HERBAL MEDICINES IN WOMEN'S LIFE

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HERBAL MEDICINES IN WOMEN'S LIFE

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Editorial: Herbal medicines in women's lives

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KEYWORDS

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Editorial on the Research Topic
Herbal medicines in women's lives

In many areas, natural products are traditionally used to improve body conditions in several periods of a women's life, including adolescent, reproductive, and menopausal periods. In the adolescent period, medical herbs are used to enhance growth. At childbearing age, medical herbs are used to treat menstruation problems, decrease vaginal infection, and increase fertility. During pregnancy, medical herbs are used to reduce discomfort and prevent miscarriage. After menopause, medical herbs are used to alleviate symptoms and delay degeneration. All the above-mentioned usages of natural products are considered to improve life quality for women. Herbs used in women' lives deserve careful study. The articles in this Research Topic involve the use of herbal medicines during childbearing and menopausal periods. The conditions involved dysmenorrhea, endometriosis, infertility, lactation, osteoporosis, and menopausal symptoms.

At child-bearing age, dysmenorrhea affects 45% of women (Iacovides et al., 2015). In several countries and areas with traditional medicines, herbs are commonly used to relieve cramps in primary and secondary dysmenorrhea. In this Research topic, the research of Su et al. discloses the herbs used for dysmenorrhea in Taiwan *via* a field investigation among traditional Chinese medicine pharmacies. Those pharmacies are popular among women in Taiwan. This research team found that Angelica sinensis (Oliv.) Diels and Ligusticum chuanxiong Hort are two important herbs in the prescriptions of Taiwanese pharmacies. Among the etiologies of dysmenorrhea, endometriosis is a common and intractable disease that causes secondary dysmenorrhea (Mehedintu et al., 2014). Several herbs are shown to inhibit the pathogenesis of endometriosis, such as inflammation, proliferation, and angiogenesis (Meresman et al., 2021). Endometriosis patients who take the herbs are also shown to have a lower surgery rate than those who do not take herbs (Su et al., 2014). Zheng et al. provide possible mechanisms of a traditional Chinese medicine formula, the ELeng Capsule. By transcriptomics combined with network analysis, Zheng et al. conclude that the potential mechanisms to treat endometriosis of the Eleng Capsule might include apoptosis and regulating angiogenesis, cytoskeleton, and epithelial-mesenchymal transition.

Fertility is also an important issue during the childbearing age. Zishen Yutai Pills is a famous formula taken by the female population who want to get pregnant in China. Li et al. conducted a metabonomics study to identify enriched pathways for Zishen Yutai Pills in women who were undergoing *in vitro* fertilization. Their results show that Zishen Yutai Pills down-regulated the chemicals of trihexosylceramide, glucosylceramide, and TG, and up-regulated chemicals of PIP3, PIP2, tauroursodeoxycholic acid, L-asparagine, L-glutamic acid, kynurenic acid, 11-deoxycorticosterone, melatonin glucuronide, and hydroxytyrosol. After pregnancy, women face miscellaneous pregnancy-related symptoms. Even during a normal pregnancy, due to the changes in hormones and the growth of the fetus, there are still common symptoms including morning sickness, abdominal bloating, waist pain, edema, varicosity, genitourinary infection, and mood swings (Nazik and Eryilmaz, 2014). However, women are usually careful in taking medicines to relieve the above symptoms because of some famous harmful effects on the fetus caused by medications. Instead, herbal products are popular among pregnant women for treating symptoms. This Research Topic includes a report by Gantner et al., who find that 52.0% of all pregnant women in Zurich suffer from mild mental disorders, but only 1.3% of them took psychoactive medications. They also find that Kalanchoe pinnata (Lam.) Pers, Lavandula angustifolia Mill, and Valeriana officinalis L. were the three herbs that pregnant women used the most. Those herbs were used to reduce stress, restlessness, and sleep disorders. After childbirth, breastfeeding provides health for both mothers and babies. In Tanzania, Millinga et al. conducted a cross-sectional survey using a structured questionnaire to investigate the use of herbal medicines during breastfeeding. They find that 53.8% of breastfeeding mothers used herbs. The most commonly used herbs were Piper nigrum L., and Cucurbita pepo L. We could compare this study with a previous one which was conducted in Taiwan that reported Angelica sinensis (Oliv.) Diels, Tetrapanax papyrifer (Hook.) K. Koch, and Hedysarum polybotrys Hand.-Mazz. as the three most commonly used herbs to promote lactation (Chao et al., 2020). At the same time, Millinga et al. reveal that higher education levels and a low breast milk supply were identified as predictors of the use of herbs among breastfeeding mothers.

There is still an important issue in a women's life, menopause. Before and after menopause, women face a menopause transition, which is a period that starts from the onset of irregular menstruation or vasomotor symptoms (Gracia and Freeman, 2018). During this time, the majority of women experience uncomfortable symptoms that are collectively called menopausal syndrome, such as vasomotor symptoms, mood changes, and sleep problems (Takahashi and Johnson, 2015). In this Research Topic, there are five articles related to female menopause published. The first one is by Lan et al., which used animal models of rats to generate the conditions of Kidney deficiency and Liver qi stagnation, both of which are pattern conditions happening to menopausal women. The authors find that the combination of Chinese herbal formulae corresponded to the two pattern conditions that corrected menopausal indices the best. The second article by Jalalvand et al. reveals that, in menopausal women, after 10 weeks' consumption of Elaeagnus angustifolia L., there were increases in thyroid-stimulating hormone, cortisol level, and the ratio of cortisol/dehydroepiandrosterone-Sulfate. However, the level of prolactin decreased. Uncomfortable joints are another issue during or after menopause, especially in Asian women (Haines et al., 2005). The third article is a double-blind, randomized controlled trial conducted in Korea by Kim et al. They report that red ginseng is an effective supplement that reduced the pain score in menopausal women with degenerative osteoarthritis in the hand. Osteoporosis is a severe condition that usually happens in menopausal women and puts them at risk of bone fractures (Li and Wang, 2018). The fourth article is about an herbal formula Shen-sui-tong-zhi formula, which has been used in China for treating musculoskeletal disorders for years. Xu et al. use a mouse model to dig out mechanisms for the bone density-protective effect provided by the Shen-sui-tong-zhi formula. The authors find that the main pathway Shen-sui-tong-zhi formula provides for its osteogenesis was the activation of β–catenin signaling in growth plate chondrocytes. Lastly, the fifth article related to female menopause is a study on Salvia miltiorrhiza, which is an herb having both "Blooding activating" and "Blooding tonifying" effects according to the ethnopharmacological usage in Chinese medicine; moreover, Salvia miltiorrhiza has various pharmacological effects and are applied in various clinical illnesses (Shi et al., 2019; XD et al., 2019). Tseng et al. reveal that among people who used herbal medicine, women used Salvia miltiorrhiza more than males. More precisely, Salvia miltiorrhiza was used mostly in women aged 35–49 years, those were before and during menopausal transition. Moreover, the most common diseases treated by Salvia miltiorrhiza were menopausal disorders combined with general symptoms. The most frequent formulae jointly used with Salvia miltiorrhiza were herbal formulae of Yan-Hu-Suo and Jia-Wei-Xiao-Yao-San.

During the whole life of a women, blood is an important issue. Women often suffer from anemia in many diseases and non-disease conditions, such as fibroids, adenomyosis, and uterine atony after delivery or during menstruation (Khafaga and Goldstein, 2019). women often suffer from dizziness, palpitation, and weakness even when not having anemia. These symptoms are called blood-deficient syndrome in traditional Chinese medicine. Blood-deficient syndrome lowers the quality of life of women. There is a noble medicinal material in Chinese medicine, donkey-hide gelatin, which is not a plant. Instead, it is an Equus asinus-derived medicinal material that is traditionally used to supply blood to the body. To test its efficacy

in blood deficiency-related symptoms, Zhang et al. perform a randomized, double-blind, and placebo-controlled clinical trial. Their results show that after taking 6 g of donkey-hide gelatin per day for 2 months, the symptom of dizziness was reduced and the quality of life was improved. Moreover, after 2 months, there were significant differences between the treatment and control groups in hematocrit and red blood cell numbers.

Besides treating illnesses, herbs provide cosmetic purposes. Asian women think white skin is beautiful and cosmetic companies always invent skin-whitening products (Spyropoulou et al., 2020). Asian women also take or apply skin-whitening herbal products. Ko et al. perform a field investigation on skin-whitening herbal prescriptions in Taiwan. They find that Wolfiporia extensa (Peck) Ginns, Glycyrrhiza uralensis Fisch., and Paeonia lactiflora Pall. were the three most frequently sold herbs used for skin-whitening by herbal pharmacies. They have the potential to become commercial products after safety and efficacy tests.

The articles included in this Research Topic provide a view of how herbal medicine plays a role and how herbs weave a whole story in a women's life. Some of these articles survey detailed mechanisms of herbal medicine, and some of them provided the first glimpse into the phenomenon of using herbal medicines. The doses used in animal models recruited in this Research Topic cover human equivalent doses, therefore, the results could be used as references for clinical application. All the articles in this Research Topic could be the basis of research tomorrow.

Author contributions

S-YS wrote the manuscript.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Exploration of the Modulatory Property Mechanism of ELeng Capsule in the Treatment of Endometriosis Using Transcriptomics Combined With Systems Network Pharmacology

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Endometriosis is a common gynecological disease and causes severe chronic pelvic pain and infertility. Growing evidence showed that traditional Chinese medicine (TCM) plays an active role in the treatment of endometriosis. ELeng Capsule (ELC) is a Chinese medicine formula used for the treatment of endometriosis for several years. However, the mechanisms of ELC have not been fully characterized. In this study, network pharmacology and mRNA transcriptome analysis were used to study various therapeutic targets in ELC. As a result, 40 compounds are identified, and 75 targets overlapped with endometriosis-related proteins. The mechanism of ELC for the treatment of endometriosis is based on the function modules of inducing apoptosis, inhibiting angiogenesis, and regulating immunity mainly through signaling molecules and interaction (neuroactive ligand–receptor interaction), immune system–associated pathways (toll-like receptor signaling pathway), vascular endothelial growth factor (VEGF) signaling, and MAPK signaling pathway based on network pharmacology. In addition, based on RNA-sequence analysis, we found that the mechanism of ELC was predominantly associated with the regulation of the function modules of actin and cytoskeleton, epithelial–mesenchymal transition (EMT), focal adhesion, and immunity-associated pathways. In conclusion, ELC exerted beneficial effects on endometriosis, and the potential mechanism could be realized through functional modules, such as inducing apoptosis and regulating angiogenesis, cytoskeleton, and EMT. This work not only provides insights into the therapeutic mechanism of TCM for treating

Abbreviations: BP, biological process; CC, cellular component; DEGs, differentially expressed genes; DL, drug-likeness; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCODE, Molecular Complex Detection; MF, molecular function; OB, oral bioavailability; PCA, principal component analysis; PPI, protein–protein interaction; QC, quality control; RT-PCR, real-time polymerase chain reaction; TCMSP, traditional Chinese medicine systems pharmacology; UPLC-Q-TOF/MS, ultraperformance liquid chromatography with quadrupole time-of-flight mass spectrometry.

endometriosis but also offers an efficient way for drug discovery and development from herbal medicine.

Keywords: endometriosis, mRNA transcriptome analysis, network pharmacology, ELeng Capsule, traditional Chinese medicine

INTRODUCTION

Endometriosis is a common gynecological disease and causes severe chronic pelvic pain and infertility, which affect the physical and mental health and quality of life of women. The pathogenesis of endometriosis has not been fully elucidated; an increasing body of research shows that it is associated with inflammation, immunity, angiogenesis, and epithelial–mesenchymal transition (EMT) (Khan et al., 2012; Morotti et al., 2017).

Currently, the treatment of endometriosis is mainly based on surgery and pharmacological treatment (Dunselman et al., 2014). Though beneficial, conventional treatments of endometriosis have significant limitations. In recent years, traditional Chinese medicine (TCM) plays an active role in the treatment of endometriosis such as dysmenorrhea, chronic pelvic pain, abnormal uterine bleeding, and infertility by regulating inflammation, immunity, and angiogenesis (Flower et al., 2012; Dunselman et al., 2014). Blood stasis syndrome in TCM is considered appearing in endometriosis. The Chinese preparation ELeng Capsule (ELC) is one of the famous *Huoxue Huayu* prescriptions and is currently used as an in-hospital preparation in the Guangdong Provincial Hospital of Chinese Medicine to relieve the symptoms of endometriosis-associated pain and dysmenorrhea for nearly 20 years. ELC is an empirical formula of Chinese herbs created by Yi Situ, a famous expert in Chinese medicine in Guangdong. The clinical practice and animal experiments have suggested that ELC could reduce dysmenorrhea and endometriosis-associated pain through inhibiting adhesion and inflammation and regulating immunity (Huang and Jiang, 2008; Xu et al., 2010). However, the mechanisms of action of ELC have not been fully characterized.

Chinese medicine compounds exist in complex mixtures and may contain thousands of compounds. Therefore, it is difficult to explain the principle of compatibility of Chinese medicine ingredients and analyze relevant results. Network pharmacology can predict the profiles of targets and pharmacological actions of herbal compounds to reveal "compounds/drugs-genes/targets-disease," which will improve current drug discovery strategies (Mihalyi et al., 2006; Rogers et al., 2009; Zeng et al., 2017). In addition, the development of multi-omics technology also provides new tools for research on TCM. The high-throughput RNA-sequencing (RNA-seq) has been used to reveal molecular mechanisms and explore biomarkers for diagnosis and treatment (Duan et al., 2018; Zhang et al., 2019). These new methods could provide the basis for clarifying the therapeutic mechanisms of herbal medicine.

In this study, RNA-sequencing combined with network pharmacology was performed to identify targets regulated by ELC treatment. Then, autologous transplantation of the endometriosis rat model was used to evaluate the *in vivo* effect of ELC on endometriosis. We aim to provide a reliable way for subsequent experimental verification and new drug research and development.

MATERIALS AND METHODS

Workflow of Network Pharmacology Combined With RNA-Sequence Approach

The workflow is shown in **Figure 1**: (A) Endometriosis model rats were established and used to verify the core targets. (B) The compounds of ELCs were identified by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS). (C) Network pharmacology was used to analyze the compounds-targets network of ELC. (D) RNA-sequencing was used to identify differentially expressed genes (DEGs). Biological functions and pathways were determined through gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Gene set enrichment analysis (GSEA) and STEM analysis were used to further analyze the genetic network and modular genetics.

The Preparation of ELeng Capsule

ELC was provided by the pharmaceutical department of The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Hospital of Chinese Medicine), Guangzhou, China. The validated information of herb/plant names with taxonomic validation was collected from The Plant List (http://www.theplantlist.org). ELC composed of E'zhu (*Curcuma phaeocaulis* Valeton), Sanleng [*Sparganium stoloniferum* (Buch.-Ham. ex Graebn.) Buch.-Ham. ex Juz. (Typhaceae)], Danshen [*Salvia miltiorrhiza* Bunge (Lamiaceae)], Chishao (*Paeonia lactiflora* Pall.), Shuizhi (*Hirudo nipponia*), Biejia (*Trionyx sinensis*), Zhike (*Citrus aurantium*), and Danggui [*Angelica sinensis* (Oliv.) Diels] (**Table 1**). The weight ratio of *C. phaeocaulis* (E'zhu), *S. stoloniferum* (Sanleng), *S. miltiorrhiza* (Danshen), *Hirudo nipponia* (Shuizhi), *Trionyx sinensis* (Biejia), *P. lactiflora* (Chishao), *C. aurantium* (Zhike), and *A. sinensis* (Danggui) is 2: 2: 5: 1: 5: 5: 4: 3.4. Each capsule weighed 0.45 g, which is equal to 1.61 g of crude drug. According to the extraction method of volatile oil recorded in the Chinese Pharmacopoeia, the volatile oil was extracted by steam distillation. The water extraction and alcohol precipitation solution was filtered and spray-dried to obtain a dry extract powder.

The original medicinal materials were purchased from Guangdong KangMei Pharmaceutical Co., Ltd. The quality of the raw herbs was controlled according to the Pharmacopoeia of the People's Republic of China (2020). The patent certificate number is 432493. ELC was obtained with the water extraction–alcohol precipitation method. The validated information of major herbs, including the location, used part, family, and genus, is shown in **Supplementary Table S1**.

UPLC-Q-TOF/MS Analysis

For the preparation of compounds, 20 ml of 50% ethanol solution was added to 1 g of medicinal powder of ELC. The mixture was

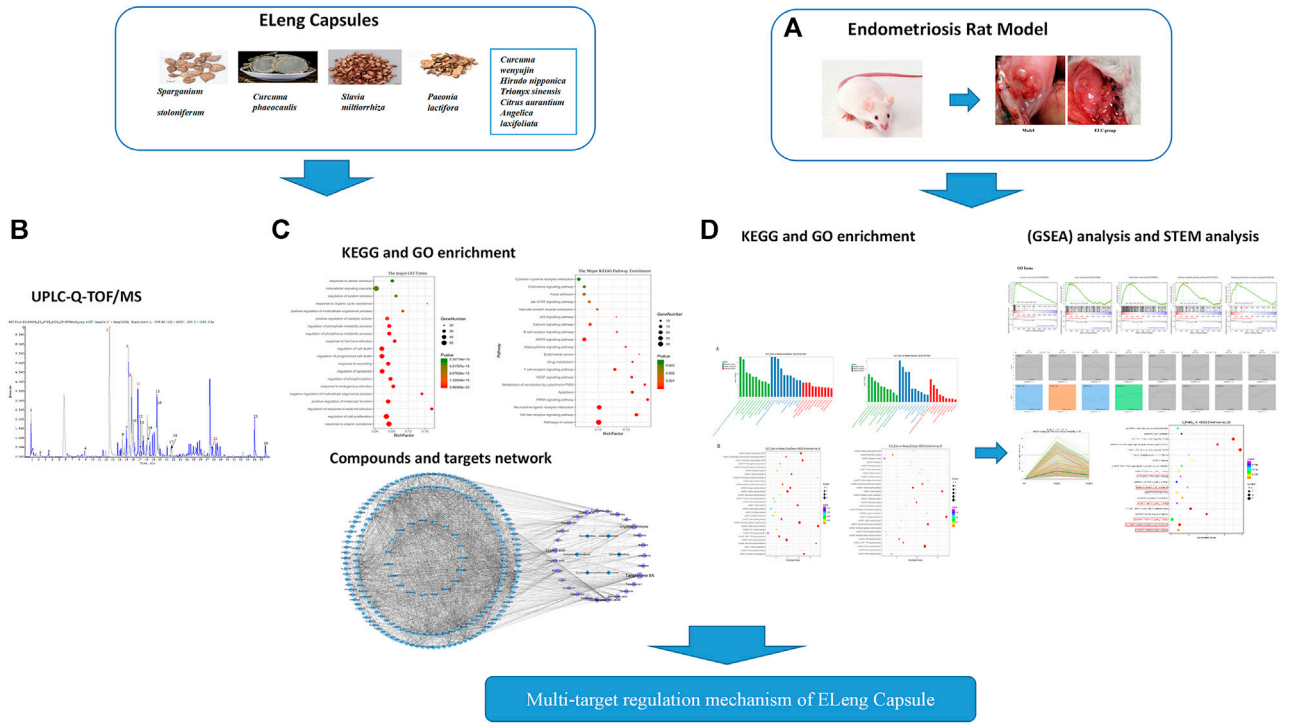

FIGURE 1 | Workflow of network pharmacology combined with the RNA-sequence approach. **(A)** Endometriosis model rats were established and used to verify the core targets. **(B)** The compounds of ELCs were identified by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS). **(C)** Network pharmacology was used to analyze the compounds-targets network of ELC. **(D)** RNA-sequencing was used to identify differentially expressed genes (DEGs). Biological functions and pathways were determined through gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Gene set enrichment analysis (GSEA) and STEM analysis were used to further analyze the genetic network and modular genetics.

TABLE 1 | The major herbs of ELeng Capsule.

Herb	Component	Effect
Curcuma phaeocaulis Valeton (E'zhu)	Ginger plant, Wen Yujin Curcuma Wenyujin Y.H. Chen et C. Ling, rhizome	Treatment of mass in the abdomen, amenorrhea due to blood stasis, distension, and pain due to stagnation of undigested food
Sparganium stoloniferum (Buch.-Ham. ex Graebn.) Buch.-Ham. ex Juz. (Typhaceae) (Sanleng)	Black-triangular plant, *Sparganium stoloniferum* Buch.-Ham, tubers	To break blood, move qi, relieve pain, and disperse accumulation
Salvia miltiorrhiza Bunge (Lamiaceae) (Danshen)	*Salvia miltiorrhiza* Bunge (Lamiaceae), dry roots, and rhizomes	To quicken blood and dispel stasis, regulate menstruation and relieve pain, nourish blood and calm spirit, cool blood and disperse swelling abscess
Hirudo nipponia (Shuizhi)	Mink animal otter, *Hirudo nipponia* Whitman, dry body	To clear heat and resolve toxin, disperse swelling and relieve pain
Trionyx sinensis (Biejia)	Cyprinidae, *Trionyx sinensis* Wiegmann, carapace	Nourish the Yin and suppress Yang, dispel stasis and dissipate knots, soften hardness
Paeonia lactiflora Pall.(Chishao)	Ranunculaceae, peony, *Paeonia lactiflora* pall, root	Treatment of maculation in epidemic diseases, spitting of blood, epistaxis, inflammation of the eye, pain in the chest and lateral thorax, amenorrhea, dysmenorrhea, mass formation in the abdomen, traumatic injuries
Citrus aurantium L.(Zhike)	*Citrus aurantium* L., a dried, immature fruit of the cultivar	Clear heat and activate blood, promoting circulation of qi and blood
Angelica sinensis (Oliv.) Diels (Danggui)	*Angelica sinensis* (Oliv.) Diels, root	To nourish blood and regulate menstruation, quicken blood, relieve pain, moisten intestines, and relieve constipation

ultrasonicated for 30 min and centrifuged at 1.2×10^4 rpm for 5 min, and the supernatant was removed. The chromatographic conditions were as follows: The UPLC device was Agilent 1290 UPLC, and the column was Agilent SB-C18, 2.1 × 100 mm, 1.8 μm. The column temperature was 30°C, and the injection volume was 5 μl. The detection wavelength was 254 nm. Phase A is 0.1% formic acid aqueous solution, and phase B is acetonitrile. Gas chromatography-mass spectrometry (GC-MS) was performed using Agilent 7890A/5975C. The column was Agilent HP-5MS, 30 m × 250 μm × 0.25 μm. The inlet

temperature was 250°C, ion source temperature was 230°C, and quadrupole temperature was 150°C. The compounds were tentatively characterized based on their retention time, mass accuracy of precursor ions, MS/MS spectra, and fragmentation pathways, referring to the SCIEX natural product HR-MS/MS Spectral Library and previous literatures. The conditions of UPLC-Q-TOF/MS are listed in **Supplementary Table S2**. Methanol and acetonitrile of chromatographic grade were supplied by Merck Chemicals (Shanghai, China). 2-Chloro-L-phenylalanine was bought from Hengbai Biotechnology (Shanghai, China).

Network Pharmacology Analysis of ELeng Capsule

Candidate Compound Database

Compounds of ELC were compiled from the STITCH database (http://stitch.embl.de/), the traditional Chinese medicine systems pharmacology (TCMSP) database (http://tcmspw.com/tcmsp.php), and the Universal Natural Products Database (UNPD) (Gu et al., 2013). The structures of compounds were retrieved from the PubChem dataset (https://www.ncbi.nlm.nih.gov/pccompound/). All three-dimensional molecular structures of active ingredients were obtained from PubChem in mol2 format.

Candidate Endometriosis-Associated Genes

The genes of endometriosis of patients were collected from GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/), and GenBank (https://ncbiinsights.ncbi.nlm.nih.gov/tag/genbank/) databases. The targets/proteins were researched in the UniProt database (https://www.uniprot.org). The three-dimensional structures of proteins related to endometriosis were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) (www.rcsb.org/pdb/home/home.do).

GO and KEGG Enrichment Analyses

Furthermore, we performed GO enrichment and KEGG pathway enrichment analyses of ELC-associated targets. We used the web-based search engine, DAVID, to determine over-represented GO terms and KEGG pathways with thresholds of an enrichment score >2, count >5, and $p < 0.05$.

Network Construction

The online search tool for recurring instances of neighboring genes (STRING, version 9.1) (http://string-db.org/) was used to predict the protein–protein interactions. The compounds-targets networks were constructed using Cytoscape software 3.7.2 (http://cytoscape.org/). The related parameters were calculated to detect significant nodes (Shannon et al., 2003).

