG, JB, and AP-J; writing—original draft preparation, AP-J, MG, and JB; writing—review and editing, KS and RM-Ż; visualization, AP-J; and supervision, KS and RM-Ż.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.738549/full#supplementary-material

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