Animal Model Establishment and Treatments

This study used female Sprague Dawley (SD) rats (age: 8 ± 1 weeks, weight: 220–230 g). The rats are from the Experimental Animal Center of Guangdong Province (Guangzhou, Guangdong, China), and the certificate number is 44007200054328. The rats were housed at 20 ± 2°C on a 12-h light/dark cycle, with *ad libitum* access to food and water, and raised in the Laboratory Animal Center of The Second Clinical Medical College of Guangzhou University of Chinese Medicine (Guangzhou, Guangdong, China). The rats were housed five per cage. The animals and the protocols were approved by the Guangdong Provincial Hospital of Chinese Medicine Committee on the Use of Live Animals for Teaching and Research (No. SZY2016007). And disposal methods were in accordance with animal ethics standards.

Surgical Operation

A model of endometriosis was established through allotransplantation in rats. All operational procedures were conducted under sterile conditions. The rats were anesthetized with 3% pentobarbital sodium prior to performing a vertical incision in the abdomen. The right uterus of each rat was removed and immediately placed in a saline solution. Briefly under sterile conditions, the endometria were separated from the myometria and cut into 0.5 × 0.5 cm pieces. The endometria were sutured onto the peritoneum close to blood vessels in each abdominal wall using a 5-0 absorbable suture. After transplantation (28 days), the growth of the ectopic endometrium was observed via gross and microscopic examination. The endometriosis rat models established successful are following criteria that endometrial explants developed into ovoid, large, fluid-filled, well-vascularized, and cystic lesions (Vernon and Wilson, 1985). The volume of the ectopic endometrium was detected by a vernier caliper with the volume formula (length × width × height × 0.52).

After 28 days of auto-transplantation, endometriosis models were successfully established. The 40 endometriosis SD rats were randomly divided into four groups: the ELC low-dose group (0.5 g/kg/d of ELC), the ELC middle-dose group (1 g/kg/d of ELC), the ELC high-dose group (2 g/kg/d of ELC), and the model group (10 ml/kg/d of 0.9% sodium chloride). The middle-dose group was equivalent to the clinical dose. For animal experiments, the interior powder (0.45 g/capsule) after removed from the shells of ELC was blended with appropriate saline as a working mixture for use. Rats were fed by gavage once a day for 28 days.

In addition, another ten SD rats were selected as the control group and fed routinely. At the end of ELC treatment, the eutopic endometrium from the control group and ectopic endometrium from the endometriosis model rats were collected. The volumes of ectopic endometrial lesions in each group before (V1) and after (V2) treatment were measured. The tissues were used for histopathology analysis, immunohistochemistry, RNA-sequencing, and quantitative polymerase chain reaction (qPCR) validation.

Hematoxylin and Eosin and Masson's Trichrome Staining

Sections from different groups were stained with hematoxylin and eosin (HE). And Masson's trichrome staining was used for

the detection of collagen fibers in tissues. The stained areas of the sections were observed under an optical microscope (Nikon, Japan) and NIS-Elements. Fibrosis analysis was performed using the ImageJ software to analyze the proportion of blue staining.

Transmission Electron Microscopy Analysis

The tissue samples were fixed immediately with 1% glutaraldehyde and 4% formalin for 6 h at 4°C and rinsed in 0.1 M cacodylate buffer overnight. Ultrathin sections were prepared with Ultratome Nova, double-stained with uranyl acetate and lead citrate, and examined under an electron microscope.

Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin-dUTP Nick-End Labeling Assay

Apoptosis was detected using the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) apoptosis detection kit (C1086, Beyotime Biotechnology, China) according to the manufacturer's instructions (n = 4 each group). The labeled apoptotic cells expressed green fluorescence under fluorescence microscopy. The Image J software was used for assessing the ratio.

Monoclonal Antibody and Microvessel Density

Vascular endothelial cells were labeled with a CD34 monoclonal antibody, and the microvessel density (MVD) was counted ($n = 6$ each group). The dilution ratio of anti-CD34 antibody (1:500, ab185732, Abcam, United States) was used. Three dense microvessel areas were selected for each slice, and the microvessels were counted by a double-blind method under high power (200×).

Immunohistochemical Staining

The sections were stained by IHC staining to detect the expression of factors in the VEGF family and α-SMA. After the antigen was repaired, primary antibodies were added at 4°C overnight, and then secondary antibodies were added at room temperature for 1 h, avoiding light. Diaminobenzidine (DAB) was used for staining, and neutral gum was used to seal pieces. The antibodies were anti-VEGFA (1:1000, ab81289, Abcam, United States), anti-VEGFB (1:1000, ab81289, Abcam, United States), anti-VEGFC (1:1000, ab81289, Abcam, United States), and α-SMA (1:1000, 14395-1-AP, Proteintech, United States) ($n = 4$ each group). The Image J software was used for assessing the mean optical density.

ELISA

The serum of abdominal aorta was prepared for analysis. Thereafter, the samples and standard samples were diluted with distilled water and applied to ELISA plates. The VEGFA (*Cloud-Clone Corp*, Wuhan, China) and VEGFB (*Cloud-Clone Corp*, Wuhan, China) concentrations were determined according to the manufacturer's instructions. Absorbance levels were measured at 450 nm using an ELISA reader.

RNA-Sequencing of ELeng Capsule

The Design of RNA-Sequencing Analysis

The model rats of the 1 mg/kg/d dose ELC group were chosen for further biological experiment because of the dose equivalent to the human dose. We randomly selected transcriptomes from eutopic endometrium tissues and ectopic endometrium tissues for analyses in the control, model, and ELC groups. Sample groups consisted of $n = 4$ eutopic endometria, including (Con_Euto), (Model_Euto), and (ELC_Euto) groups, $n = 4$ model group ectopic endometriotic lesions (Model_Ecto), and $n = 5$ ELC group ectopic endometriotic lesions (ELC_Ecto). A crossover comparison was performed in the following three paired groups to identify genes that were differentially regulated in the model group and ELC group: Con_Euto *vs.* Model_Ecto groups; Con_Euto *vs.* ELC_Ecto groups; and Model_Ecto *vs.* ELC_Ecto groups.

Gene Ontology Terms and Kyoto Encyclopedia of Genes and Genomes Analyses

Preparation of transcriptome libraries and sequencing were performed by Shanghai OE Biotech Co. (Shanghai, China). Raw data (raw reads) were processed using Trimmomatic (Bolger et al., 2014). Multiple hypothesis testing correction for the treatment effect was performed using the false discovery rate (FDR) method. GO and KEGG enrichment analyses of differentially expressed genes (DEGs) were, respectively, performed using R studio. The GO analysis provides three structured networks of defined terms to describe gene product attributes: cellular compartment (CC), biological process (BP), and molecular function (MF). Pathway analysis was applied to determine the significant pathways of DEGs according to KEGG, MapSplice, and Reactome Functional Interaction network and external interaction databases (Reactome database). Fisher's exact test was used to identify significantly enriched pathways, and the threshold of significance was defined as $p < 0.05$ and FDR <0.05.

Gene Set Enrichment Analysis

In this study, 1,000 genes of permutations were set to generate a null distribution for the enrichment score in the hallmark gene sets and functional annotation gene sets. The publicly available GSEA software package (www.broad.mit.edu) was used for leading edge analysis to examine genome-wide expression profiles (Subramanian et al., 2005). Nominal $p < 0.05$, FDR <0.25, and gene set size >100 were defined as the cut-off criteria. The aim of this analysis was to determine whether the members of the identified gene ontology and KEGG pathways were randomly distributed throughout the ranked gene list or concentrated at the top or bottom.

Trend Modular Analysis (STEM Analysis)

Short Time-series Expression Miner (STEM) is a software program, which could be designed for the analysis of short time-series microarray gene expression data (Ernst and Bar-Joseph, 2006). This approach was used to identify the profiles of the "up to down" model from Con_Euto to Model_Ecto to

ELC_Ecto. The results of STEM analysis could help discover the regulation mechanism of ELC.

Construction of the Protein–Protein Interaction Network

The STRING database provides comprehensive information regarding interactions between proteins. Subsequently, the PPI network was visualized using Cytoscape (version 3.7.2; National Institute of General Medical Sciences, Bethesda, MD, United States) (Shannon et al., 2003). The PPI network was used to filter modules based on the Molecular Complex Detection (MCODE) plugin in Cytoscape with the following conditions: degree cut-off = 2; k-core = 2; node score cut-off = 0.2; and max depth = 100.

Quantitative Reverse Transcription-PCR

qRT-PCR was performed to validate the gene expression data obtained from deep sequencing. Total mRNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. The first strand of cDNA was synthesized using primers designed in our laboratory. The RT product was amplified using SYBR Green on a 7500 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, United States). All samples were run in triplicate, and the relative gene expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method. The sequencing accessions of the primers were myogenin (Myog), SET and MYND domain containing 1 (Smyd1), SIX homeobox 1 (Six1), calcium voltage-gated channel subunit alpha1 S (Cacna1s), eukaryotic translation elongation factor 1 alpha 2 (Eef1a2), ryanodine receptor 1 (Ryr1), actinin alpha 2 (Actn2), myogenic differentiation 1 (Myod1), mitogen-activated protein kinase 12 (Mapk12), and myosin heavy chain 4 (Myh4). Gene expression levels were normalized to that of ACTB. The primer sequences are shown in **Table 2**.

Statistical Analysis

Data were analyzed using the Prism software (version 7.0; GraphPad Prism, San Diego, California, United States). All experimental data are presented as the mean ± standard error of the mean. The qPCR data were analyzed using two-tailed Student's *t*-test. Unless otherwise indicated, $p < 0.05$ denotes statistical significance.

RESULTS

UPLC-Q-TOF/MS Results for ELC

In this study, the compounds of ELC were identified by UPLC-Q-TOF/MS. According to the UPLC-Q-TOF/MS combined with the data obtained from the literature and databases, another 26 compounds were identified in the ELC compound preparation. Representative fingerprint chromatograms of ELC are displayed in **Supplementary Figures S1–S3**. The identified compounds of ELC are shown in **Supplementary Table S3**. In our previous study, a total of 14 compounds were identified based on GC-MS, namely, eucalyptol, D-camphor, isoborneol, L(−)-borneol, α-terpineol, β-elemene, γ-elemene, α-humulene, germacra, curcumenol, β-cyclocostunolide, curcumenone, pulmonary zederone, and ent-kaurene (**Supplementary Figure S4** and **Supplementary Table S4**). Combined with our previous study results, we have identified a total of 40 compounds in ELC.

Network Pharmacology Analysis

The Compounds' Associated Targets of ELeng Capsule

Furthermore, based on the data obtained from the network pharmacology–related databases, we identified 27 potential compounds with 194 potential targets based on STITCH, TCMSP, and UNPD datasets. The result showed that these major targets were involved in angiogenesis, inflammation, immunity, cell adhesion, cell invasion, and other modules. **Supplementary Table S5** shows the major compounds and targets of ELC. Combined with the target and previous research evidence, the results implied that tanshinone IIA, cryptotanshinone, rosmarinic acid, danshensu, tanshinone I, paeoniflorin, gallic acid, linoleic acid, γ-elemene, hesperetin, palmitic acid, naringin, *etc*., may be compounds that play the major role in endometriosis (**Table 3**).

Furthermore, we had collected 1,289 endometriosis-related genes/targets from GeneCards, GenBank, and OMIM databases. A total of 75 targets of ELC overlapped with endometriosis-related proteins. Information on these targets is provided in

TABLE 2 | Primer sequences used for real-time PCRs.

No.	Gene symbol	Forward primer	Reverse primer
1	Myog	CGACCTGATGGAGCTGTA	GGTGGACAGGAAGGTAGT
2	Smyd1	ACCGTCTATTTAACAAGGAAGC	GCACCGTGGCATTTACTA
3	Six1	ATTAGTGAGGGAAACAAGTGC	GTTTGTTGCGTTACTAACATCG
4	Cacna1s	CACCTGGTTCACCAACTTTAT	CTGATTCCTCATGGAGTCG
5	Eef1a2	CCAGCAAATACCCTCAACC	GTCTTCTCCTTGCCCATTC
6	Ryr1	AGCCGTATGTACCTGAGT	GTGGCGTCTTCCTGTAATC
7	Actn2	CCAGCGCCATGAATCAGATA	CTCCTCCTGGATCATGTACTC
8	Myod1	GACAGCAGGTGTGCATTC	TAGTAGCTCCATGTCCCAGT
9	Mapk12	CAGTGGACATTTGGTCTGTTG	TGGTCCAGGTGGTCATTG
10	Myh4	CAAGGTGAAGAACGCCTA	TCCAGCTCGTGGATATGC
11	ACTB	GCGAGTACAACCTTCTTGC	TATCGTCATCCATGGCGAAC

TABLE 3 | Mechanism of the compounds with potential therapeutic properties.

Herb name	Compound name	Molecular formula	Potential targets based on network pharmacology	Major mechanism	References
Salvia miltiorrhiza	Salvianolic acid A	C26H22O10	AKT1,BCL2,CDKN3, EIF3L,F10,PRSS1,CASP3, COL7A1,F7,PTPN6,CCND1	Anti-thrombosis; anti-fibrosis	Xu et al. (2018)
	Tanshinone IIA	C19H18O3	ACHE,ADRA1A,ADRB1, ADRB2,CASP3,CHRM1, F2,OPRM1,CHRM2,DPP4, RXRA,PTGS2,CHRM5,CHRNA7, OPRD1,CHRM3,CHRM4,DRD1, NFKB3,CYP1A1,EDN1,BCL2, FOS,TP53,CYP1A2,CYP3A4, ITGB3,JUN,MMP9	Reduce the expression of AGT, REN, ACE, ANGII, and AT2 in DRG neurons; reduce the VEGF/VEGFR2 pathway and CD146	Qi Zhang et al. (2016); Luo et al. (2021)
	Cryptotanshinone	C19H20O3	ADRA1A,ADRA1B,ADRA1D, ADRB1,ADRB2,APP,BCL2L1, BIRC5,CHRM1,CHRM3, CHRM4,CHRNA7,PTGS1,DRD1, CHRM5,PTGS2,CA2,OPRD1, CHRM2,TOP2A,OPRM1, NCOA2,PGR,GABRA1, NFKB3,STAT1,CCND1, TNF,EDN1	Anti-tumor, anti-inflammatory, neuroprotective, cardioprotective, visceral protective, anti-metabolic disorders; anti-tumor effects; STAT3-related pathways	Wu et al. (2020)
	Rosmarinic acid	C18H16O8	F2,ESR1,AR,PPARG,PTGS2, DPP4,PRSS1,NFKB3,IKBKB, CDKN3,EIF3L,MAPK1,CASP3, STAT1,CCL13,MGAM,IL2,NFATC3, CCND3,IL4R,IL5,CCL3, CD80,CD86,CCL11, CCR6,IDO1,SNCA, IGHG1,C3,C5	Anti-cancer properties; inhibit the proliferation of primary HESCs and T-HESCs	Yesil-Celiktas et al. (2010); Ferella et al. (2018)
	Salvianic acid A	C9H10O5	ACHE,ACTB,ADRA1D,ADRA2A, ADRA2B,ADRA2C,ADRB1, ADRB2,COL1A1,COL3A1,F2, TGFB1,HMOX1,PLAU		
	Tanshinone I	C18H12O3	F2,AR,PTGS2,RXRA,DPP4, HSP90AB1,PIK3CG,PRSS1,VEGFA, ICAM1,VCAM1		
	Linolenic acid	C18H30O2	PTGS1,PTGS2,NCOA2, O3FAR1,FADS2,FADS1,PPARA, PPARG,CD36,PLA2G6,PLA2G2A, PLA2G1B,PNPLA8		
Paeonia lactiflora Pall. (Chishao)	Paeoniflorin	C23H28O11	TNF,IL6,CD14,LBP, TLR4,HSF1,IL8	Relieve pain; anti-inflammatory through inhibiting TLR4/MMP-9/2/IL-1β signaling pathway	Fan et al. (2018); Chen et al. (2020)
	Paeoniflorigenone	C17H18O6	GABRA1	Induce apoptosis; suppress proliferation	Huang et al. (2017)
	γ-Elemene	C15H24	CHRM2,PTGS1,PTGS2,RXRA,ADRA1A,RXRA, GABRA2,GABRA1,GABRA6,PTGS1,CHRM3	Analgesic effects; anti-tumor	Chen et al. (2021)
Citrus aurantium L. (Zhike)	Neoeriocitrin	C27H32O15	TOP2A	Induce apoptosis; regulation of the MAPK and Akt signal transduction pathways	Park et al. (2017)
	Narirutin	C27H32O14	TOP2A	Cell signal transduction pathways in cancer	Memariani et al. (2020)

(Continued on following page)

TABLE 3 | (*Continued*) Mechanism of the compounds with potential therapeutic properties.

Herb name	Compound name	Molecular formula	Potential targets based on network pharmacology	Major mechanism	References
	Naringin	C27H32O14	TOP2A,CDKN3,TNF,RASGRF1, RAF1,PPARA,DPP4, PPARG,AKT1,NQO1,MMP9,BMP2,CCK,GHSR,IL8	Anti-tumor through cell signal transduction pathways in cancer (JAK–STAT pathway, PI3-kinase/Akt/mTOR signaling pathway, Notch pathway, NF-κB and cox-2 pathway, Wnt pathway, MAPK-ERK pathway, TGF-β signaling pathway); regulate angiogenesis	Memariani et al. (2020)
	Hesperetin	C16H14O6	PTGS1,ADRB1,PTGS2,HSP90AB1, PIK3CG,PRKACA,NCOA2,CAMTA3, CYP71B35,TAG1,AT4G35090,CAT1, AT1G20620,RHC1A,CYP86A8, CYP86A2,CYP86A7	Promote cisplatin-induced apoptosis in gastric cancer	He et al. (2020)
	Limonin	C26H30O8	CYP3A4	Induce apoptosis	Rahman et al. (2015)
Sparganium stoloniferum (Buch.-Ham. ex Graebn.) Buch.-Ham. ex Juz. (Typhaceae) (Sanleng)	Gallic acid	C7H6O5	PTGS1,PTGS2,MAOB,PGR,PTPN6, TOP2A,HSP90AB1,PIK3CG, CASP9,CASP3,TP53,FASN,FASLG, MGST1,CYP3A43	Analgesic effects	
	Sparstolonin B	C15H8O5	TLR2,TLR4	A potential therapeutic agent for toll-like receptor–mediated inflammatory disorders	Yepuri et al. (2019); Jin et al., 2018
	Sanleng acid	C18H34O5		Anti-tumor activity	
	β-Elemene	C15H24	PTGS2,GABRA2,RXRA NET,CHRM2,GABRA1,GABRA6, PTGS1,CHRM3,CHRM1,ADRA1A, CHRNA7,NCOA2,GABRA5, BCL2,CDKN3,EIF3L,RB1, TP53,TEP1,RUNX1T1, CRK2,CCNB1,RHOA	Analgesic effects; anti-tumor activity; induce apoptosis	Bi et al. (2018); Chen et al. (2021)
Curcuma phaeocaulis Valeton (E'zhu)	Curdione	C15H24O2	CYP3A4	Anti-tumor activity	Chen et al. (2021)
	Isoborneol	C10H18O	GABRA6,PGR,CYP2C8, GABRA2,CHRM2,GABRA1, CHRM3,CHRM1,PTGS2, GABRA5,NET,ADRA1A	Anti-inflammatory and analgesic effects	Wang et al. (2017)
	Germacra	C15H24	PTGS2,RXRA,NET,GABRA1,MAOB	Analgesic effects	
	Borneol	C10H18O	CYP2C8,GABRA2,GABRA5,CHRM2, GABRA1,IGHG1,GABRA6,PTGS1, PTGS2,NET	Anti-inflammatory and analgesic effects	Wang et al. (2017)
	Zederone	C15H18O3	NOS2,CHRM3,F2,CHRM1, ADRB2,GABRA1,CHRNA7	Analgesic effects	Faiz et al. (2015)

Supplementary Figure S5 and **Supplementary Table S6**. Following cytoHubba analysis, the PPI network revealed that VEGFA, IL6, TP53, PTGS2, AKT1, MMP9, MAPK1, JUN, CASP3, IL10, *etc.*, could be the major relevant targets.

GO Enrichment and KEGG Pathway Analyses

The BP in GO terms is related to cell death, apoptosis, proliferation, *etc.* We also found that some targets are related to the GO terms of smooth muscle hyperplasia and regulation of smooth muscle cell-matrix adhesion. And the main KEGG pathways include signaling molecules and interaction (neuroactive ligand–receptor interaction and cytokine–cytokine receptor interaction), immune system [toll-like receptor (TLR) signaling, B cell receptor signaling, and T cell receptor pathways], and other signal transduction pathways [PPAR signaling pathway, metabolism of xenobiotics by cytochrome P450, vascular endothelial growth factor (VEGF) signaling pathway, and calcium signaling pathway]. Thus, the core compounds of ELC may be involved in regulating inflammation and immunity, reducing adhesion and angiogenesis, and inducing cell apoptosis. The major GO terms and KEGG pathways are shown in **Figures 2A,B**. The network of major targets and compounds from the database is shown in **Figure 2C**.

Potential Mechanism of ELeng Capsule in Endometriosis Rat Model

The Effect of ELeng Capsule on Pathology and Ultramicro-Pathology

To further assess the obtained results of network pharmacology analysis, we successfully established a rat model in endometriosis. We mainly examined the effects of ELC in inducing apoptosis and inhibiting angiogenesis and fibrosis.

Figure 3A shows the changes in lesions after modeling. After the treatment, the average value of tissue in ELC groups was lower than that in the model group, while the difference was not statistically significant ($p > 0.05$) (**Supplementary Table S7**). Compared with that in the model group, the lesion volumes before and after ELC treatment in the ELC middle-dose group changed significantly ($p = 0.028 < 0.05$). These results showed that ELC may reduce the volume of ectopic lesions to a certain extent in endometriosis rat models. The HE staining revealed the formation of local glands in the lesions of the model and ELC group rats (**Figure 3B**). Compared with the eutopic endometrium in the control group, the ectopic endometrium in the model group had a thinner endometrium, intact glandular epithelial cells, and a loose arrangement.

The ultra-structures have been examined using an electron microscope. In the model group, many microvilli endometrial glandular epithelial cell surfaces and long villi can be seen. In the ectopic tissue in the ELC group, the microvilli were reduced. And the mitochondria were swollen in the cytoplasm, and autophagy and apoptotic bodies were observed (**Figure 3C**). **Figures 3C iii,iv** show the apoptotic body, and **Figures 3C v,vi** show the autophagosome. This result suggested that ELC treatment may be related to the regulation of autophagy and apoptosis.

ELeng Capsule Could Promote Apoptosis in Ectopic Endometrial Tissues

The distribution of green fluorescence included glandular epithelial and mesenchymal cells. As shown in **Figure 3D**, the nuclei of positive staining apoptotic cells emitted green fluorescent signals in the ectopic endometrium tissues. Compared with that in the model group, the apoptotic area in the middle-dose group of ELC increased significantly ($n = 4$, $p < 0.05$). This result suggested that ELC could participate in the process of cell apoptosis.

ELC Could Reduce the MVD and the Expression of VEGFA and VEGFC in Ectopic Endometrial Tissues

As shown in **Figure 4A**, a significantly increased MVD was observed in ectopic lesions compared with the corresponding eutopic endometria and normal endometria. The ectopic endometria in the middle-dose group exhibited the highest MVD, and normal endometria exhibited the lowest MVD. The MVD in the ectopic lesion was significantly higher than that in the eutopic endometrium ($n = 6$, $p = 0.015 < 0.05$). The expression of CD34 in high-, middle-, and low-dose groups of ELC was significantly lower than the model group expression.

Figure 4B shows the expression of VEGFA, VEGFB, and VEGFC in the cytoplasm and membrane of glandular epithelial cells and mesenchymal cells in ectopic lesions in the endometriosis rat model. The VEGF gene expressions of mesenchymal cells were weaker than those of glandular epithelial cells. The expression of VEGFA in middle-dose and low-dose groups decreased compared with that in the model group ($p < 0.05$). There was no significant difference between the model group and the ELC high-, middle-, and low-dose groups in the VEGFB expression ($p < 0.05$). Compared with that in the model group, the expression of VEGFC in ectopic lesions significantly decreased in the high-, middle-, and low-dose groups of ELC, and the differences were statistically significant ($p < 0.05$). These results suggested that ELC may inhibit angiogenesis by reducing the expression of VEGFA and VEGFB.

In addition, compared with that in the control group (34.838 ± 1.403 pg/ml, $n = 8$), the VEGFA level in serum increased in the model group (38.866 ± 2.706 pg/ml, $n = 8$) ($p = 0.008 < 0.05$). Compared with that in the model group, the serum VEGFA level in the high-dose group (35.345 ± 4.205 pg/ml, $n = 8$) and low-dose group (35.024 ± 2.662 pg/ml, $n = 8$) of ELC significantly decreased (**$p = 0.020$, ***$p = 0.012$). There was no significant difference in the serum of VEGFB expression in different groups of endometriosis model rats ($p > 0.05$). Thus, the regulation effect of ELC may be mainly localized in ectopic lesions (**Figure 4C** and **Supplementary Table S8**).

ELeng Capsule Could Reduce the Local Fibrosis in Ectopic Lesions

The results of Masson's trichrome staining showed that the ectopic lesions were fibrotic. Compared with that in the model

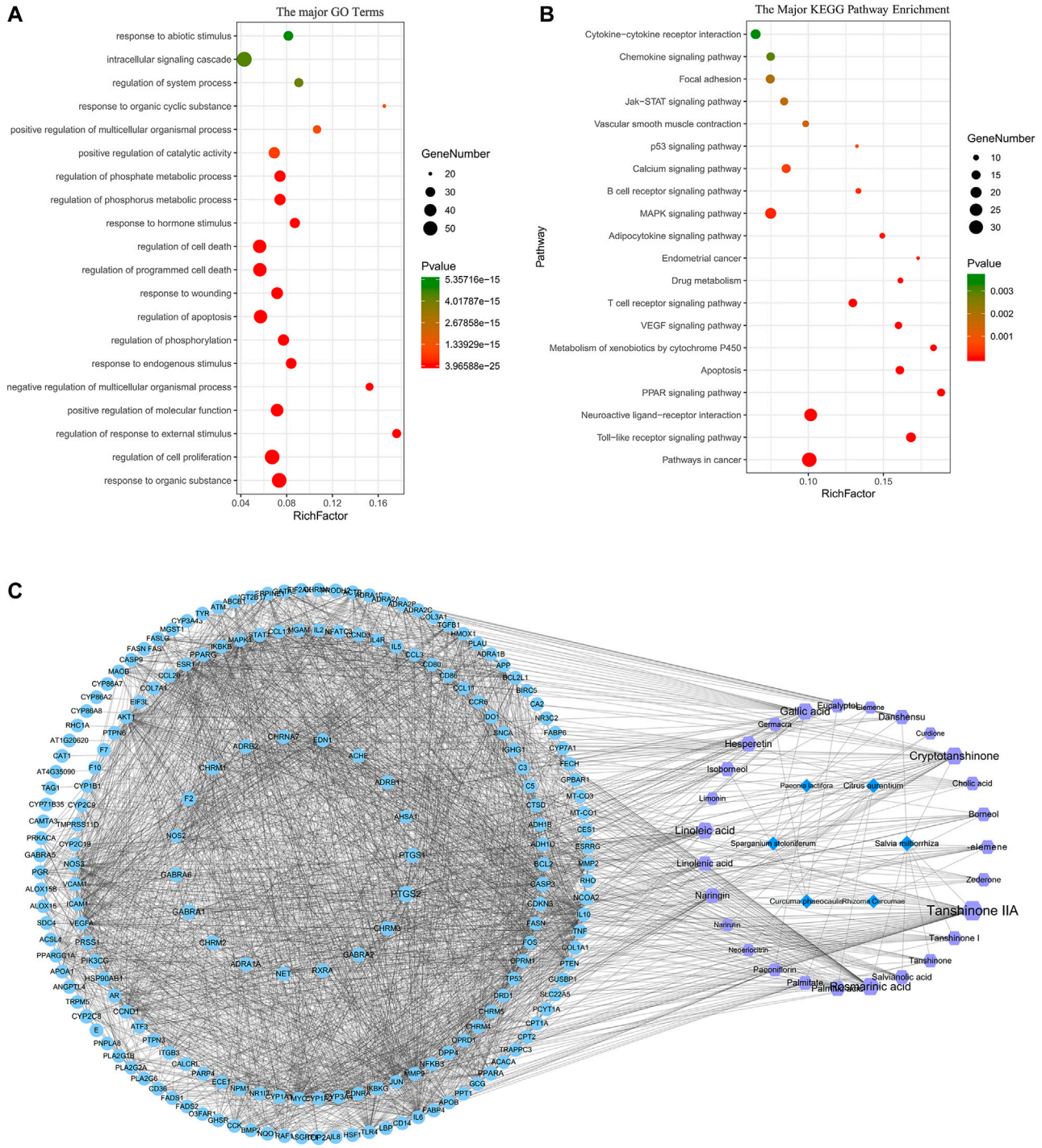

FIGURE 2 | Network pharmacology analysis of ELeng Capsule. **(A)** The major GO terms of BP of potential targets of ELC. **(B)** The 20 major KEGG pathways of potential targets of ELC. **(C)** "Herb–compound–target-signaling pathway" network of ELC. The light blue nodes represent targets (genes); the purple nodes represent compounds; the dark blue nodes represent herbs.

group, the positive area of fibrosis decreased in the high-, middle-, and low-dose groups of ELC, and the difference was statistically significant ($p < 0.05$). The result showed that ELC could reduce the degree of fibrosis in endometriosis model rats in ectopic lesions. The results of Masson's trichrome staining are shown in **Figure 5**. In addition, the expression of α-SMA in ectopic lesions of model rats increased ($p < 0.05$). After ELC treatment, the expression of α-SMA in ELC groups (high-, middle-, and low-

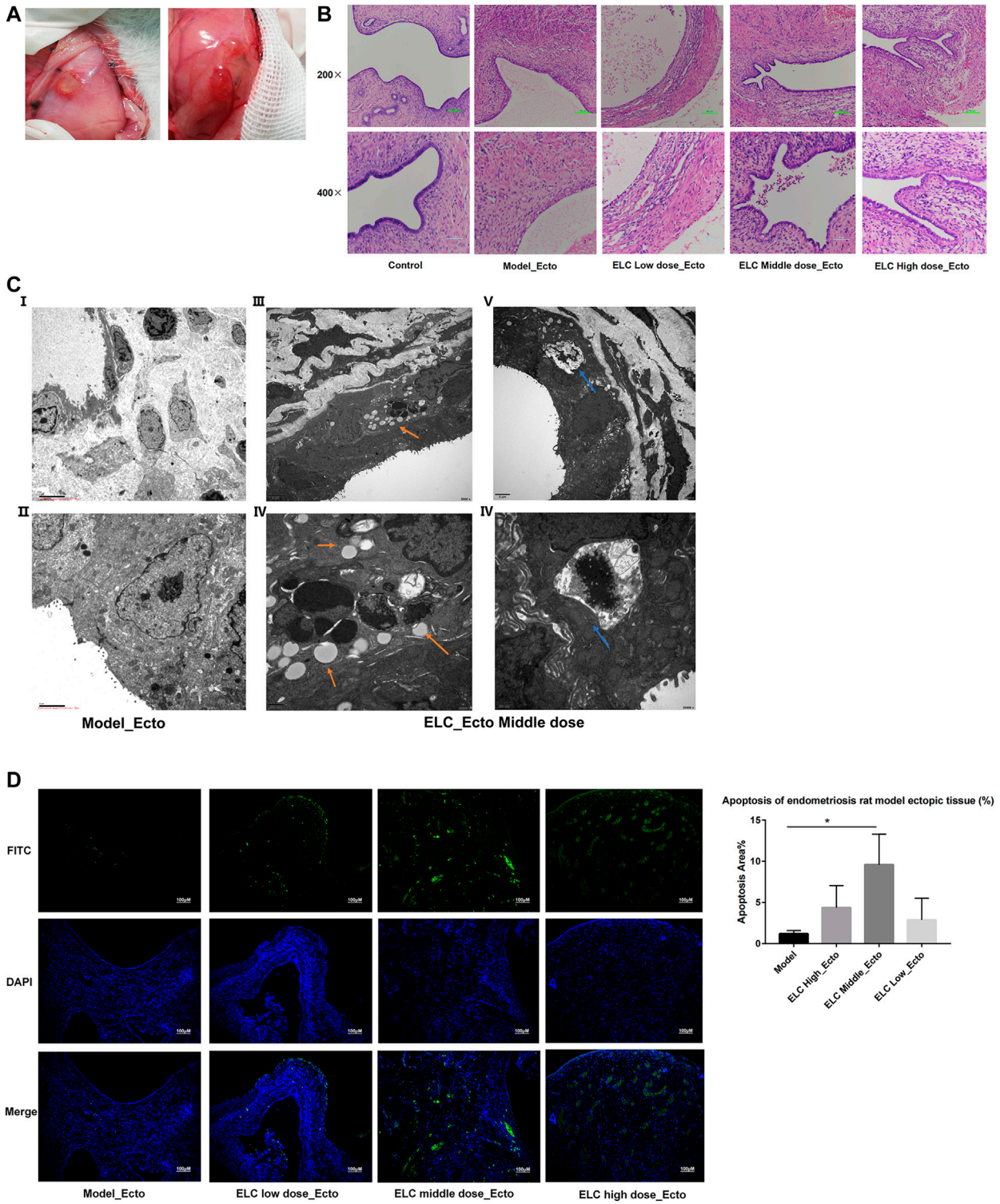

FIGURE 3 | Pathological microstructure and ultrastructure. **(A)** Graphs of ectopic endometrium lesions in endometriosis rat models. **(B)** Microstructure of the ectopic endometrium by HE staining (200× and 400×). **(C)** Ultrastructure of ectopic endometriotic lesions. The blue arrow indicates autophagosome. The orange arrow shows the structure of apoptosis bodies. **(D)** Detection of apoptosis was using the TUNEL assay (100x). Apoptosis in ectopic endometria of different groups was observed by the TUNEL assay. DAPI-stained nuclei appeared in blue. Green-stained tissue appeared in green due to the presence of apoptotic cells. The apoptotic index (%) of ectopic endometrial tissues was significantly higher in middle doze group (n = 4).

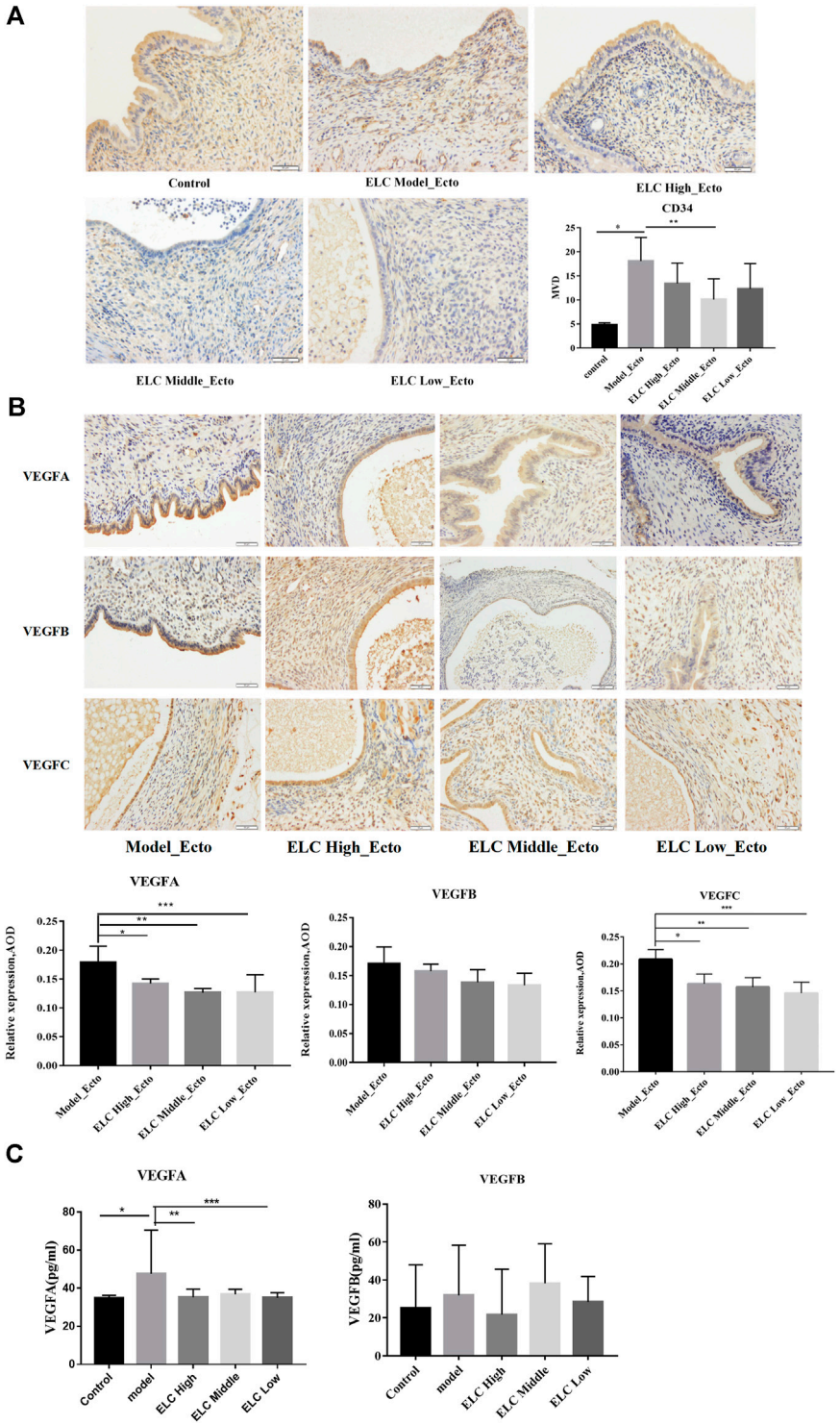

FIGURE 4 | MVD and expression of VEGF in ectopic endometrial tissues. **(A)** Compared with that in the control group eutopic endometrium, the MVD in the ectopic endometrium in the model group increased ($n = 6$, $^{*}p = 0.001 < 0.05$). Compared with that in the model group, the MVD in the ectopic endometrium in the ELC middle-dose group decreased ($^{**}p = 0.018 < 0.05$). **(B)** The results suggested that the expression of VEGFA was statistically significant ($p = 0.014$). $^{*}p = 0.031 < 0.05$; $^{**}p = 0.004 < 0.05$; $^{***}p = 0.005 < 0.05$. There was no significant difference in VEGFB expression among different groups ($p > 0.05$). Compared with that in the model group, the expression of VEGFC was reduced in ELC groups. $^{*}p = 0.005$; $^{**}p = 0.002$; $^{***}p = 0.000$. **(C)** The expression levels of VEGFA in serum were statistically significant ($F = 2.742$, $p = 0.044 < 0.05$). $^{*}p = 0.008$; $^{**}p = 0.020$; $^{***}p = 0.012$. There was no significant difference in the expression levels of VEGFB in serum among different groups ($F = 0.674$, $p = 0.614 > 0.05$). Values are represented as mean ± SD, $n = 4$. $p < 0.05$ as determined by one-way ANOVA.

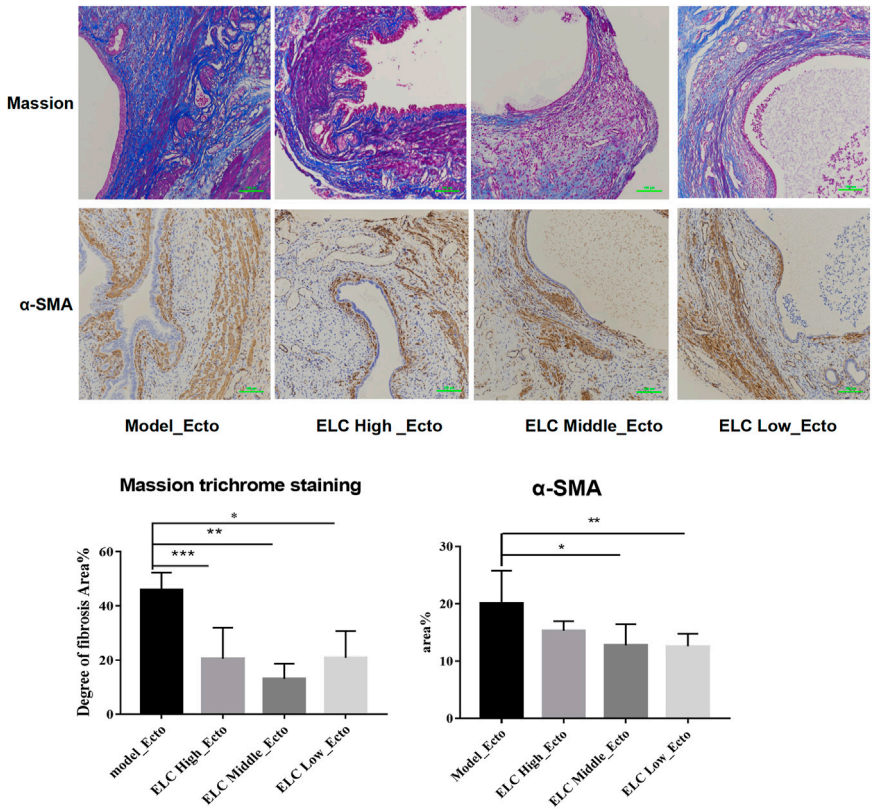

FIGURE 5 | Result of fibrosis in ectopic lesions in endometriosis model rats. Values are represented as mean ± SD, $n = 4$. $p < 0.05$ as determined by one-way ANOVA. The Masson staining showed local fibrosis after modeling (×200). The percentage of fibrosis was positive by Masson staining of ectopic lesions in tissue sections. Compared with the model group, the ELC group has a lower degree of ELC fibrosis. Values are represented as mean ± SD ($n = 4$) (*$p < 0.05$). ELC could reduce the degree of fibrosis of the lesion. Model group: 45.86 ± 6.42%, ELC High_Ecto: 20.56 ± 11.41%, ELC Middle_Ecto: 13.06 ± 5.68%, and ELC Low_Ecto: 20.87 ± 9.93%. *$p = 0.0068$; **$p = 0.0009$; ***$p = 0.0074$ ($p < 0.05$). Compared with that in the control eutopic endometrium, the fibrosis area (Area%) of the model group and ELC group increased significantly ($p = 0.0457 < 0.05$). Compared with that in the model group, the fibrosis area ratio was reduced in the ELC middle-dose group and low-dose group. *$p = 0.040$; **$p = 0.0346$ ($p < 0.05$).

dose groups) was all reduced compared with that in the model group ($p < 0.05$). These results suggest that ELC could reduce the fibrosis process of ectopic lesions by inhibiting the expression of α-SMA in ectopic lesions.

RNA-Sequencing Analysis of Endometriosis Rat Model Characteristics and the Treatment With ELeng Capsules

The Differentially Expressed Gene Screening Analysis

We further analyzed the potential mechanism of ELC by RNA transcriptome. According to the results of the principal components analysis, there is no difference in Con_Euto, Model_Euto, and ELC_Euto groups. These suggested that ELC may not affect the eutopic endometrium in endometriosis rat models. There were a total of 1,461 DEGs in Con_Euto vs. Model_Ecto groups, 557 DEGs in Model_Ecto vs. ELC_Ecto groups, and 1,097 DEGs in Con_Euto vs. ELC_Ecto groups (FC-1.5). **Supplementary Figure S6** shows the PCA of five groups (A), Venn analysis of more than five groups (B), upregulated and downregulated DEGs (C), and heatmap illustration (D).

In this study, there were 1,048 upregulated DEGs and 413 downregulated DEGs between Model_Ecto and Con_Euto groups. These DEGs mainly participate in the processes such as inflammation, cytoskeleton, EMT, and angiogenesis. In the ELC_Ecto *vs.* Model_Ecto group analysis, a total of 66 and 491 upregulated and downregulated DEGs, respectively, were identified, reflecting the differential expression of related genes after treatment with ELC.

GO and KEGG Enrichment Analyses of Model_Ecto vs. Con_Euto

We analyzed the characteristics of the rat endometriosis model based on our RNA-sequence data based on the DEGs of Model_Ecto *vs.* Con_Euto. As shown in GO terms, the upregulated genes were most significantly enriched in the CC of extracellular region, the BP of muscle contraction, and the MF of actin filament binding, fibronectin binding, calcium ion binding, *etc*. The actin-associated GO terms may relate to the development of ectopic lesions of endometriosis (**Figure 6A**). The major upregulated KEGG analysis pathways were extracellular matrix–receptor

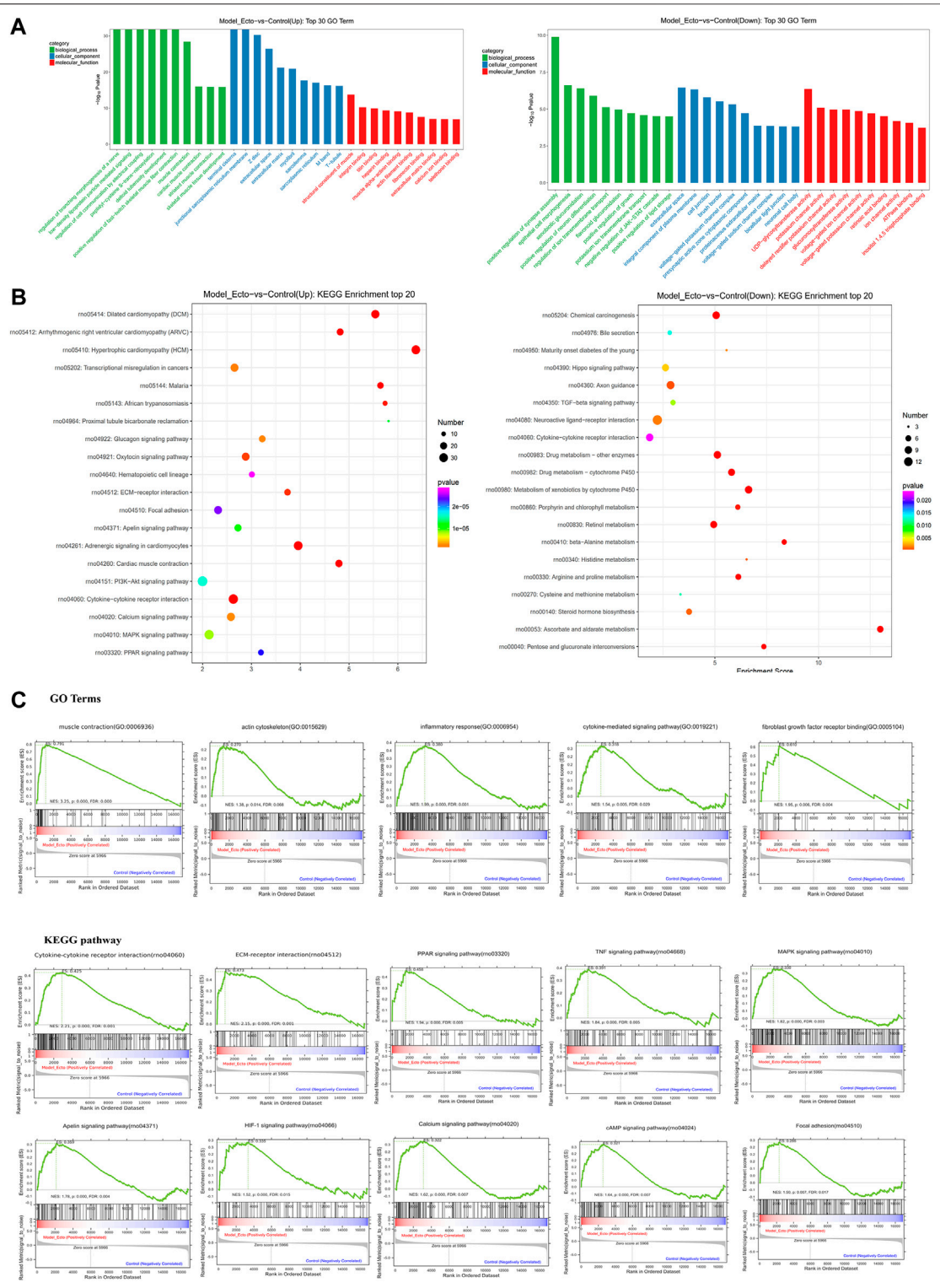

FIGURE 6 | RNA-sequencing reveals the characteristic of endometriosis rat models induced by autotransplantation. Transcriptome characteristics of endometriosis model rats were found through comparing the ectopic endometrium in model rats and the eutopic endometrium in control group rats. **(A)** Results of GO enrichment analysis for upregulated DEGs and downregulated DEGs reversed by ELC ectopic endometrium groups and model ectopic endometrium groups. **(B)** KEGG analysis of upregulated and downregulated genes. KEGG pathway analysis of upregulated and downregulated genes in the ectopic endometrium in rat models *vs.* control eutopic endometrium. The gene ratio refers to the ratio of the number of target genes associated with a KEGG pathway to the total number of genes in the pathway. **(C)** GSEA in endometriosis rat models showed enrichment of GO analysis and KEGG pathway. The normalized enrichment score (NES), *p*-value, and false discovery rate (FDR) are indicated for each gene set. GSEA revealed a significant enrichment of gene signatures associated with endometriosis ($p < 0.05$).

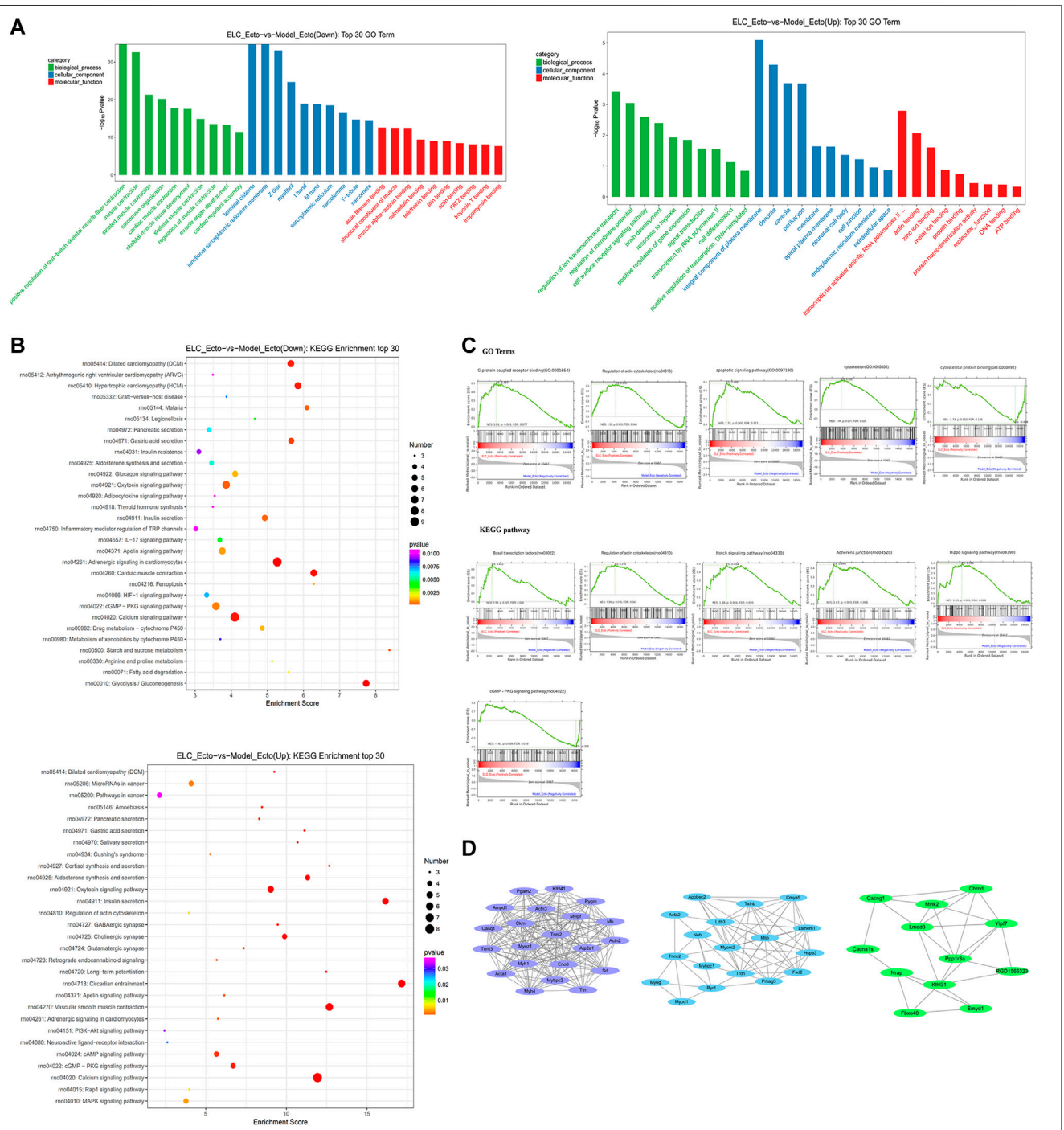

FIGURE 7 | RNA-sequencing reveals the transcriptome profile of gene expression changes in the treatment with ELC. The related genes and pathways regulated by ELC were found through comparing the DEGs between the Model_Ecto group and the ELC_Ecto group. **(A)** Results of gene ontology enrichment analysis for upregulated DEGs and downregulated DEGs reversed by ELC ectopic endometrium groups and model ectopic endometrium groups. **(B)** Results of KEGG enrichment analysis between ELC_Ecto groups and Model_Ecto groups. **(C)** The results of GSEA revealed a significant enrichment of gene signatures associated with ELC treatment ($p < 0.05$). **(D)** MCODE network of cluster analysis of the potential targets network in ELC treatment.

(ECM–receptor) interaction, p53 signaling pathway, endocrine resistance, interleukin-17 (IL-17) signaling pathway, chemokine signaling pathway, cytokine–cytokine receptor interaction, *etc.* (**Figure 6B**).

Furthermore, utilizing data from the GeneCards dataset, we found that there were 113 upregulated and 28 downregulated DEGs in Model_Ecto *vs.* Con_Euto overlapped with endometriosis genes. The upregulated genes associated with

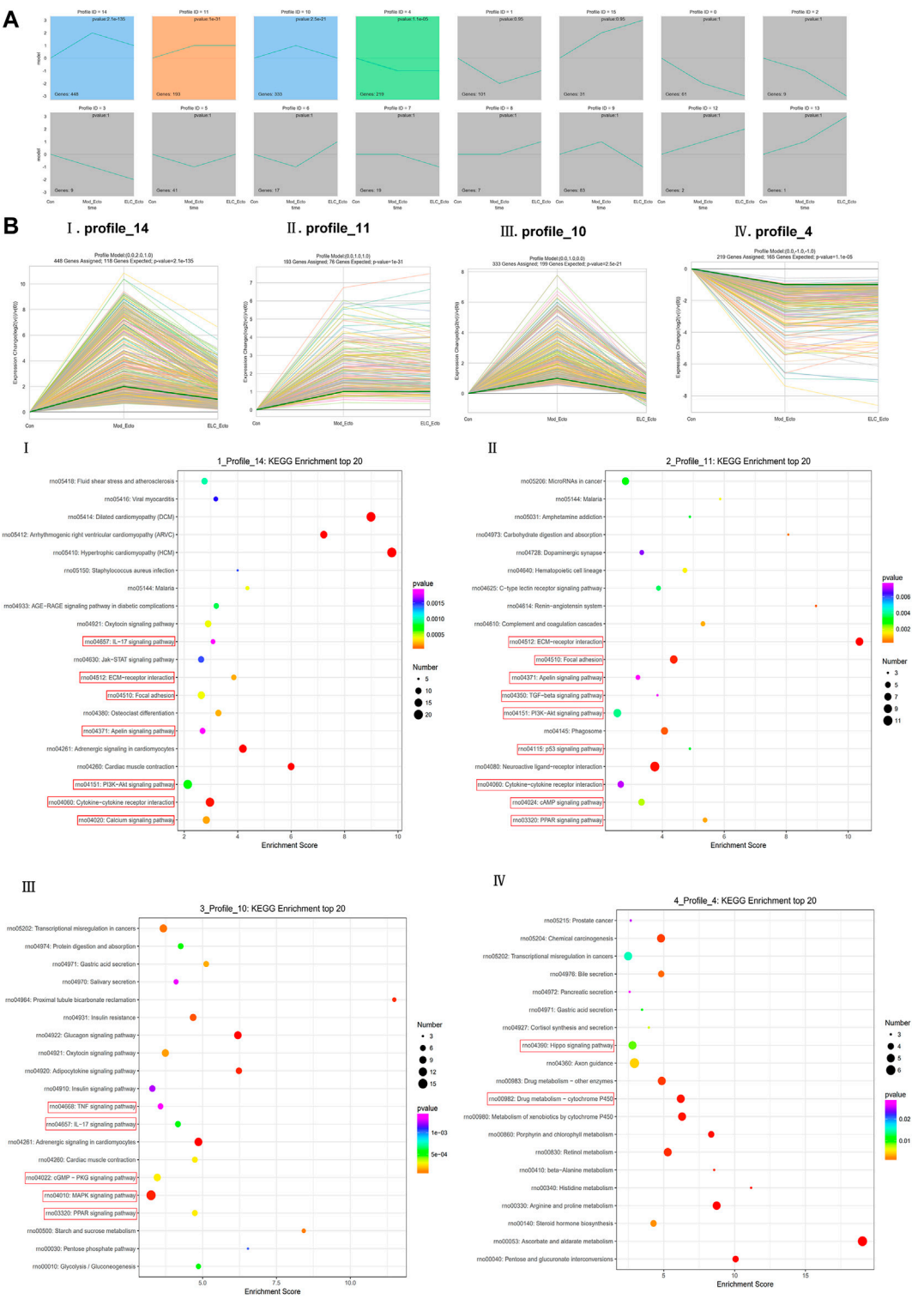

FIGURE 8 | STEM analysis of ELC treatment in endometriosis rats. **(A)** Trend chart of overall STEM analysis. **(B)** Four statistically significant trends. The results of gene cluster analysis were statistically significant in profiles 14, 11, 10, and 4 ($p < 0.05$). The profile_11 panel **(Bii)** and profile_4 panel **(Biv)** could be the regulatory genes of endometriosis model development ($p < 0.05$). The series test of the profile_14 panel **(Bi)** and profile_10 panel **(Biii)** showed that the significant clusters were considered potential profiles that could be affected by ELC treatment ($p < 0.05$). **(i)** $p = 2.1E-135$, **(ii)** $P = 1E-31$, **(iii)** $p = 2.50E-21$, and **(iv)** $p = 0.000011$.

endometriosis in humans are related to cytokine–cytokine receptor interaction, phosphatidylinositol 3 kinase–Akt (PI3K–Akt) signaling pathway, pathway in cancer, MAPK signaling pathway, ECM–receptor interaction, Ras signaling pathway, toll-like receptor (TLR) signaling pathway, IL-17 signaling pathway, p53 signaling pathway, forkhead box protein O signaling pathway, focal adhesion, etc. Based on the above analyses, the rat model of endometriosis may be suitable for investigating the transcriptome level.

In addition, based on GSEA, neuroactive ligand–receptor interaction, cell adhesion molecules, and regulation of actin cytoskeleton are closely related to the occurrence and development of endometriosis. The development of endometriosis is related to the GO terms of "skeletal muscle fiber," "endodermal cell differentiation," "regulation of signaling receptor activity," "positive regulation of myoblast differentiation," "response to cytokine," "chemokine-mediated signaling pathway," "positive regulation of smooth muscle cell migration," etc. The KEGG pathways of GSEA showed that the peroxisome proliferator–activated receptor signaling, tumor necrosis factor signaling, MAPK signaling, apelin signaling, hypoxia-inducible factor-1 signaling, PI3K–Akt signaling pathway, and focal adhesion are related to endometriosis (**Figure 6C**) ($p < 0.01$, FDR <0.25).

GO and KEGG Enrichment Analyses of ELC_Ecto vs. Model_Ecto

We mainly focused on the DEGs in ectopic lesions of endometriosis rats after the intervention of ELC based on DEGs of ELC_Ecto *vs.* Model_Ecto. The BP, CC, and MF GO terms suggested muscle- and troponin-associated regulation, which could be related to ELC treatment. The major enriched GO BPs were positive regulation of fast-twitch skeletal muscle fiber contraction, muscle contraction, striated muscle contraction, etc. The major enriched GO CCs were terminal cisterna, junctional sarcoplasmic reticulum membrane, Z disc, etc. The major enriched GO MFs were actin filament binding, structural constituent of muscle, actin binding, etc. (**Figure 7A**). These results revealed that the treatment with ELC could be related to the regulation of troponin and cytoskeleton.

We further analyzed the KEGG pathways of ELC_Ecto vs. Model_Ecto. The downregulated DEGs were mainly enriched in the following pathways: calcium signaling, apelin signaling, cyclic guanosine monophosphate–protein kinase G (cGMP–PKG) signaling, 5′ adenosine monophosphate–activated protein kinase signaling, hypoxia-inducible factor-1 (HIF-1) signaling, MAPK signaling, PI3K–Akt signaling pathway, focal adhesion, *etc.* (**Figure 7B**).

Based on GSEA, we also found other signaling pathways, including the Notch signaling pathway, adherens junction, Hippo signaling pathway, and regulation of actin cytoskeleton, which were related to the treatment of endometriosis with ELC (FDR <0.25) (**Figure 7C**). ELC may inhibit fibrosis and EMT by regulating the aforementioned pathways. The core genes established in the network may be related to the regulation by ELC treatment. Following MCODE analysis in Cytoscape, we selected three major modules for module network visualization (**Figure 7D**). The core nodes continued to be associated with genes related to actin, cytoskeleton, and fibrosis.

STEM Analysis of Differential Expression Patterns

The results of the gene cluster analysis were statistically significant in profile_14, profile_11, profile_10, and profile_4 ($p < 0.05$) (**Figure 8A**). After the development of the endometriosis model, actin-associated DEGs in the Model_Ecto and ELC_Ecto groups were upregulated; these may be the regulatory genes for the development of the endometriosis model (profile_11 and profile_4). The series test of profile_14 and profile_10 showed that the significant clusters were considered potential profiles that could be affected by treatment with ELC ($p < 0.05$). Several actin-related and microfilament proteins were upregulated in the model group and downregulated after treatment, suggesting that the overall regulation mechanism of ELC treatment in the ectopic endometrium is related to the regulation of actin cytoskeleton (**Figure 8B**).

Protein–Protein Interaction Network

We explored the relationship between the endometriosis-related genes and downregulated genes after treatment with ELC. We constructed network relationships between the core genes of the two groups of DEGs and analyzed the possible network relationships through relevant pathways. We selected the calcium signaling pathway, cGMP–PKG signaling pathway, apelin signaling pathway, HIF-1 signaling pathway, AMPK signaling pathway, GnRH signaling pathway, and associated DEGs to construct the network (**Figure 9A**). The hub downregulated genes closely related to treatment with ELC were as follows: ACTN3, ACTN2, MYOM2, myoglobin, RYR1, MYOG, MYH7, MYOD1, sarcalumenin, myosin light chain kinase 2 (MYLK2), SMYD1, MAP3K7, MAPK12, MYH4, CACNA1S, EEF1A2, and CACNG1.

Identifying the Potential Genes in ELC Treatment

The expression ratios of these DEGs are determined by qPCR (**Figure 9B** and **Supplementary Table S9**). The genes, which are related to tumors, cytoskeleton, and cell potential, have numerous biological functions and may be involved in the development of endometriosis. EEF1A2 encodes an isoform of the alpha subunit of the elongation factor-1 complex and may be critical in the development of ovarian cancer (Worley et al., 2015). Targeting EEF1A2 and plitidepsin to release protein kinase R may trigger the extrinsic pathway of MAPK and nuclear factor-κB–dependent activation, leading to tumor cell death (Losada et al., 2018). RYR1 is the core factor of the calcium signaling pathway. The ryanodine receptor calcium release channel is central to the cytoplasmic calcium signaling pathway (Dulhunty et al., 2018).

DISCUSSION

Endometriosis is a common and difficult gynecological disease. Even now, its exact mechanisms are still not clearly understood, and treatment strategies still need to be further improved. A growing body of evidence showed that the mechanism of Chinese medicine in the treatment of endometriosis could be related to inhibiting

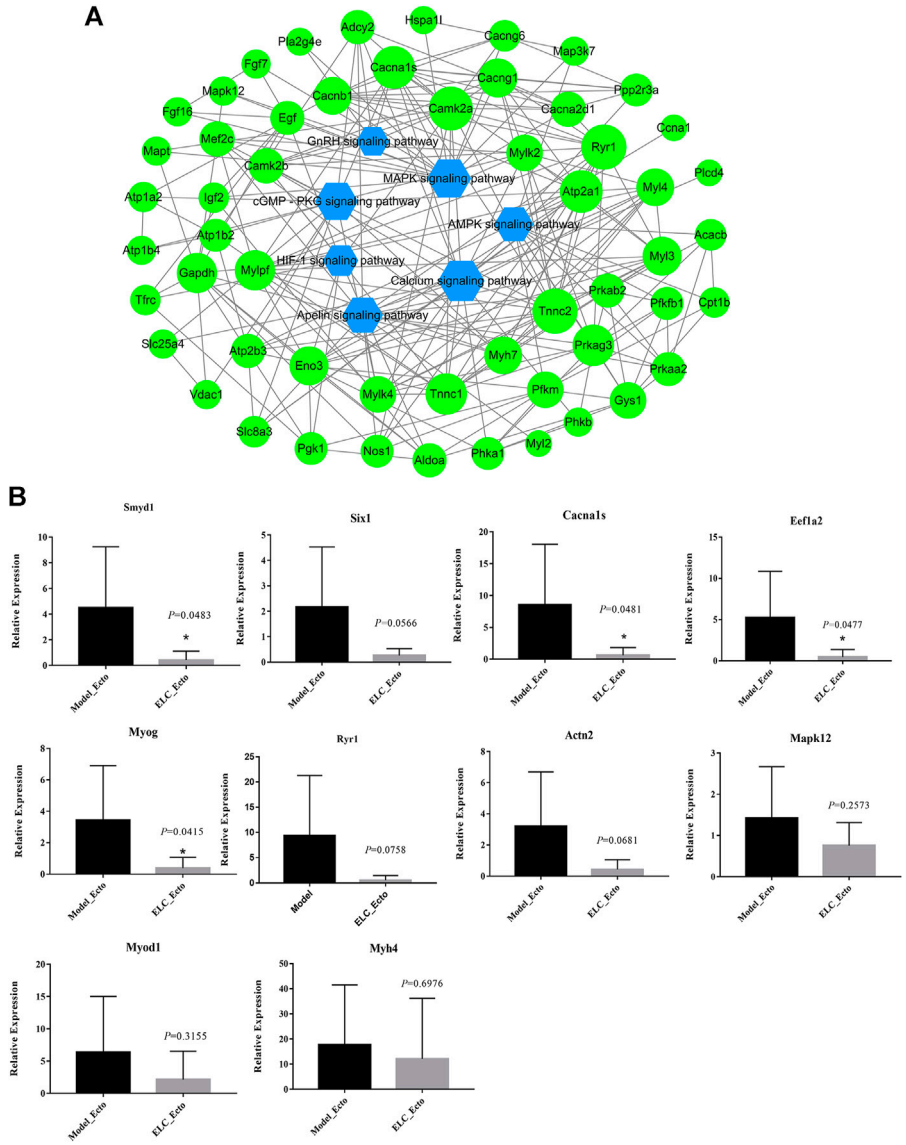

FIGURE 9 | DEG effect of ELeng Capsules in the endometriosis model rats. **(A)** Network of major KEGG pathways and targets of Model_Ecto vs. ELC_Ecto downregulated DEGs. **(B)** Expression of genes in ectopic endometrium tissues in endometriosis rat models detected by qRT-PCR and shown by the expression fold changes. ACTB was used as the internal control. Data are shown as mean ± SD, $^{*}p < 0.05$.

inflammation, enhancing the immune response, regulating angiogenesis-related pathways, and inducing apoptosis (Weisheng et al., 2019). Overall, ELC has the benefits of activating blood circulation, removing blood stasis, and relieving pain. In this study, we investigated the regulated genes of ELC by network pharmacology and RNA-sequencing and found the characteristics of a rat model of endometriosis as well.

The Characteristics of Endometriosis Rat Model

In the present study, we compared the expression of genes in endometriotic lesions in a rat model and the eutopic endometria of normal rats by RNA-sequence. We found that upregulated DEGs between Model_Ecto and Con_Euto were significantly enriched in several pathways, including focal adhesion, ECM–receptor interaction, calcium signaling pathway, and cytokine–cytokine receptor interaction. The results of RNA-seq indicate the EMT and fibrosis in ectopic endometrium lesions in endometriosis. Another study of the rat endometriosis model suggested that osteopontin, Lyn, Vav1, Runx1, and l-selectin play important roles in the pathogenesis of endometriosis based on gene expression profiling (Konno et al., 2007).

EMT and fibroblast-to-myofibroblast transdifferentiation as well as increases in cellular contractility, collagen production, and

smooth muscle metaplasia lead to fibrosis (Zhang et al., 2016; Liu et al., 2018). These pathological changes may be triggered by infection, mechanical damage, and inflammation and induce EMT in the mesothelium (Albertsen and Ward, 2017). Endometriotic tissue is often induced in rodents via transplantation through surgery or intraperitoneal injection of uterine tissue fragments. The time of collection in rat models is 8 weeks after modeling in our study and the lesion has begun to undergo fibrosis. Thus, the model could reflect the fibrosis and EMT characteristics of endometriosis.

Furthermore, tissue remodeling genes in cytoskeleton, smooth muscle contraction, cellular adhesion, tight junctions, and O-glycan biosynthesis were the most significant to lesions (Sohler et al., 2013). The roles of actin and cytoskeleton in the development of endometriosis, as well as the relationship with cell adhesion, invasion, and fibrosis (Zhan et al., 2016), also attracted our attention.

In summary, the rat models of endometriosis could represent the characteristics of endometriosis to a certain extent (Gu et al., 2020) and could contribute to the molecular pathology of peritoneal endometriosis. Although animal models cannot completely recapitulate the human disease process, they could help understand the complex and interactive roles of the endometrial phenotype, the peritoneal microenvironment, and pathogenic genes, which collectively determine an individual's risk of developing endometriosis (Bruner-Tran et al., 2018).

The Potential Mechanism of ELC Treatment

In this study, we had identified 40 compounds in ELC, established the compounds and targets network, and performed further analysis of the potential mechanism involved in treatment with ELC by network pharmacology and RNA-sequence. Interestingly, we found that compounds in ELC could relieve endometriosis-associated pain and regulate the neuroactive ligand–receptor interaction, metabolism of xenobiotics by cytochrome P450, and TLR signaling, VEGF signaling, and calcium signaling pathways. Furthermore, some targets belonged to more than one compound, which suggested that these uniform targets might be the foundation of synergistic therapeutic effect.

The reported efficacy of compounds is related to the mechanism of ELC in endometriosis treatment. Previous phytochemical investigations indicated that the main constituent of *C. phaeocaulis* and *S. stoloniferum* could present anti-tumor and anti-inflammatory activity. Sparstolonin B could serve as a potential therapeutic agent for the treatment of TLR-mediated inflammatory disorders (Yepuri et al., 2019) and also alleviate neuropathic pain by selectively suppressing TLR2 and TLR4 (Jin et al., 2018). β-Elemene, a terpenoid from *C. phaeocaulis*, possesses broad-spectrum anti-tumor activity and is effective against several types of tumors (Bi et al., 2018). Borneol and isoborneol are the monoterpenoid compounds with effective anti-inflammatory and analgesic effects (Wang et al., 2017). Zederone as an analgesic principle could be used to relieve pain in rheumatic disorders in mice (Faiz et al., 2015). The above compounds are suggested to be the effective compounds for the analgesic effect in ELC. Furthermore, *C. phaeocaulis*– and *S. stoloniferum*–medicated serum might suppress TGF-β1–induced EMT in triple-negative breast cancer by decreasing the phosphorylated Smad3 pathway *in vitro* (Yin et al., 2018). *C. phaeocaulis* and its terpenoids (β-elemene, germacrone, curdione) could be the potential anti-cancer drugs (Chen et al., 2021).

S. miltiorrhiza and *P. lactiflora* Pall. are the herbal medicine that has long been used for the treatment of blood stasis and dysmenorrhea. S. *miltiorrhiza* has the effects of promoting blood circulation, eliminating blood stasis, and relieving pain. The active compounds of *S. miltiorrhiza* include tanshinone I, tanshinone IIA, salvianolic acid, and dihydrotanshinone (MEIm et al., 2019). Salvianolic acid A has several pharmacological actions such as anti-thrombosis and anti-fibrosis (Xu et al., 2018). Tanshinone IIA could reduce the VEGF/VEGFR2 pathway and CD146 *in vitro* and *in vivo* and regulate angiogenic function in human umbilical vein endothelial cells (Zhang et al., 2017). Tanshinone IIA could also improve the paw withdrawal threshold to reduce the mechanical hyperalgesia and regulate the DRG renin angiotensin system (RAS) by reducing the protein expression of AGT, REN, ACE, ANGII, and AT2 in DRG neurons (Chen and Gong, 2020). Tanshinone IIA could also inhibit ectopic endometrial stromal cell (EESC) proliferation and migration through the extracellular matrix (ECM)–receptor interaction pathway and estrogen signaling pathway based on iTRAQ analysis (Luo et al., 2021). Rosmarinic acid is a potential natural compound with anti-cancer properties, as demonstrated in various human cancer cell lines (Yesil-Celiktas et al., 2010). Cryptotanshinone could enhance anti-tumor activity by targeting STAT3-related receptors and targeting NF-κB–related pathways (Wu et al., 2020). It also could inhibit the proliferation of primary HESCs and T-HESCs and induce cell cycle arrest of the latter in the G2/M phase *in vitro* (Ferella et al., 2018). *P. lactiflora* Pall. has hematopoietic functions, anti-inflammatory activity, and immunological properties. In *P. lactiflora*, paeoniflorin exerts anti-inflammatory effect through multiple targets (Zhou et al., 2020) and inhibits the plantar incision–induced microglia TLR4/MMP-9/2/IL-1β signaling pathway and suppresses postoperative pain (Fan et al., 2018). Paeoniflorin, as the major compound in Guizhi Fuling prescription, might play a critical role in the anti-endometriosis effect based on the gray correlation analysis strategy (Chen et al., 2020). Furthermore, paeoniflorigenone could induce apoptosis and suppress proliferation (Huang et al., 2017).

Furthermore, the use of *C. aurantium* is mainly focused on improvement of qi stagnation and remission of pain. And *C. aurantium* may possess anti-tumor activity as well. Naringenin could induce apoptosis and endoplasmic reticulum stress through regulation of the MAPK and Akt signal transduction pathways in End1/E6E7 and VK2/E6E7 cells (Park et al., 2017). Limonin could induce apoptosis, thereby affecting the growth of SNU449 and HCT-15 tumor cells (Rahman et al., 2015). Hesperetin could promote cisplatin-induced apoptosis in gastric cancer through upregulating the expression of tensin homolog (PTEN) (He et al., 2020). Naringin and its aglycone naringenin have shown anti-

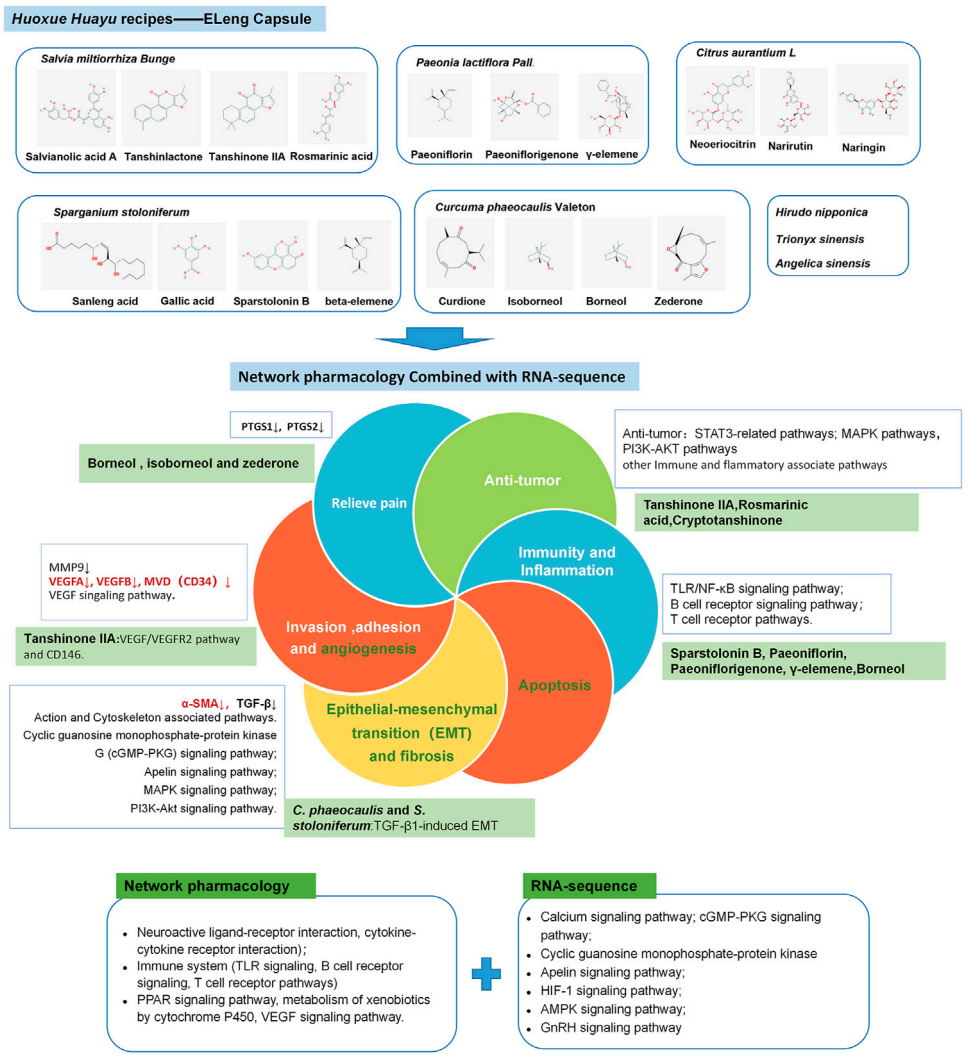

FIGURE 10 | Potential multiple target mechanisms of ELeng Capsule.

carcinogenic activities through cell signal transduction pathways in cancer (JAK–STAT pathway, PI3-kinase/Akt/mTOR pathway, Notch pathway, NF-κB and cox-2 pathway, Wnt pathway, MAPK-ERK pathway, TGF-β pathway) (Memariani et al., 2020). In summary, ELC has the characteristic of multi-target regulation, which may regulate angiogenesis and induce apoptosis based on network pharmacology. Our experimental validation also provides evidence of these effects.

These compounds of ELC may exert new synergistic regulatory effects. In order to further explore the regulatory mechanism of ELC in the transcription level, we further conducted RNA-sequence analysis. The results suggested that the DEGs are related to the cytoskeleton, EMT, fibrosis, muscle fibrosis, and MAPK signaling pathway after ELC treatment, which expanded our understanding of the regulatory effect of ELC. Interestingly, we found that the transcriptome analysis and network pharmacology only partially overlap. And this discrepancy may be related to the comprehensive regulation of a variety of compounds, drug responses of experimental animals, differences in regulation of transcription and translation levels, *etc.* The comprehensive regulation mechanism of herbal medicine still needs to be further studied.

Regulation of cytoskeleton and EMT process is also one of the important approaches for endometriosis treatment. These pathological processes are more closely related to abdominal endometriosis and deep infiltration of endometriosis patients (Ping et al., 2016). In addition, through the GSEA of the ELC_Ecto group and the Model_Ecto group, the results of the KEGG enrichment analysis revealed a relationship with the Notch signaling pathway and the Hippo signaling pathway. The hyperactivation of the ADAM17/Notch signaling pathway could result in an increase in fibrosis, which is associated with deep infiltrating endometriosis (DIE) (Gonzalez-Foruria et al., 2017).

In other enriched KEGG pathways of ELC regulation, apelin, as a ligand of the APJ receptor, has functions in angiogenesis and

cell proliferation and is a vasoactive and regulatory peptide (Luo et al., 2018). And apelin expression in the eutopic and ectopic endometria changes periodically (Ozkan et al., 2013). The DEGs in the apelin signaling pathway are related to muscle contraction, calmodulin binding, and the myosin complex. Moreover, the kinase-associated pathways are associated with endometriosis. A genome-wide association study analysis revealed that multiple pathways, new variants in MAP3K4, and several pathways linked to MAPK are associated with endometriosis (Uimari et al., 2017). The serine/threonine kinase Akt and extracellular regulatory kinase signaling pathways can synergistically support deep endometriosis by enhancing the proliferation and survival of endometrial stromal cells (ESCs) in the *in vitro* fibrotic microenvironment (Matsuzaki and Darcha, 2015). The above-mentioned pathways, as related pathways for endometriosis, may participate in the regulation process of ELC. The potential mechanism of ELC is shown in **Figure 10**.

Based on this research, we also have a new discovery about the mechanism of action of Chinese medicine for removing blood stasis. At present, current research on the role of TCM in promoting blood circulation and removing phlegm is focused on apoptosis, inflammatory immunity, and angiogenesis in endometriosis. These results suggest that endometriosis is associated with EMT and that there are differences in differentially expressed proteins among various syndromes in TCM (Wen et al., 2018). Several natural compounds suggested the treatment of cancer, inflammatory, and fibrosing diseases through the regulation of the EMT process (Avila-Carrasco et al., 2019). The mechanism for the regulation of cytoskeleton and EMT through TCM is lacking. And the mechanism of ELC on EMT and fibrosis needs further investigation in terms of compound, single herb, and prescription optimization.

Limitation

There are several limitations in this study. Firstly, in the HPLC/GC-MS analysis of TCM, only the small molecule compounds derived from plants in ELC were analyzed. The three source animals were not analyzed. Secondly, we obtained our results using rat endometriosis models. Although we have shown that rats/mice are a good animal model for studying endometriosis, they cannot reflect the natural course of the human disease. Further research on cells is warranted to clarify the mechanism involved in the intervention with ELC and study the relationship between the regulation of the cytoskeleton and troponin and the presence of endometriosis.

CONCLUSION

In this study, we have explained the treatment mechanism of ELC using transcriptome analysis and network pharmacology. We hypothesized that ELC may regulate inflammation, immunity, cell adhesion, and cytoskeleton-related genes, influence the process of EMT, and consequently affect the development of lesions. Combined techniques may also offer an efficient method of drug discovery from herbal medicine.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Guangdong Provincial Hospital of Chinese Medicine Committee on the Use of Live Animals for Teaching and Research (SZY2016007).

AUTHOR CONTRIBUTIONS

All authors were responsible for the study concept and design. WZ drafted the paper. JWa helped draft the paper. WZ, JWa, JWu, YH, and TW participated in animal experiments. WZ and JWa performed analysis of transcriptome results and disease model characteristics. WZ participated in network pharmacology analysis. LC and XL designed and supervised the study. WZ and JWa contributed equally to this work. The author(s) 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.2021.674874/full#supplementary-material

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CLINICAL TRIAL
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The Effects of *Elaeagnus angustifolia* L. on the Thyroid-Stimulating Hormone, Dehydroepiandrosterone-Sulfate, Prolactin and Cortisol Levels in Post-Menopausal Women: A Double-Blind, Randomized, and Placebo-Controlled Study

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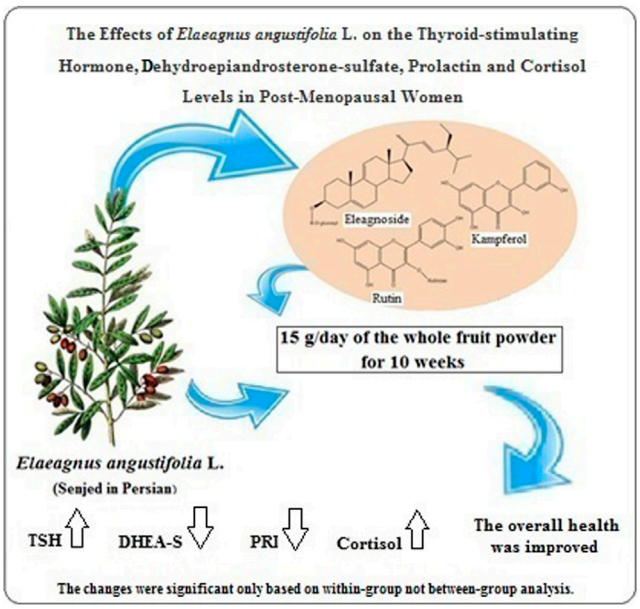
Menopause is the last menstrual period associated with a decline in ovarian steroid secretion and follicular loss. Hormone profile changes during menopause include a decrease in the production of estrogen, dehydroepiandrosterone (DHEA), and prolactin (PRL), and an increase in thyroid-stimulating hormone (TSH) and cortisol. Herbal medicines are considered as alternatives to hormone therapy. The studies on postmenopausal women have shown that *Elaeagnus angustifolia* L. (called "Senjed" in Persian) has some efficacy in improving sex hormone and lipid profiles, joint pain, and cardiovascular function, as the decrease in luteinizing hormone, low-density lipoprotein, and heart rate was significant. The aim of the present study was to evaluate the effects of *E. angustifolia* on TSH, DHEA-S, PRL, and cortisol levels and their ratios in postmenopausal women. It is assumed that the eventual effects of hormones on the brain and other tissues are determined by the balance between interdependent hormones. In the present randomized double-blinded placebo-controlled trial (https://en.irct.ir/search/result?query=IRCT20170227032795N4), fifty-eight postmenopausal women were randomly assigned to one of two medicinal herb (15 g of the whole *E. angustifolia* fruit powder) and placebo (7.5 g isomalt + 7.5 g cornstarch) groups. After 10 weeks of the treatment, the serum levels of TSH, DHEA-S, PRL, cortisol hormones, and their ratios were measured. The increase in the TSH, and cortisol levels, and cortisol/DHEA-S ratio and the decrease in prolactin and DHEA-S and the PRL/TSH, PRL/cortisol, and DHEA-S/TSH ratios after *E. angustifolia* consumption were significant only based on within-group but not on the between-group analysis. Based on between-group analyses, the changes in the

hormone profile were not significant in the placebo group. According to Iranian tradition and folklore, *E. angustifolia* fruit is a symbol of female fertility. Therefore, its consumption is highly recommended to maintain health in the elderly, especially women. However, the observed outcomes about the effect of *E. angustifolia* on menopause were not completely in line with the Iranian folklore. *E. angustifolia* consumption did not significantly affect the hormone profile and ratios at the end of the ten-week trial, possibly due to the small sample size, short time, and the fact that our participants were postmenopausal women.

Keywords: menopause, *Elaeagnus angustifolia* L., TSH, DHEA-S, prolactin, cortisol, traditional medicine, Iran



GRAPHICAL ABSTRACT | The effects of *Elaeagnus angustifolia* L. on the thyroid-stimulating hormone, dehydroepiandrosterone-sulfate, prolactin and cortisol levels in post-menopausal women. The changes were significant only based on within-group not between group analysis.

INTRODUCTION

Menopause is one of the inevitable components of aging, including permanent menstrual cycles and loss of ovarian function. During this period, postmenopausal women experience decreased estrogen production in the ovary. Menopause occurs after 12 consecutive months of amenorrhea, indicating the end of fertility. Menopause typically occurs in midlife women, during their late 40s or early 50s, signaling the end of the fertile phase of a woman's life (Davis et al., 2015).

Menopause symptoms can be divided into several categories: symptoms of the central nervous system, including hot flashes, sleep disorders, anxiety, depression, and migraines. Symptoms of the urogenital system are vaginal dryness and sexual dysfunction, such as decreased libido and dyspareunia. Postmenopausal women may also experience symptoms such as weight gain, osteoporosis, and joint pain (Monteleone et al., 2018; Wilhelms et al., 2019).

Hormones' profile changes during menopause include the decrease in the production of dehydroepiandrosterone (DHEA) (Labrie et al., 2017) and prolactin (PRL) (Kwon et al., 2014) and an increase in thyroid-stimulating hormone (TSH, also known as thyrotropin) and cortisol (Pearce, 2007; Kalleinen et al., 2008). In females, estrogen is mostly synthesized by ovarian follicles (Merchenthaler, 2018). During menopause, ovarian function decreases, leading to decreased estrogen levels. DHEA is one of the main precursors of androgen that turns into estrogen and testosterone. DHEA-S, the sulfated form of DHEA, is the most abundant steroid hormone in serum (Scheffers et al., 2015). Estrogen stimulates the transcription of the PRL gene (Binart, 2017) and increases the serum levels of thyroxine-binding globulin (TBG), thereby reducing TSH. Consequently, in postmenopausal women, estrogen and TBG levels decrease; thus, TSH increases (Patisaul and Jefferson, 2010). It is assumed that the eventual effects of hormones on the brain and other tissues are determined by the balance between interdependent hormones (Maninger et al., 2009). In other words, hormone ratios are considered a straightforward way to analyze the effect of two independent hormones simultaneously (Sollberger et al., 2016). The changes in some hormone ratios in some diseases have

been studied. For instance, some studies have shown that the PRL/cortisol ratio increased in autoimmune diseases such as systemic lupus erythematosus (Koeller et al., 2004), rheumatoid arthritis (Zoli et al., 2002), and Hashimoto's disease (Legakis et al., 2001). However, to the best of our knowledge, few or no studies have been done on the ratios of TSH, DHEA-S, PRL, and cortisol in postmenopausal women.

There are various treatments for menopausal and postmenopausal women, including hormone replacement therapy (HRT), drug and non-drug therapies. Hormone replacement therapy includes the therapy with estrogen alone or estrogen combined with progesterone. Selective serotonin reuptake inhibitors (e.g., citalopram, paroxetine, and sertraline) and selective serotonin-norepinephrine reuptake inhibitors (e.g., venlafaxine and desvenlafaxine) are common types of postmenopausal drug therapies (Reviewed in Akhlaghi et al., 2015 and Yasui et al., 2003). Exercise therapy, meditation, yoga, aromatherapy, acupuncture, and behavior therapy are examples of non-drug therapies for menopause (Reviewed in Mintziori G et al., 2015). However, herbs have been a popular and well-known method of treating menopausal symptoms for centuries (Eden, J. A. 2012). Phytoestrogens are nonsteroidal, plant-derived compounds that are structurally or functionally similar to mammalian estrogens (Patisaul and Jefferson, 2010). Phytoestrogens are polyphenolic compounds and have antioxidant activity (Rodríguez-Landa et al., 2018). Phytoestrogens selectively bind to estrogen receptors, ERα and ERβ, like estrogens and are known to be effective in treating menopausal symptoms, such as osteoporosis, hot flashes and vasomotor symptoms, and anxiety and depression symptoms (Sirotkin and Harrath, 2014; Rodríguez-Landa et al., 2018).

E. angustifolia L. (Russian olive), known as Senjed in Persian, has high antioxidant activity and flavonoids in fruits (peel, flesh, and seeds) (Faramarz et al., 2015; Hamidpour et al., 2017). Senjed is used in Iranian traditional medicine (ITM) in the treatment of abdominal distension, diarrhea, jaundice and hemorrhoids, and for the heart and lungs, knee osteoarthritis, and joint pain (Mahboubi, 2018; Emaminia et al., 2020; Shabani et al., 2020).

In folkloric Iranian traditional medicine, *E. angustifolia* is recommended for the elimination of side effects of menopause. Desirable effects of *E. angustifolia* have been investigated in some cases such as wound repairing (Moezzi et al., 2009), the treatment of knee osteoarthritis (Ali Shiri et al., 2007), analgesic and anti-inflammatory effects in mice (Hosseinzadeh et al., 2003; Karimi et al., 2010), and symptomatic oral lichen planus (Taheri et al., 2010). The present study is a continuation of the previous works by Emaminia et al. (2020) and Shabani et al. (2020) and aims to investigate the effect of ripe *E. angustifolia* whole fruit on the serum level of TSH, DHEA-S, PRL, cortisol, and their ratios in postmenopausal women.

MATERIALS AND METHODS

Elaeagnus angustifolia L. Preparation

E. angustifolia fruits were products of Damghan's gardens (Semnan province, Iran) in October 2017. The *E. angustifolia* specimen was kept in the herbarium of Damghan university [Voucher number, Amirahmadi:1842 (DU000584)]. After the authors of this paper, Dr. Atefe Amirahmadi (Ph.D. of Plant Biosystematics, School of Biology, Damghan University) and Dr. Anna Abodolshahi (Ph.D. of Food science and technology (Food Safety Research Center, Semnan, Iran), confirmed the quality and health of the fruits, the whole fruit powder was prepared as previously described (Fantasia and Sutherland, 2014; Nikniaz et al., 2015). Isomalt from Puyakabak Manufacturing and Trading Company (Batch No 1604112, Tehran, Iran) and corn starch from the Bijan Pharmacy (Tehran, Iran) were also prepared with food grade. The powders were further sealed into packs separately, each pack containing 15 g of *E. angustifolia* whole fruit and isomalt + cornstarch powder (1:1 ratio).

Herbal medicine and placebo packaging were coordinated by a person who did not play a role in this project in two packages with different color schemes (red and blue, respectively). Supervisors and project students and those involved in clinical trials were unaware of the nature of these packages until the end of designing, receiving the latest test results, and analyzing the data.

The blue (placebo) and red (herbal medicine) packages were stored in a refrigerator for food before delivery to the participants, and they were suggested to store their packages in the refrigerator before consumption. **Figure 1** shows the flowchart of participant's recruitment and retention.

Study Design

This study follows the CONSORT 2010 checklist of clinical trials (Schulz KF et al., 2010) and is a part of a double-blind, randomized placebo-controlled clinical trial with the ethical code Abzums.Rec.1396.162 and registration code IRCT20170227032795N4 in the clinical trial system. Among the postmenopausal women referring to the gynecology clinic of the Kamali hospital (Karaj, Alborz, Iran), 60 postmenopausal women were invited after gynecologist confirmation, personal interview, explanation of the purpose and method of the study, and receiving informed written consent.

Subject Selection

Inclusion Criteria

The postmenopausal women were invited to participate in the study based on inclusion and exclusion criteria. The inclusion criteria included menopausal women aged 40–70 years and serum cholesterol levels between 200 and 300 mg/ml. In the initial assessment, blood pressure, heart rate, total cholesterol, and FSH levels were evaluated to confirm being menopause. Women who were included in the study did not have a risk factor for not using medication to reduce their lipid level or did not have any history of receiving hormone injections in the last 6 months, and also had no history of daily intake or allergy to *E. angustifolia* (Emaminia et al., 2020; Shabani et al., 2020).

Exclusion Criteria

Exclusion criteria included metabolic disorders such as diabetes, cardiovascular and renal diseases, consuming alcohol, and psychiatric drugs, smoking cigarette, and hookah (Emaminia et al., 2020; Shabani et al., 2020).

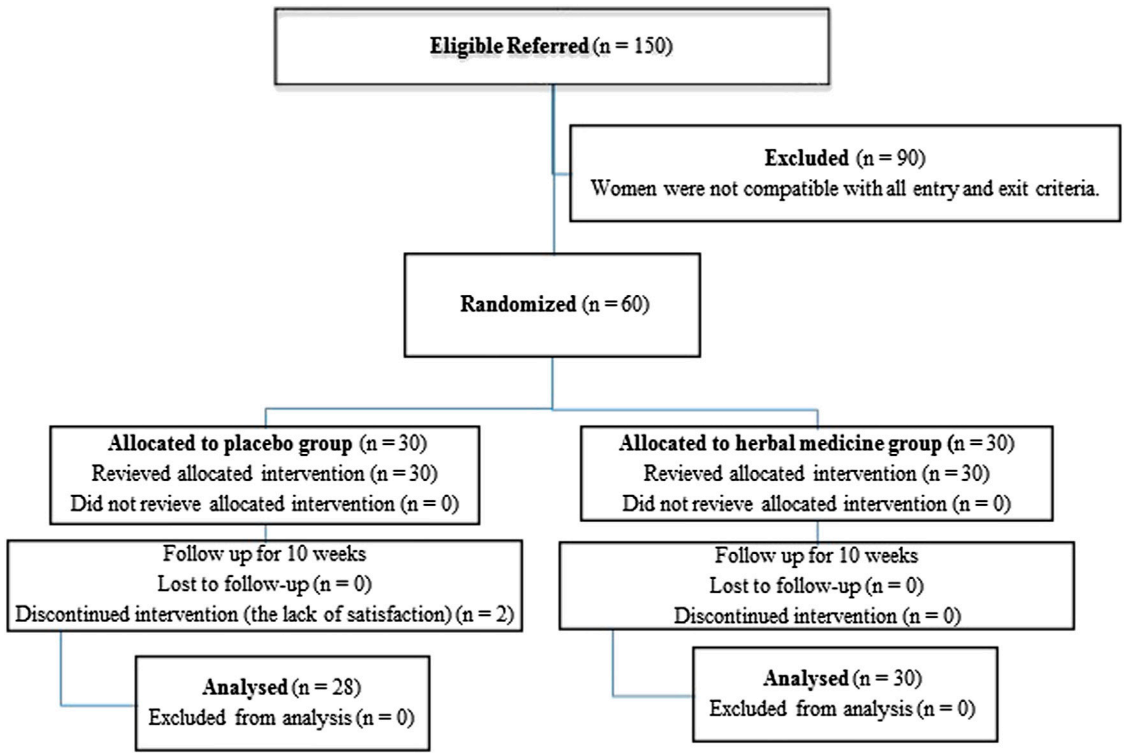


FIGURE 1 | Flow chart for the participants' enrollment, randomization, and retention. Participants in the herbal medicine and placebo groups received 15 g/day of *E. angustifolia* L. fruit powder and (7.5 g corn starch + 7.5 g isomalt)/day, respectively.

Randomization and Experimental Groups

A simple random selection method was used for assigning the postmenopausal women into two control and intervention groups, each with 30 participants. A person who did not participate in this trial did this randomization. The numbers 1–60 were written on paper of the same color and shape. The paper was put in a pot after folding. Then, the bowl components were stirred. The paper was taken randomly by a person who did not have a role in this project. The first 30 numbers that came out of the bowl were intended for people who were supposed to be in the blue group. The 30 numbers remaining in the bowl were considered as the red group. Each study group took 15 g of herbal medicine (whole *E. angustifolia* fruit powder) or placebo (a combination of 7.5 g of corn starch and 7.5 g of isomalt) equally for 10 weeks every day. The dosage and duration of Senjed and placebo consumption were selected based on studies on the effect of this fruit on osteoarthritis and the lipid profile of obese women (Ali Shiri et al., 2007; Moezzi et al., 2009), and the ineffectiveness of cornstarch and isomalt on blood glucose and lipid profiles (Karimi et al., 2010; Ebrahimi et al., 2014; Nikniaz et al., 2015).

Participants were recommended to eat the packages' contents after breakfast and with milk, if possible (Nikniaz et al., 2016). Two people involved in this clinical trial, Farzaneh Emaminia and Mahzad Shabani, were in touch with participants twice a week regularly to record the participants' general condition, satisfaction, or dissatisfaction.

TABLE 1 | Calculation of the sample size based on the difference in change (pre-post) of the levels of thyroid-stimulating hormone (TSH), dehydroepiandrosterone-sulfate (DHEA-S), prolactin (PRL).

Parameter	SD	Difference	Power	Sample size
TSH	2.7	2	80	29
DHEA-S	40	30	80	28
PRL	180	150	80	23

Data Collection and Sampling

For data collection, fasting blood samples were taken, and an interview questionnaire was filled, which was done in Iranzamin Lab (Karaj) in two steps, including at the beginning and the end of the trial after 10 weeks of consumption of herbal medicine/placebo and the ending stage.

Hormone Profile

Blood analysis was done for four hormones, including TSH, DHEA-S, PRL, and cortisol. To measure hormones, the serum sample was prepared from 3 ml of patients' fasting blood samples. Enzyme-Linked Immunosorbent Assay (ELISA) and commercial kits of Bio Karpira Company (Iran) were used to measure the PRL and TSH hormones. An automated

TABLE 2 | Comparison of the baseline characteristics of participants between two study groups.

Variable	**Category**	**Groups**		**Total (*n* = 58)**	***p*-value**
		Herbal medicine (*n* = 30)	**Placebo (*n* = 28)**		
Age	Numerical	56.63 ± 5.43	54.07 ± 6.90	55.39 ± 6.26	0.121[t]

Variables	**Category**	**Total**	**Groups**				**Total**		***p*-value***
			Herbal medicine		**Placebo**				
			Num	**Percentage**	**Num**	**Percentage**	**Num**	**Percentage**	
Education	Uneducated	1	4	13.3%	2	7.1%	6	10.3%	0.677[tt]
	1–6 years	2	16	53.3%	17	60.7%	33	56.9%	
	6–12 years	3	8	26.7%	5	17.9%	13	22.4%	
	12+	4	2	6.7%	4	14.3%	6	10.3%	
Job	Yes	1	2	6.7%	4	14.3%	6	10.3%	0.415[**]
	No	2	28	93.3%	24	85.7%	52	89.7%	
Type of delivery	Natural childbirth	1	23	76.7%	23	60.7%	40	69.0%	0.265[tt]
	Cesarean delivery	2	2	6.7%	7	25.0%	9	15.5%	
	Both	3	5	16.7%	2	7.1%	7	12.1%	
	None of them	4	0	0.0%	2	7.1%	2	3.4%	
Lactation period	No	1	0	0.0%	3	10.7%	3	5.2%	0.466[tt]
	Less than 6 months	2	2	6.7%	3	10.7%	5	8.6%	
	Less than 1 year	3	3	10.0%	0	0.0%	3	5.2%	
	More than 1 year	4	25	83.3%	22	78.6%	47	81.0%	
Exercise	No	1	14	46.7%	13	46.4%	27	46.6%	0.280[tt]
	Every week	2	6	20.0%	1	3.6%	7	12.1%	
	Twice in week	3	6	20.0%	3	10.7%	9	15.5%	
	Every day	4	4	13.3%	11	39.3%	15	25.9%	
Consumption of any type of booster pill	Yes	1	17	56.7%	12	42.9%	29	50.0%	0.293*
	No	2	13	43.3%	16	57.1%	29	50.0%	
Harmful habits	No	1	27	90.0%	26	92.9%	53	91.4%	0.701[tt]
	Cigarette	2	1	3.3%	0	0.0%	1	1.7%	
	Hookah	3	1	3.3%	2	7.1%	3	5.2%	
	Alcoholic drinks	4	1	3.3%	0	0.0%	1	1.7%	
Misadventure event over 5 years ago	Yes	1	17	56.7%	18	64.3%	35	60.3%	0.553*
	No	2	13	43.3%	10	35.7%	23	39.7%	

N, the number of participants; t: t *test, tt: Mann Whitney test, *, Chi-Square test, **, Fisher's test. A* p*-value < 0.05 was considered statistically significant.*

TABLE 3 | Comparison of the levels of thyroid-stimulating hormone (TSH), dehydroepiandrosterone-sulfate (DHEA-S), prolactin (PRL), and cortisol and their ratios between the two study groups.

a) Parameters with non-normal distribution. Data are shown as median ± interquartile range (IQR)

Variables (unite)	Time	Herbal medicine groups (*n* = 30)	Placebo groups (*n* = 28)	*p-value*
		Median ± IQR	Median ± IQR	
TSH (μIU/ml)	Pre-treatment	2.51 ± 2.12	2.12 ± 1.77	0.597[tt]
	Post-treatment	2.93 ± 2.90	2.61 ± 1.55	0.780[tt]
	Change score	0.63 ± 2.33	0.04 ± 2.62	0.857[tt]
	Within groups	**0.007[w]**	0.374[w]	—
DHEA-S (μg/ml)	Pre-treatment	52.30 ± 42.20	36.20 ± 37.72	0.486[tt]
	Post-treatment	17.85 ± 33.85	19.50 ± 36.41	0.950[tt]
	Change score	−32.26 ± 48.49	−14.73 ± 38.92	0.857[tt]
	Within groups	**<0.001[w]**	**0.015[w]**	—
PRL (mIU/L)	Pre-treatment	193.10 ± 98.33	191.41 ± 114.66	0.656[tt]
	Post-treatment	161.50 ± 145.06	159.76 ± 221.52	0.905[tt]
	Change score	−53.00 ± 177.23	−68.55 ± 187.95	0.938[tt]
	Within groups	**0.015[w]**	**0.063[w]**	—
PRL/TSH	Pre-treatment	73.21 ± 105.38	99.74 ± 85.79	0.656[tt]
	Post-treatment	29.17 ± 61.02	43.56 ± 100.54	0.905[tt]
	Change score	−31.11 ± 105.46	−41.14 ± 114.23	0.938[tt]
	Within groups	**0.007[w]**	**0.015[w]**	—
PRL/DHEA-S	Pre-treatment	3.99 ± 5.16	4.89 ± 5.76	0.597[tt]
	Post-treatment	7.62 ± 180.80	7.79 ± 1,323.56	0.905[tt]
	Change score	2.77 ± 182.55	3.10 ± 1,324.24	0.938[tt]
	Within groups	0.070[w]	0.084[w]	—
PRL/Cortisol	Pre-treatment	31.62 ± 26.77	18.76 ± 13.10	0.155[tt]
	Post-treatment	12.67 ± 14.79	9.91 ± 15.58	0.780[tt]
	Change score	−15.16 ± 30.60	−8.21 ± 26.64	0.857[tt]
	Within groups	**<0.001[w]**	**0.034[w]**	—
Cortisol/DHEA-S	Pre-treatment	0.15 ± 0.24	0.29 ± 0.23	0.145[tt]
	Post-treatment	0.56 ± 20.31	0.63.±95.29	0.605[tt]
	Change score	0.35 ± 20.48	0.32 ± 95.41	0.938[tt]
	Within groups	**<0.001[w]**	**0.015[w]**	—
DHEA-S/TSH	Pre-treatment	19.77 ± 32.16	18.76 ± 23.31	0785[tt]
	Post-treatment	5.15 ± 9.21	6.77 ± 15.25	0.905[tt]
	Change score	−13.30 ± 28.60	−7.73 ± 22.95	0.857[tt]
	Within groups	**<0.001[w]**	**0.015[w]**	—
Cortisol/TSH	Pre-treatment	2.91 ± 2.93	4.33 ± 6.02	0.153[tt]
	Post-treatment	3.49 ± 3.70	5.06 ± 5.61	0.780[tt]
	Change score	0.18 ± 2.87	1.06 ± 6.37	0.938[tt]
	Within groups	0.586[w]	0.767[w]	—

b) Parameters with the normal distribution. Data are shown as mean ± standard deviation

Variables (unite)	Time	Herbal medicine groups (*n* = 30)	Placebo groups (*n* = 28)	Mean difference	95% Confidence interval of the difference		*p-value*
		Mean ± SD	Mean ± SD		Lower	Upper	
Cortisol (μg/dl)	Pre-treatment	7.82 ± 3.70	10.77 ± 5.76	−2.957	−5.489	−0.425	0.145[t]
	Post-treatment	11.42 ± 5.12	13.28 ± 6.06	−1.862	−4.810	1.086	0.780
	Change score	3.60 ± 6.02	2.51 ± 7.99	1.095	−2.613	4.804	0.938
	Within groups	**0.006[p]**	0.135[p]	—	—	—	—

Between-group p*-values show the significance of differences in the studied parameters between two study groups before and after the trial period. Within-group* p*-values show the significance of differences in the studied parameters of each study group before and after the trial period. Change score* p*-values compare between* p*-values of each studied parameter. t, T-test; tt, Mann-Whitney test; w, Wilcoxon test;* p*, Pair t-test. Bold figures show that the difference is significant (*p *< 0.05).*

Immulite 2000 (Siemens, United States) working on the Chemiluminescence technique was used to measure DHEA-S and cortisol hormones.

Statistical Analysis

Statistical analysis was performed using SPSS software version 25. In the initial analysis, the normality of data distribution was assessed with the Shapiro-Wilk test. The groups were compared with Independent Samples *t*-test if the parameter distribution was normal, and for the parameter with abnormal distribution, the Mann-Whitney test was used. The within-group comparisons were performed using a paired sample *t*-test or Wilcoxon test for data with the normal or abnormal distribution. The *p*-values of within-group analyses indicate the importance of the difference in the studied parameters in each research group of herbal medicine or placebo before and after the trial period. Between-group *p*-values indicate the importance of the difference in the studied parameters between herbal medicine and placebo groups before and after the trial. Change score *p*-value was used to compare between-group *p*-values of each studied parameter. A p-value < 0.05 was considered statistically significant. Since we had several outcomes, all *p*-values were adjusted using the Benjamini-Hochberg correction method to control the multiple comparisons problem (Benjamini et al., 1995).

Based on the central limit theorem (CLT) (Kwak and Kim, 2017), the sample means that the probability distribution of the studied characteristics in a sample size of 30 is close to the normal probability distribution. The sample size was also calculated based on the difference in (pre-post) change of TSH, DHEA-S, and PRL that have been considered main outcomes. Among these items, the change in TSH provided the largest sample. The final sample size was calculated based on this parameter. To have a power of 80% (type II error of 20%) and type I error of 5% to detect a difference of changes as big as 2, when the standard deviation of TSH was believed to be 2.7 in each group, a sample size of 29 was needed (**Table 1**). We included 30 samples in each group to compensate for the probable missing.

RESULTS

In this clinical trial, the parameters studied in postmenopausal women in both herbal medicine and placebo groups were measured before treatment and at the end of the trial. In the placebo group, two menopausal women refused to continue their cooperation. Therefore, data were reported for 58 postmenopausal women in two groups after 10 weeks of treatment. In both herbal medicine (*E. angustifolia*) and placebo (isomalt and cornstarch) groups, the women had a normal distribution based on age, as previously reported (Emaminia et al., 2020; Shabani et al., 2020). As shown in **Table 2**, all 58 participants' average age was 55.39 years; which was 56.63 years in the herbal medicine group (n = 30) and 54.07 years in the placebo group (n = 28), respectively. The difference between the two study groups was not significant in this regard (p = 0.121). There was no statistically significant difference between the two herbal medicine and placebo groups in terms of baseline characteristics such as education, job, type of delivery, lactation period, exercise, consumption of any booster pill, harmful habits, and misadventure events in the past 5 years (**Table 2**).

Table 3 shows the results of the effects of herbal medicine and placebo treatment on the studied hormones. Considering within-group *p*-values, the TSH amount showed a significant increase in the herbal medicine (p = 0.007) group after 10 weeks of treatment, but this change in the placebo group was not significant. By assessing the changes between the two study groups, the changes in the amount of TSH were not significant before and after treatment (change score *p*-value = 0.857).

The DHEA-S amount showed a significant decrease in both herbal medicine and placebo groups after ten-week treatment (within-group *p*-values < 0.001 and = 0.015, respectively). However, this hormone's changes were not significant before and after treatment between the two study groups (change score *p*-value = 0.857).

The herbal medicine group's increase in cortisol levels was significant, but not in the placebo group (within-group *p*-values = 0.006 and 0.135, respectively). The cortisol changes were not significant between the herbal medicine and placebo groups at the end of the trial (change score *p*-value = 0.938).

The decrease in PRL levels was significant only in herbal medicine group but not in placebo group after ten-week treatment (within-group *p*-values = 0.015 and 0.063, respectively). However, the hormone level changes were not significant between the two study groups at the end of treatments (change score *p*-value = 0.938).

For hormone ratios, considering within-group *p*-values, the PRL to TSH ratio showed a significant decrease in both herbal medicine (p = 0.007) and placebo (p = 0.015) groups at the end of the trial. However, assessing the change score *p*-values showed that the decrease in this ratio was not significant between the two study groups (p = 0.938).

The increase in the ratio of PRL to DHEA-S was not significant in herbal medicine and placebo groups (within-group *p*-values = 0.070 and 0.084, respectively). The change in this ratio was not significant between the two study groups (change score *p*-value = 0.938) after ten-week treatment.

In within-group *p*-value analysis, PRL to cortisol ratio showed a significant decrease in both herbal medicine (p < 0.001) and placebo (p = 0.034) groups at the end of the trial. However, the decrease in this ratio was not significant between the two study groups (change score *p*-value = 0.857).

The increase in the cortisol to DHEA-S ratio was significant in both herbal medicine and placebo groups after ten-week treatment (within-group *p*-values <0.001 and 0.015, respectively). The changes in this ratio were not significant between the two study groups (change score *p*-value = 0.938).

The decrease in the ratio of DHEA-S to TSH was significant in the herbal medicine and placebo groups (within-group *p*-values < 0.001 and 0.015, respectively), but not between two study groups (change score *p*-value = 0.857).

Considering within-group *p*-values, cortisol to TSH ratio did not change significantly in both herbal medicine (p = 0.586) and

placebo ($p = 0.767$) groups. Also, the change in this ratio was not significant between the two study groups at the end of the trial ($p = 0.938$).

DISCUSSION

Menopause is a stage in women's life that affects their quality of life. To treat menopausal symptoms, lifestyle changes or hormonal and non-hormonal therapies are suggested, each with its benefits and risks. Hormone therapy can lead to certain diseases, such as cancer (Fantasia and Sutherland, 2014). Accordingly, women turn to complementary and alternative therapies (Dietz et al., 2016). In Iranian folklore medicine, oral intake of *E. angustifolia* fruit powder is considered useful for osteoarthritis, enuresis, liver, stomach, intestine, heart and lungs wounds and asthma (Reviewed in Mahboubi, 2018). Furthermore, according to Iranian tradition and folklore, *E. angustifolia* fruit symbolizes love, happiness, wisdom, and female fertility. The exocarp, endocarp, and seed of *E. angustifolia* fruit are recommended to strengthen skin, muscle, and skeletal function, respectively. Accordingly, its consumption is highly recommended to maintain health in elderly, especially women. In the present study and the continuation of our previous studies (Emaminia et al., 2020; Shabani et al., 2020), the effect of *E. angustifolia* on some serum hormones was studied in postmenopausal women for the first time.

In our previous study, i.e., the study by Emaminia et al. (2020), we showed that the increase in FSH and estradiol levels, and the decrease in progesterone levels were not significant after *E. angustifolia* consumption. However, the LH level decreased significantly. Moreover, the improvement in joint pain was significant in postmenopausal participants. In our another study, i.e., the study by Shabani et al. (2020), we showed that a ten-week treatment with *E. angustifolia* significantly lowered LDL-C level and heart rate in postmenopausal participants without significant change in the glycemic profile.

According to between-group analyses, in the current study, after 10 weeks of treatment with *E. angustifolia*, no significant changes were observed in the studied parameters. However, in the within-group analyses, the levels of TSH, cortisol, and cortisol/DHEA-S increased significantly after taking *E. angustifolia*. At the same time, there was a significant decrease in the levels of DHEA-S, PRL, PRL/TSH, PRL/cortisol, and DHEA-S/TSH at the end of the trial. The changes in the ratios of PRL/DHEA-S and cortisol/TSH were not significant.

High TSH levels can have adverse effects on cardiac function. Decreased thyroid hormones (T3 and T4) can lead to increased atherogenic markers total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) (Wanjia et al., 2012). TSH level may increase with age (Bremner et al., 2012; Leng and Razvi, 2019). Due to the low individuality index of TSH, it is not possible to determine an accurate TSH upper limit at the individual level from population data. With the current upper limit of the serum TSH reference range in the elderly, overdiagnosis of hypothyroidism is possible (Leng and Razvi, 2019). In addition to levels of thyroid hormones, particularly free T4, other studies are needed to define an age-specific TSH reference range as part of clinical practice (Bremner et al., 2012; Leng and Razvi, 2019). In the current study, the levels of thyroid hormones were not measured. However, based on our previous study, it can be concluded that the insignificant 16.73% increase in the TSH level from 2.51 μIU/ml before treatment to 2.93 μIU/ml at the end of the trial had no negative impact on the lipid profile and cardiovascular function in postmenopausal participants. This is because treatment with *E. angustifolia* had no significant effect on TC and triglyceride (TG) levels, systolic and diastolic blood pressure, but significantly decreased heart rate and LDL-C (Shabani et al., 2020).

In the current study, the increase in cortisol levels and the decrease in DHEA-S levels were significant only in within-group but not between-group comparisons. The cortisol/DHEA-S ratio also increased significantly in the herbal medicine and placebo groups only in within-group but not between-group analyses (**Table 3**).

DHEA is a multifunctional hormone with immune enhancing, breast-cancer preventive, and antiaging effects (Yen et al., 1998; Ferrari et al., 2001a; b). DHEA is a precursor to androgens and estrogen and its conversion into estrone is the major source of estrogen in postmenopausal women (Rodwell et al., 2018). DHEA-S is the most abundant steroid hormone in serum (Ruan et al., 2017) and can modulate lipid and glucose metabolism (Yen and Laughlin, 1998). As DHEA-S levels decrease, pain threshold and tolerance decrease (De Abreu Freitas., 2012). However, despite these changes, postmenopausal women reported a reduction in pain after *E. angustifolia* consumption (Emaminia et al., 2020).

In our previous studies, i.e., the study by Emaminia et al. (2020), we showed that the estradiol (E2) and testosterone (T) levels increased insignificantly by 28.29 and 59.59%, respectively, after treatment with *E. angustifolia*. (Emaminia et al., 2020). Furthermore, a calculation using the current data and the result obtained from the study by Emaminia et al. (2020) showed a decrease in the ratios of DHEA-S/E2 (65.10%) and DHEA-S/P (47.35%), and DHEA-S/T (54.53%), and an increase in E2/T (26.45%) and E2/P (13.81%) ratios. In the current study, the DHEA levels were not measured. However, the 65.86% obvious reduction in DHEA-S in the herbal medicine group could be due to the conversion of DHEA to progesterone, testosterone, estradiol, and oxygenated DHEA metabolites rather than conversion into DHEA-S in the peripheral tissues (El Kihel et al., 2012; Rodwell et al., 2018) which requires the assessment of key enzymes in these pathways as well as other DHEA metabolites.

In our previous study, a 45.98% decrease in progesterone hormone (P) level was seen after treatment with herbal medicine which was significant in within-group ($p = 0.049$), but not in between-group (change score p-value = 0.273) analyses (Emaminia et al., 2020). One of the reasons for the increase in cortisol in the current study might be the conversion of progesterone into cortisol, which requires the assessment of related enzymes in this pathway. It is noteworthy to mention that the level of TC did not change significantly after ten-week treatment with *E. angustifolia* as shown in our previous study, i.e., the study by Shabani et al. (2020).

Cortisol increases lipolysis, which actually breaks down triglycerides into glycerol and fatty acid. It also reduces glucose consumption and insulin sensitivity (PNandhini et al., 2019). In the previous study, TG levels decreased after *E. angustifolia* treatment, although this change was not significant (change score *p*-values = 0.065). But, fasting blood glucose (within-groups *p*-value = 0.002, change score *p*-value = 0.303) and insulin (change score *p*-value = 0.04) increased; however, both remained within normal range (Shabani et al., 2020). These glycemic profile changes due to herbal medicine consumption can be related to the effect of herbal medicine on cortisol. Understanding the exact mechanism of these changes in the hormonal and glycemic profile of postmenopausal women requires further studies.

The relationship between cortisol and DHEA-S is usually considered in tandem. These two critical adrenal steroids have opposite effects on the central nervous system (Yen and Laughlin, 1998; Ferrari, et al., 2001a; b). The neurosteroid DHEA-S has anti-glucocorticoid, antihypertensive, anti-inflammatory, and neuroprotective effects (Maninger et al., 2009). Although cortisol secretion is generally well maintained with age, DHEA-S levels progressively decrease with aging. The molar ratio between cortisol and DHEA-S also increases with age (Yen and Laughlin, 1998; Ferrari et al., 2001b).

The treatment with both *E. angustifolia* and placebo increased the cortisol/DHEA-S ratio which was only significant in the within-group but not between-group analyses (**Table 3**). High serum cortisol/DHEA-S ratio (≥0.2 μg/dl) is a risk factor for sarcopenia in elderly diabetic patients. Chronic stress often increases cortisol secretion and decreases DHEA-S secretion (Phillips et al., 2010). Therefore, the ratio of DHEA-S/cortisol decreases (Yanagita et al., 2019). A decrease in DHEA-S levels and an increase in cortisol/DHEA-S ratio have been suggested as a possible mechanism in cancer-related mortality (Phillips et al., 2010). An increased cortisol/DHEA-S ratio was also shown in schizophrenia (Markopoulou et al., 2009) and treatment-resistant depression (Ritsner, 2009). DHEA and DHEA-S levels increase significantly in response to acute psychosocial stress along with significantly elevated cortisol, heart rate, and systolic and diastolic blood pressure in both men and women. However, the ability to increase these levels during acute psychosocial stress decreases with age. The molar ratio between cortisol and DHEA-S increases with stress (Lennartsson et al., 2012). The postmenopausal women in the present study did not suffer from specific metabolic diseases such as diabetes or neurodegenerative diseases such as schizophrenia. Furthermore, we showed previously that ten-week consumption of *E. angustifolia* reduced significantly heart rate without any significant change in blood pressure (Shabani et al., 2020). Accordingly, it can be concluded that the decreased DHEA-S level and increased cortisol/DHEA-S ratio, which were not significant, did not negatively affect on the participants and did not indicate an increased stress in them.

Postmenopausal women are at greater risk of developing atherosclerosis and hypertension. Evidence has shown that high levels of PRL may accelerate vascular aging in menopause and play a key role in increasing the prevalence of hypertension after menopausal transition (Amirzadegan et al., 2019). There has also been a relationship between high plasma PRL and cardiovascular mortality (Byberg et al., 2019). Anxiety is also associated with high levels of PRL (Barry et al., 2015). The amount of PRL in early postmenopausal women is different from that in the late postmenopausal women (Grattan and LeTissier, 2015). Ten weeks of *E. angustifolia* treatment caused a 16.36% decrease in PRL, which was only significant in within-group but not between-group comparisons (**Table 3**). Regarding the impact of hyperprolactinemia on cardiovascular function, the beneficial effects of *E. angustifolia* on heart rate and LDL-C level (Shabani et al., 2020) might be attributed to the decreased PRL level after *E. angustifolia* consumption.

The ratio of PRL/TSH in herbal medicine and placebo groups significantly decreased only in within-group but not between-group comparisons (**Table 3**). Studies suggest that pituitary hormones such as growth hormone, FSH, TSH, and PRL have important roles in regulating bone. Osteoporosis is also associated with thyroid dysfunction in older women. Fracture risk has been reported to be associated with low serum TSH levels (Colaianni et al., 2013). In hypothyroidism, the hypothalamus increases TSH production in the pituitary gland by producing thyrotropin-releasing hormone (TRH), which is required for normal physiological elevation of thyroxine levels. Prolonged hypothyroidism causes hyperplasia of pituitary thyrotropic cells. TRH has a poor excitatory effect on pituitary lactotrophic cells, which might result in a slight to moderate increase in PRL. In other words, in hypothyroidism, the concentrations of both TSH and PRL increases. In a report of a 67-year-old woman with a large pituitary mass resulting in a very high level of TSH, the PRL level also showed a significant increase (Ansari and Almalki, 2016). In postmenopausal women, plasma PRL levels decrease in parallel with the decrease in plasma estradiol. So, postmenopausal women have lower baseline plasma PRL levels than premenopausal women have (Markianos et al., 1996; Pałubska et al., 2017).

As far as we know, no studies have been done on the ratio of TSH and PRL in postmenopausal women. In the present study, the increase in TSH level and the decrease in PRL and also PRL to TSH ratio in herbal medicine group were only significant in the within-group analysis but not between two study groups at the end of the trial (**Table 3**). PRL deficiency reduces the risk of cardiovascular disease (Byberg et al., 2019). Increased TSH levels also lead to hypercholesterolemia and hypertriglyceridemia (Wanjia et al., 2012). Although the decrease in PRL was not significant after treatment with herbal medicine, we previously showed that *E. angustifolia* treatment also significantly reduced LDL-C and improved cardiovascular function and lipid profile in postmenopausal women (Shabani et al., 2020). Therefore, it can be concluded that the overall effect of changes in the two hormones TSH and PRL after treatment with herbal medicine on the lipid profile and cardiovascular function was positive.

In both within-and between-group analyses, PRL/DHEA-S hormonal ratio changes were not significant in the two study groups (**Table 3**). In studies on the relationship between hormones and sexual function, and the relationship between sex hormones and the quality of life, DHEA was the only hormone that had a significant negative relationship with sexual function, which was inversely related to sexual desire levels (Peixoto et al., 2019). Given that DHEA is an indirect

testosterone precursor, the relationship between this hormone and sexual desire is expected to be positive. However, in the study mentioned above, higher DHEA levels were associated with lower sexual desire (Peixoto et al., 2019). It has been suggested that because DHEA and DHEA-S are important precursors for the production of estrogen and androgens, DHEA treatment may be a physiological strategy to reduce the symptoms of hormone deficiency in postmenopausal women (Davis et al., 2011). Another study reported that TC and DHEA-S declined steadily between the ages of 20 and 45 years, which was associated with a slight change in TC but a persistent decrease in DHEA-S in menopause. An age-related decline in DHEA-S level in women with polycystic ovary syndrome (PCOS) was also shown (Schmidt et al., 2011). PRL is also an important stress-induced hormone that increases in postmenopausal women who suffer from acute illness. A study with a specific focus on postmenopausal women showed no significant difference between postmenopausal women and the control group who were healthy postmenopausal women in the PRL levels (Raj et al., 2016). Based on our knowledge, no studies have been conducted on PRL to DHEA-S ratio. In the present study, the decrease in the amount of both hormones after treatment with herbal medicine was only significant in within-group comparisons but not between-group comparisons. The increase in PRL to DHEA-S ratio in both study groups was not significant at the end of the trial in any of the within-) or between-group comparisons (**Table 3**). In our previous study, i.e., the study by Emaminia et al. (2020), we showed that *E. angustifolia* consumption increased testosterone levels in postmenopausal women, though this increase was not significant (Emaminia et al., 2020). Since postmenopausal women in this study did not suffer from certain stress or acute illness (**Table 2**), the results obtained in this study are not unexpected. It should also be noted that study participants did not report any change in their sexual desire (unpublished data) at the end of the trial in the questionnaire.

The reduction in PRL/cortisol ratio was significant in both study groups only in within-group but not between-group analyses (**Table 3**). However, considering the increased PRL/cortisol ratio in some autoimmune diseases (Legakis et al., 2001; Zoli et al., 2002; Koeller et al., 2004), it can be concluded that the observed decrease in PRL/cortisol ratio seems to favorably reduce the likelihood of autoimmune diseases in our study's participants.

The DHEA-S/TSH ratio decreased after both herbal medicine and placebo treatment which was significant in within-group but not between-group comparisons (**Table 3**). Studies have shown that DHEA-S does not decrease uniformly with age but rather increases before menopause (Crawford et al., 2009). Tagawa et al. (2000) showed that serum concentrations of DHEA-S decreased in hypothyroidism but increased in hyperthyroidism. It was suggested thyroid hormones regulate the serum concentration of DHEA and DHEA-S. In patients with thyroid dysfunction, serum concentration of cholesterol also changes significantly; that is to say, hypercholesterolemia is associated with hypothyroidism and hypocholesterolemia is associated with hyperthyroidism (Reviewed in Tagawa et al., 2000). DHEA and thyroid hormones were not measured in our study, and the decrease in DHEA-S and increase in TSH were significant only in within-group comparisons (**Table 3**). In another study, we showed that 10 weeks of treatment with *E. angustifolia* did not change the levels of TC and TG but significantly decreased LDL-C and increased insulin levels. However, the insulin level remained in the normal range (Shabani et al., 2020). Thus, the increase in insulin levels after treatment with herbal medicine is probably unrelated to DHEA and TSH and is probably more affected by cortisol, which decreases glucose consumption and insulin sensitivity (Markianos et al., 1996). To our knowledge, the ratio of DHEA-S to TSH has not been studied in postmenopausal women or other study groups. Considering the changes in the lipid profile after *E. angustifolia* consumption (Shabani et al., 2020), it can be concluded that the small changes in TSH and DHEA-S levels observed in the current study are not indicative of hypothyroidism or thyroid dysfunction in postmenopausal participants However, measurement of thyroid hormones and TRH is required to understand the exact mechanism. Moreover, the significance and relevance of these changes to carbohydrate and fat metabolism need further investigation.

In both within-and between-group comparisons, the treatment with neither *E. angustifolia* nor placebo affected cortisol/TSH ratio (**Table 3**). However, this ratio was significantly higher in the placebo group than the herbal medicine group at the beginning of the trial (between-group p-value = 0.046). In the present study, the increase in cortisol after 10 weeks of treatment was significant only in the herbal medicine group and not in the between-group comparison (**Table 3**). As we know, the ratio of cortisol to TSH has not been investigated so far. Given the role of cortisol in stress and the role that both hormones play in regulating carbohydrate and lipid metabolism, it can be said that postmenopausal women's metabolic status did not change significantly in terms of this hormonal ratio.

CONCLUSION

The current trial was the continuation of our studies previously conducted and the first ethnopharmacological study on the effect of *E. angustifolia* on the profile of TSH, DHEA-S, PRL, cortisol, and their ratios in the postmenopausal women. The observed outcomes about the effect of *E. angustifolia* on menopause were not completely in line with the Iranian folklore. The ten-week consumption of *E. angustifolia* had no significant effects on the level of the studied hormones or their ratios. A larger sample size study on premenopausal and menopausal participants in the long term is necessary to evaluate the effect of *E. angustifolia* on menopausal symptoms in terms of the studied hormone.

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1- Damghan University, Damghan, Iran. 2- Alborz University of Medical Sciences, Karaj, Iran.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Whenever is necessary, we could provide our raw data in excel format without personal information for the participants. Requests to access these datasets should be directed to arezaei@du.ac.ir.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Abzums.Rec.1396.162 ethical code and IRCT20170227032795N4 registration code. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FJ, MS, and FE have made contributions to data collection. All authors contributed to the analysis, interpretation of data, and drafting of the manuscript with input from other team members. AR and BB involved in the conception and design of the study. However, all authors involved in the critical revision of the manuscript and approved the final manuscript.

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Metabonomics Study on the Infertility Treated With Zishen Yutai Pills Combined With *In Vitro* Fertilization-embryo Transfer

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Zishen Yutai Pills (ZYP) is a safe and well quality-controlled TCM preparation with promising effects in many fields of reproduction, including prevention of miscarriage, increase of pregnancy rate during *in vitro* fertilization-embryo transfer (IVF-ET). The plasma of patients was collected from a clinical trial, namely, "Effect of Traditional Chinese Medicine vs placebo on live births among women undergoing *in vitro* fertilization, a multi-center randomized controlled trial." Plasma samples were analyzed with metabonomics method. UPLC-MS technology was used to establish the plasma metabolic fingerprint. Multivariate statistical analysis was applied for comparing the differences of plasma metabolites between ZYP group and placebo group, 44 potential metabolites were screen out and identified. Pathway analysis was conducted with database mining. Compared with placebo, chemicals were found to be significantly down-regulated on HCG trigger day and 14 days after embryo transplantation, including trihexosylceramide (d18:1/26:1), glucosylceramide(d18:1/26:0), TG(22:6/15:0/22:6), TG(22:4/20:4/18:4). Compared with placebo, some chemicals were found to be significantly up-regulated on HCG trigger day and 14 days after embryo transplantation, i.e., PIP3(16:0/16:1), PIP2(18:1/18:1), tauroursodeoxycholic acid, L-asparagine, L-glutamic acid, kynurenic acid, 11-deoxycorticosterone, melatonin glucuronide, hydroxytyrosol. These metabolites were highly enriched in pathways including sphingolipid metabolism, alanine, aspartic acid and glutamic acid metabolism, aminoacyl tRNA biosynthesis, taurine and hypotaurine metabolism. This study revealed metabolic differences between subjects administered with ZYP and placebo. Relating metabolites were identified and pathways were enriched, providing basis on the exploration on the underlying mechanisms of ZYP combined with IVF-ET in the treatment of infertility.

Keywords: traditional Chinese medicine, Zishen Yutai Pills, metabonomics, infertility, embryo transfer

INTRODUCTION

Infertility has been one of the major conditions affecting the well-being of human worldwide (Inhorn and Patrizio, 2015). Nowadays, *in vitro* fertilization-embryo transfer (IVF-ET) is a common procedure, helping couples with fertility problems to achieve parenthood (Kissin et al., 2014). However, according to previous report from the European Annual Conference on Reproduction, the success rate of IVF-ET was only 30–40%, and the rate of pregnancy rate was even lower (Wyns et al., 2020). There is still a bottleneck in the improvement of pregnancy outcome.

In IVF-ET, oocytes quality and endometrial receptivity are the two most important factors affecting the outcome of embryo transfer (Bu et al., 2016; Kristensen et al., 2017). Prior to IVF-ET, multiple mature oocytes could be obtained through controlled ovarian hyperstimulation (COH). Poor ovarian response to gonadotropin leads to defects in both the quality and quantity of oocytes, and eventually lead to a low pregnancy rate (Vaiarelli et al., 2018). It is also well acknowledged that embryo implantation requires good endometrial receptivity. Although a clear definition on endometrial receptivity is still absent, many literatures have put forward that endometrial thickness could be an important indicator for endometrial receptivity (Mahajan and Sharma, 2016). A thickness of endometrium below 8 mm is a risk factor for pregnancy loss during IVF-ET (Bu et al., 2016).

Traditional Chinese medicine is an important complementary therapy in the IVF-ET (Smith et al., 2010). Zishen Yutai Pills (ZYP) is a safe and well quality-controlled TCM preparation with promising effects in many fields of reproduction, including prevention of miscarriage, increase of pregnancy rate (Zhu et al., 2002; Gao et al., 2015b; Ma et al., 2018; Cao et al., 2020).

ZYP contains 15 Chinese traditional medicine herbs, i.e., Cuscutae Semen (the ripe dried seed of *Cuscuta Chinensis* Lam.), Ginseng Radix et Rhizome (the dried root and rhizome of *Panax ginseng* C. A. Mey.), Dipsaci Radix (the dried root of *Dipsacus asper* Wall. ex DC.), Taxilli Herba (the dried leafy stem and branch of *Taxillus chinensis* (DC.) Danser), Eucommiae Cortex (the dried bark of *Eucommia ulmoides* Oilv.), Morindae Officinalis Radix (the dried root of *Marinda officinalis* How), Cervi Cornu Degelatinatum (the residue after water extraction of ossified antler of *Cervus nippon* Temminck), Codonopsis Radix (the dried root of *Codonopsis pilosula* (Franch.) Nannf.), Atractylodis Macrocephalae Rhizoma (the dried rhizome of *Atractylodes macrocephala* Koidz.), Asini Corii Colla (solid glue prepared by stewing and concentrating from the hide of *Equus asinus* L.), Lycii Fructus (the dried ripe fruit of *Lycium barbarum* L.), Rehmanniae Radix Praeparata (the steamed and dried root of *Rehmannia glutinosa* (Gaertn.) DC.), Polygoni Multiflori Radix Praeparata (the steamed and dried root of *Polygonum multiflorum* Thunb.), Artemisiae Argyi Folium (the dried leaf of *Artemisia argyi* Lévl. et Vant.), and Amomi Fructus (the dried fruit of *Amomum villosum* Lour.) (Cao et al., 2020). The formula of ZYP is listed as shown in **Table 1**, including detailed formula and the amounts of raw materials contained in the daily dose. In addition, production process of ZYP complies with the relevant requirements of law of China's Drug Administration and GMP. Production process is normative and controllable to ensure the quality consistency of each batch of products.

According to previous research, different methods were applied in the quality control of ZYP. The contents of five components, namely, loganic acid, chlorogenic acid, loganin, sweroside, and asperosaponin VI, were determined in ZYP by high performance liquid chromatography (HPLC) (Ma et al., 2018). In another report, ultrahigh performance liquid chromatography coupled with charged aerosol detector (UPLC-CAD) fingerprint and multi-components quantitative analysis was developed and validated for quality evaluation of ZYP. Fifty-two characteristic peaks were selected to evaluate the similarities among different batches of ZYP (Cao et al., 2020). Both methods could be the proof of the stability of ZYP, due to the consistency in both contents of components and fingerprint chromatogram.

Previous reports have shown that during IVF-ET, the administration of ZYP on the third day of the menstrual cycle

TABLE 1 | Standard prescription of ZYP and raw material amount used in daily dose.

Medicine material	Standard prescription amount/g	Raw material amount used in daily dose/g
Cuscutae semen	800	9.60
Ginseng radix et rhizoma	50	0.60
Dipsaci radix	480	5.76
Taxilli herba	480	5.76
Eucommiae cortex	290	3.48
Morindae officinalis radix	190	2.28
Cervi cornu degelatinatum	140	1.68
Codonopsis radix	580	6.96
Atractylodis macrocephalae rhizoma	240	2.88
Asini corii colla	30	0.36
Lycii fructus	190	2.28
Rehmanniae radix praeparata	480	5.76
Polygoni multiflori radix praeparata	240	2.88
Artemisiae argyi folium	140	1.68
Amomi fructus	70	0.84

According to the clinical protocol, ZYP is given at a dose of 15 g/day.

before COH could increase the thickness of endometrium and improve the quality of oocytes, leading to an increased pregnancy rate (Zhu et al., 2002). *In vivo* experiments also indicated that ZYP could increase the thickness of endometrium in peri-implantation mice, increase the expression of β3 mRNA, and promote the expression of HOXA10 gene in the endometrium of mice (Gao et al., 2015a). However, the mechanisms of ZYP, especially during the procedure of IVF-ET, still requires further exploration.

The mechanisms of traditional Chinese medicine are hard to decipher due to its complexity of chemical components and the pharmacological interactions among components (Kibble et al., 2015). However, metabonomics could be a useful tool under such conditions because this method focus on the biological effects of treatment from a holistic perspective, making it a promising tool in the exploration of traditional Chinese medicine (Wang et al., 2012; Li et al., 2020).

In this study, plasma samples were collected during a clinical trial entitled "Effect of Traditional Chinese Medicine vs placebo on live births among women undergoing *in vitro* fertilization, a multicenter randomized controlled trial" (registration number: ChiCTR-TRC-14004494, abbreviated as ZYP-RCT). UPLC-MS technology was used to establish the fingerprint of plasma metabolites, and metabonomics analysis was carried out on the plasma of patients from both the ZYP group and the placebo group with multivariate statistical analysis and pathway enrichment, providing evidence for its possible application in IVF-ET for the treatment of infertility.

MATERIALS AND METHODS

Drug Used in ZYP-RCT

ZYP (China National Medical Products Administration Permit No. Z44020008) was obtained from Guangzhou Baiyunshan Zhongyi Pharmaceutical Co. Ltd. (Guangzhou, China). Four different batches of commercial product ZYP were used in the current ZYP-RCT (Batch No. 20130301, 20141101, 20150301, 20150802).

All the voucher specimens were deposited at Guangdong Provincial Hospital of Chinese Medicine (Guangzhou, China). Chemical profiles and quantitative determination were reported in **Supplementary Material**.

Plasma Sample Collection

A total of 94 patients participated in the ZYP-RCT, including 46 cases in placebo group and 48 cases in ZYP group. This RCT was registered on Clinical Trial Registration in China (http://www.chictr.org.cn), with a registration No. Chictr-TRC-14004494. The treatment protocol was based on best practice (Chen et al., 2019b). Elbow vein plasma (6 ml) was collected from all subjects after an overnight fast and placed in EDTA anticoagulant tube. After centrifugation at 3,000 rpm for 15 min, plasma was collected and stored under −80°C for further analysis (Li et al., 2020).

Standard protocol of ZYP-RCT was provided in **Supplementary Material**. The plasma samples were collected at four time points, i.e., baseline (**T1**), Gn-starting day (**T2**), HCG trigger day (**T3**) and 14 days after ET (**T4**). A total of 329 plasma samples were included in this metabonomics study.

According previous reports, ZYP has promising effects in the prevention of miscarriage. In addition, Zishen Yutai pill could also be applied in reproductive assistant technology as a complementary medicine, especially for those advanced maternal age women (≥35 yr old). Therefore, metabonomics study was also carried out in the following subgroups, namely, the advanced maternal age group (≥35 yr old, abbreviated as AMA group), and the abortion history group (abbreviated as AH group). The statistical results of clinical samples are shown in **Table 2**, with clinical characteristics from both groups provided as well.

The plasma samples were collected at four time points, i.e., baseline (**T1**), Gn-starting day (**T2**), HCG trigger day (**T3**) and 14 days after ET (**T4**).

Instruments and Reagents

Ultimate™ 3000 high performance liquid chromatograph was applied in LC analysis. Mass spectrometry was carried out in Q Exactive™ Plus Mass Spectrometry with ESI ionization source (Thermo Fisher Scientific Ultimate™). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and formic acid from FLUKA company (Buchs, Switzerland). All other reagents were commercially available. Ultrapure water (18.2 mΩ) was prepared using Millipore-Q ultra-pure water system (Millipore, France).

Plasma Sample Preparation

Before metabonomics analysis, samples were placed and thawed under 4°C. Plasma (100 μl) was then transferred into EP tube (1.5 ml). After adding acetonitrile (400 μl), sample was vortexed for 2 min and then centrifuged at 13,000 rpm at 4°C for 20 min. Supernatant (400 μl) was collected and then transferred into EP tube thereafter. Water (200 μl) was added in EP tube. The sample was vortexed for 30 min and centrifuged for 20 min at 13,000 rpm under 4°C. Finally, the sample was placed in an injection vial (2 ml) for sample analysis (Li et al., 2020).

LC-MS Analysis and Methodology Investigation

Detailed conditions for LC-MS analysis were reported in **Supplementary Material**. Methodology investigation was also conducted, including precision, stability and repeatability (**Supplementary Material**).

Data Acquisition and Processing

Metabonomics raw data were collected with Xcalibur™ software (Thermo Scientific), and the profilings of metabolic fingerprints were obtained. The raw data were processed by Progenesis QI software. The quantitative information of all metabolites in each sample was obtained after chromatographic peak recognition, peak alignment, and normalization.

Multivariate Statistical Analysis

After data processing, the data of each sample was imported into Simca-p14.1 software. Unsupervised analysis method was performed in the present study, i.e., principal component analysis (PCA). Supervised analysis methods were also performed, including partial least-squares discrimination analysis (PLS-DA) and orthogonal partial least-squares discrimination analysis (OPLS-DA). The contribution of different metabolites in the sample clustering was obtained. The

TABLE 2 | Clinical characteristic of subjects in ZYP-RCT on baseline and sample grouping.

	Placebo		ZYP		
Subjects in whole (*n*)	46		48		
Subjects in AMA (*n*)	22		20		
Subjects in AH (*n*)	31		31		
	Characteristics of Placebo		**Characteristics of ZYP**		***p***
Age (yr)	33.35 ± 4.44		33.38 ± 4.29		0.976
AMA	37.27 ± 2.00		37.60 ± 1.88		0.589
AH	33.16 ± 4.73		33.06 ± 4.15		0.932
Height (cm)	157.83 ± 3.92		158.17 ± 5.12		0.717
AMA	157.77 ± 2.84		157.42 ± 5.16		0.784
AH	158.26 ± 4.37		159.27 ± 5.07		0.408
Weight (kg)	53.7 ± 7		53.7 ± 6.75		0.996
AMA	54.41 ± 5.94		51.74 ± 5.79		0.154
AH	54.68 ± 6.77		54.93 ± 7.09		0.886
BMI	21.53 ± 2.46		21.46 ± 2.48		0.897
AMA	21.85 ± 2.20		20.86 ± 1.86		0.133
AH	21.81 ± 2.34		21.65 ± 2.54		0.797
Thickness of endometrium (mm)	7.8 ± 2.2		7.56 ± 2.94		0.672
AMA	7.95 ± 2.55		7.54 ± 3.24		0.647
AH	7.87 ± 2.31		7.27 ± 2.75		0.361
Duration of infertility (yr)	5.11 ± 4.09		5.23 ± 3.53		0.877
AMA	6.44 ± 4.71		7.20 ± 3.79		0.573
AH	4.81 ± 4.2		4.94 ± 3.33		0.901
Sampling numbers					

	T1	T2	T3	T4	T1	T2	T3	T4	Total
Whole	36	45	43	38	32	50	43	43	329
AMA	16	20	21	18	14	21	16	18	144
AH	27	37	35	28	20	36	31	31	245

AMA, the advanced maternal age group (≥35 yr old); AH, patients with abortion history.

differences of plasma metabolites between the subjects in ZYP group and placebo group were compared.

Identification of Metabolites and Pathway Analysis

The compounds were ranked according to their variable importance in projection (VIP) value in multivariate statistical analysis, and those with significant difference ($p < 0.05$) were screened out. These metabolites were identified according to the isotope matching results of tandem mass spectrometry, and searched from different database, including HMDB, METLIN, KEGG.

Identification of characteristic metabolites was carried out using Thermo Scientific™ Compound Discoverer™. Potential compounds and pathway analysis were also performed on online and local databases, including mzCloud™, ChemSpider™ and KEGG.

Receiver Operating Characteristic (ROC) Curve

MetaboAnalyst 3.0 (www.metaboanalyst.ca), a metabonomics data analysis platform, was applied to draw the ROC curves and multivariate exploratory ROC of the potential biomarkers (Xia et al., 2015). ROC curves were analyzed using metabolites in **T3**, in all subjects, distinguish the ZYP and the placebo. **T3** was selected because at this time points, embryos would be collected. The value of area under the curve (AUC) above 0.7 suggested good predict ability of potential biomarkers.

RESULTS

Metabolic Fingerprint of Plasma

The positive ion mode and negative ion mode were used to establish the plasma metabolic fingerprint profiling of ZYP-RCT, as shown in **Figure 1**.

Methodology Investigation

The quality control (QC) sample obtained by mixing 20 μl serum of each group of samples, processed as sample preparation. One QC sample was injected six times continuously to test the precision of the instrument, 10 m/z peaks with the highest peak intensity were selected to calculate the relative standard deviations (RSD) value, the RSD value was less than 10% ($n = 6$). One QC sample was injected into LC-MS after every 10 serum samples running to ensure the stability in the metabonomics raw data acquisition, the RSD value of 10 *m/z* peaks with the highest peak intensity was less than 10% in the 26 injections. The results showed that the metabonomics method had a good precision, the instrument stability was excellent, and the acquired data were reliable as well (**Supplementary Material**).

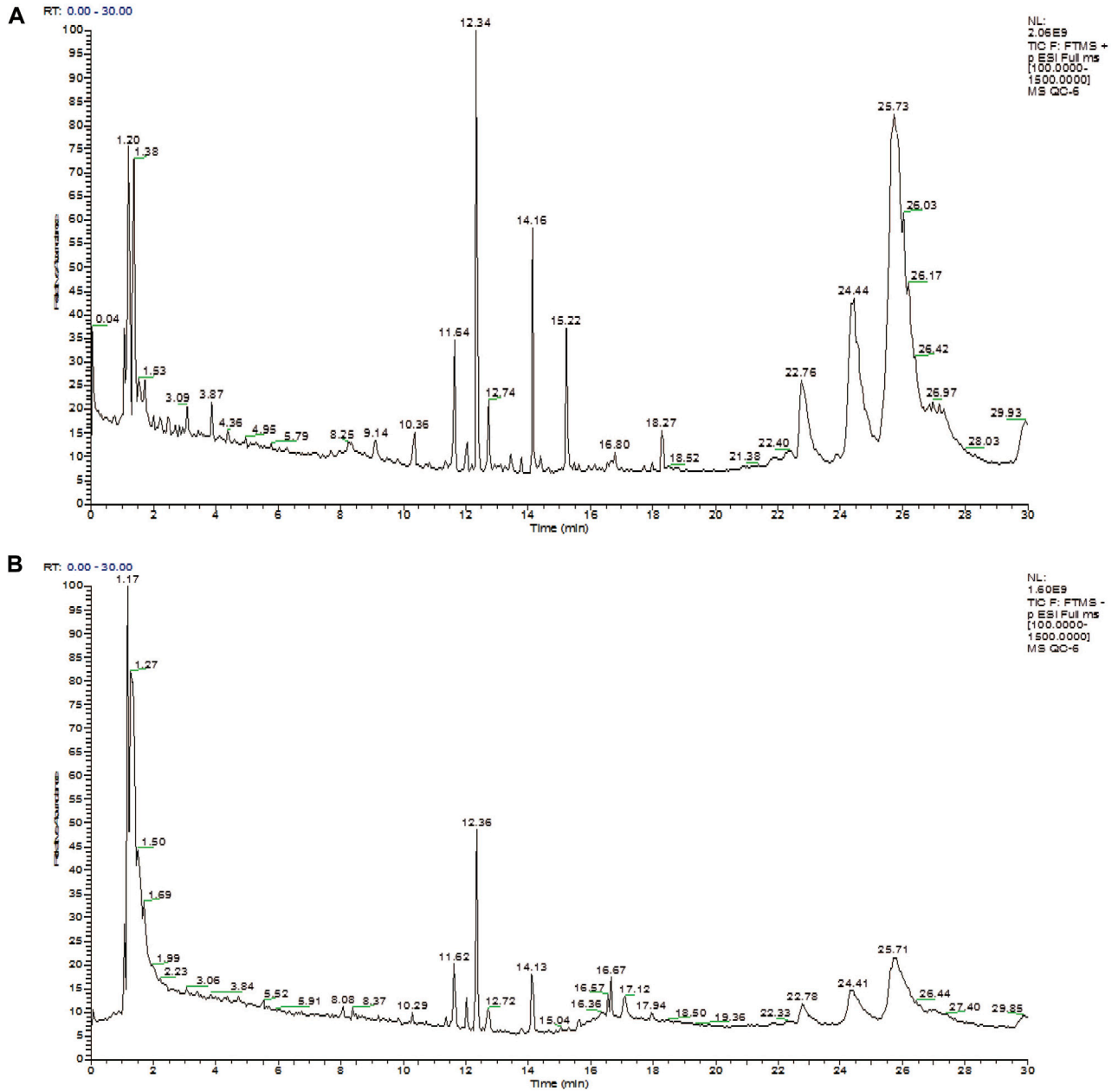

FIGURE 1 | Total ion chromatography (TIC) profiling of plasma sample (**A**: positive ion mode, **B**: negative ion mode).

Multivariate Statistical Analysis in Whole Dataset

PCA, PLS-DA and OPLS-DA analysis were performed on 329 plasma samples and QC samples. Metabolic status of ZYP group and placebo group were well clustered at each time point (**Figures 2A**, **B**, **3**). In **Figures 2A,B**, within-group distributions of metabolites were observed using PCA. In **Figure 3**, the plasma metabolites of ZYP group and placebo group achieved good overlap on baseline, which showed that there was no significant difference between the two groups before treatment, making these two sets of data comparable. After treatment, the distribution of each time point was different.

As can be seen in **Figures 2A**, **B**, **3A**,**C**,**E**,**G**, each group was also well distinguished and clustered, with the changes in each group were well distinguished among different sampling times. The values of R2X(cum), R2Y(cum), Q2(cum) were 0.453, 0.923 and 0.674, respectively in PLS-DA (positive ion mode). The values of R2X(cum), R2Y(cum), Q2(cum) were 0.520, 0.933 and 0.673, respectively in PLS-DA (negative ion mode). The values of R2X(cum), R2Y(cum), Q2(cum) were 0.418, 0.830 and 0.600, respectively in OPLS-DA (positive ion mode). The values of

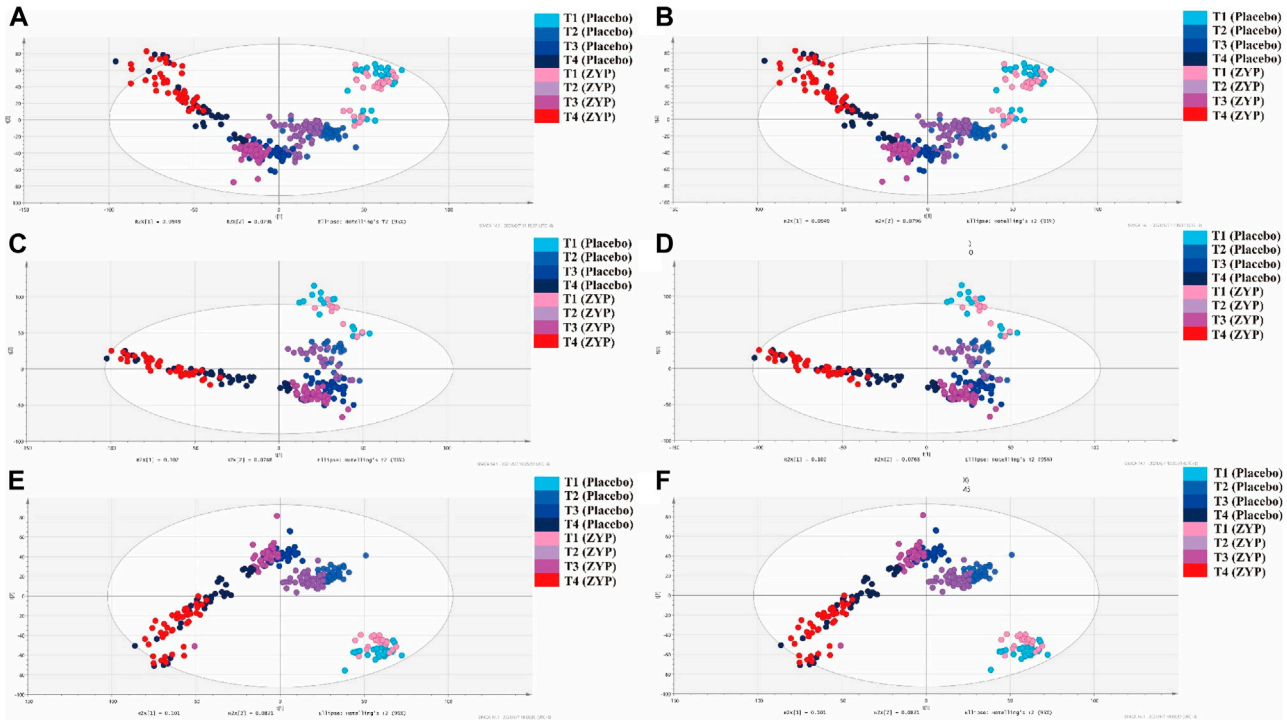

FIGURE 2 | PCA analysis on plasma samples in whole dataset and two subgroups. **(A)** PCA analysis of whole dataset in positive mode. **(B)** PCA analysis of whole dataset in negative mode. **(C)** PCA analysis of AMA subgroup in positive mode. **(D)** PCA analysis in AMA subgroup in negative mode. **(E)** PCA analysis in AH subgroup in positive mode. **(F)** PCA analysis in AH subgroup in negative mode. ■: T1 (Placebo), ■: T2 (Placebo), ■: T3 (Placebo), ■: T4 (Placebo), ■: T1 (ZYP), ■: T2 (ZYP), ■: T3 (ZYP), ■: T4 (ZYP).

R2X(cum), R2Y(cum), Q2(cum) were 0.400, 0.715 and 0.544, respectively in OPLS-DA (negative ion mode). The statistical parameters demonstrated both regression methods were satisfying. No over fitting was observed by permutation test with 100 iterations (**Figures 3B,D,F,H**), implying that all models were reliable.

The results showed that both the placebo group and the ZYP group achieved good clustering in the four observation time points, and the metabolic fingerprints could effectively distinguish the differences of the changes at each observation time point, and the ZYP group was more closely clustered than the placebo group, suggesting that the ZYP group had a strong specificity of metabolic changes.

Multivariate Statistical Analysis of ZYP and Placebo Group

In this section, multivariate statistical analysis of ZYP and placebo group was conducted respectively. Detailed information of grouping was shown in **Table 2**. Score plots showed that the metabolic status of plasma samples in ZYP group was well distinguished (**Figures 4**, **5**), indicating that the plasma metabolic status had changed significantly at each time point. The values of R2X(cum), R2Y(cum), Q2(cum) were 0.331, 0.969 and 0.900, respectively in PLS-DA (positive ion mode). The values of R2X(cum), R2Y(cum), Q2(cum) were 0.376, 0.958 and 0.900, respectively in PLS-DA (negative ion mode). The values of R2X(cum), R2Y(cum), Q2(cum) were 0.315, 0.953 and 0.886, respectively in OPLS-DA (positive ion mode). The values of R2X(cum), R2Y(cum), Q2(cum) were 0.377, 0.950 and 0.890, respectively in OPLS-DA (negative ion mode). No overfitting was observed as shown in the permutation test results in **Figures 4**, **5** in 100 iterative tests, implying that the models were reliable.

Identification of Metabolites and Metabolic Pathway Analysis

According to the methods mentioned above, 44 metabolites were screened out and identified. The identification results of each metabolite are shown in **Table 4**.

Metabolites with significant changes were analyzed by metabolic pathway impact index, including sphingolipid metabolism, alanine, aspartic acid and glutamic acid metabolism, aminoacyl tRNA biosynthesis, taurine and hypotaurine metabolism, nitrogen metabolism, glutamine and glutamic acid metabolism, arginine and proline metabolism, tryptophan metabolism, steroid hormone biosynthesis, glycerin and phospholipid metabolism pathway (**Figure 6**).

Changes of Metabolites in the AMA Subgroup

A total of 245 plasma samples were selected from all samples in AMA subgroup, and the detailed sampling information was

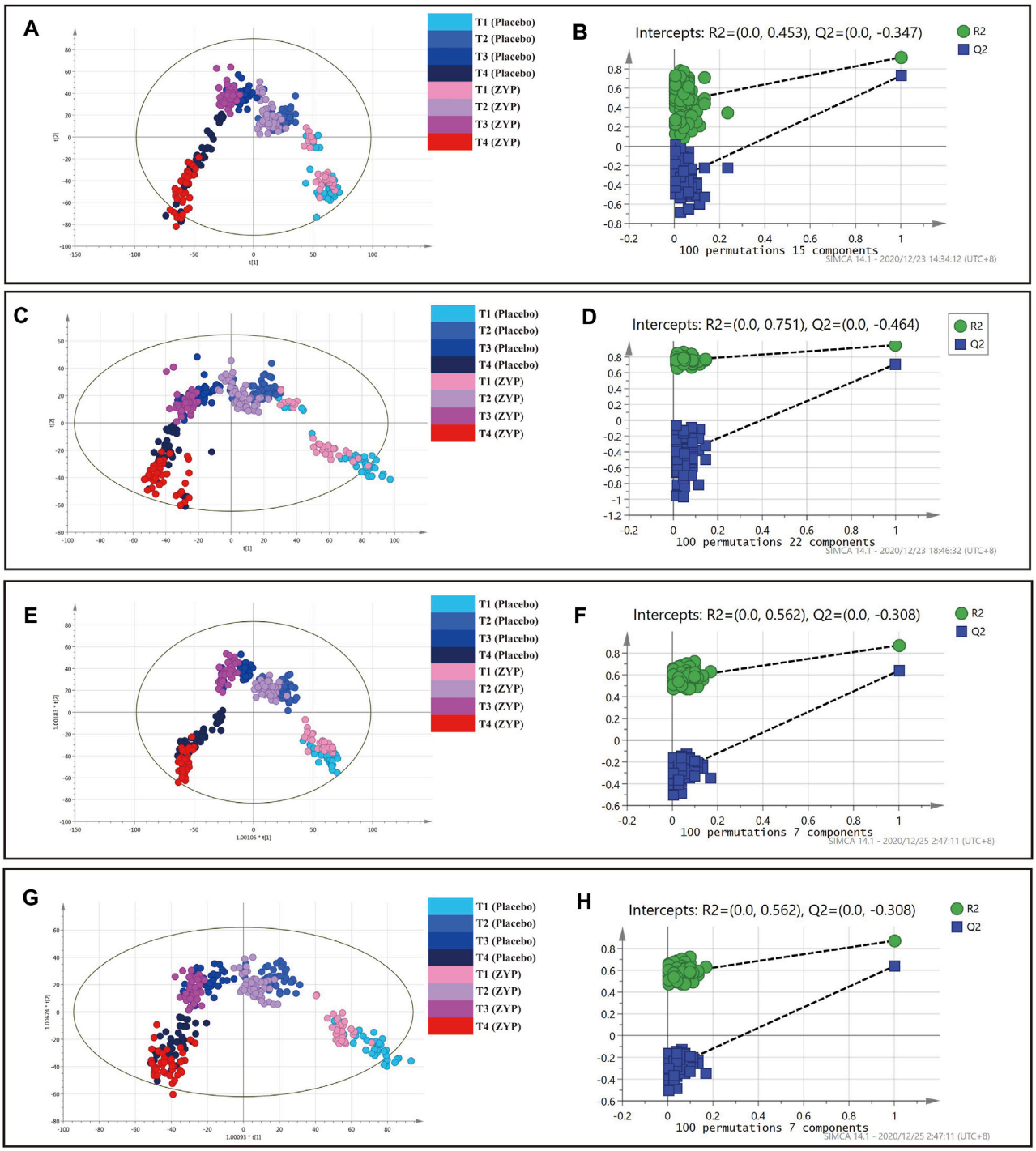

FIGURE 3 | PLS-DA and OPLS-DA analysis on plasma samples in whole dataset. **(A)** PLS-DA analysis in positive mode. **(B)** Permutation test of PLS-DA analysis in positive mode. **(C)** PLS-DA analysis in negative mode. **(D)** Permutation test of PLS-DA analysis in negative mode. **(E)** OPLS-DA analysis in positive mode. **(F)** Permutation test of OPLS-DA analysis in positive mode. **(G)** OPLS-DA analysis in negative mode. **(H)** Permutation test of OPLS-DA analysis in negative mode. ■: T1 (Placebo), ■: T2 (Placebo), ■: T3 (Placebo), ■: T4 (Placebo), ■: T1 (ZYP), ■: T2 (ZYP), ■: T3 (ZYP), ■: T4 (ZYP).

reported in **Table 2**. In **Figures 2C, D**, within-group distributions of metabolites were observed using PCA.

As can be seen from **Figure 7**, metabolites including sphinganine, trihexosylceramide (d18:1/26:1), glucosylceramide (d18:1/26:0) had undergone a decline during the whole process. For PIP3(16:0/16:1), PIP2(18:1/18:1) and tauroursodeoxycholic acid, increased levels of these metabolites in subjects administered with ZYP were observed on **T3** and **T4**, compared

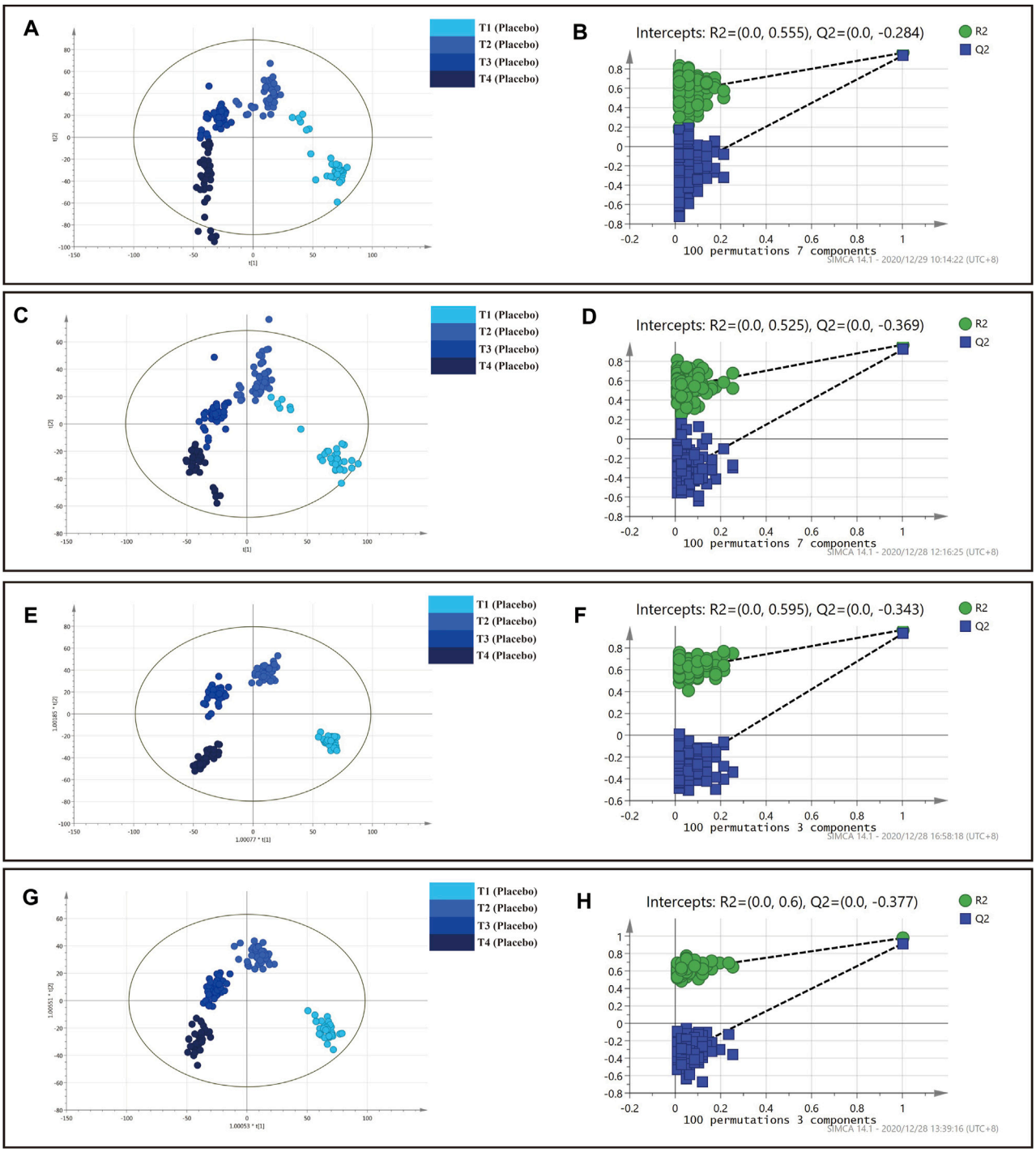

FIGURE 4 | PLS-DA and OPLS-DA analysis on plasma samples in placebo group. **(A)** PLS-DA analysis in positive mode. **(B)** Permutation test of PLS-DA analysis in positive mode. **(C)** PLS-DA analysis in negative mode. **(D)** Permutation test of PLS-DA analysis in negative mode. **(E)** OPLS-DA analysis in positive mode. **(F)** Permutation test of OPLS-DA analysis in positive mode. **(G)** OPLS-DA analysis in negative mode. **(H)** Permutation test of OPLS-DA analysis in negative mode. ■: T1 (Placebo), ■: T2 (Placebo), ■: T3 (Placebo), ■: T4 (Placebo).

with placebo group. Significant decline was also observed in TGs throughout the whole IVF-ET procedure. And the contents of TGs in ZYP group were significantly lower than that of placebo group, at **T2**, **T3** and **T4**. In **T3** and **T4**, subjects treated with ZYP had elevated levels of L-asparagine and L-glutamic acid compared with placebo group. A similar trend was observed in four metabolites including kynurenic acid, 11-deoxycorticosterone, melatonin glucuronide, and

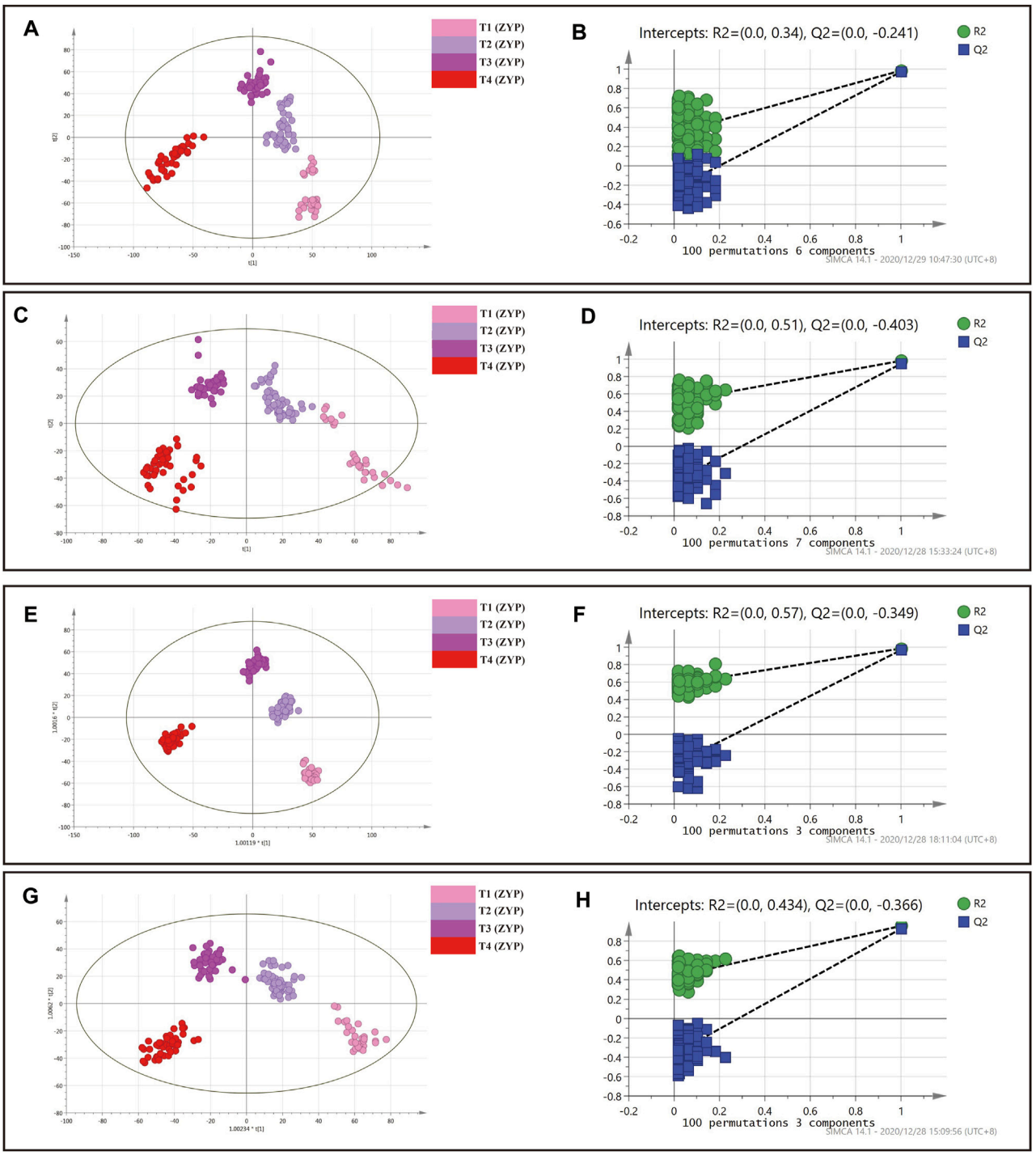

FIGURE 5 | PLS-DA and OPLS-DA analysis on plasma samples in ZYP group. **(A)** PLS-DA analysis in positive mode. **(B)** Permutation test of PLS-DA analysis in positive mode. **(C)** PLS-DA analysis in negative mode. **(D)** Permutation test of PLS-DA analysis in negative mode. **(E)** OPLS-DA analysis in positive mode. **(F)** Permutation test of OPLS-DA analysis in positive mode. **(G)** OPLS-DA analysis in negative mode. **(H)** Permutation test of OPLS-DA analysis in negative mode. ■: T1 (ZYP), ■: T2 (ZYP), ■: T3 (ZYP), ■: T4 (ZYP).

hydroxytyrosol. In addition, elevated levels of these metabolites in subjects administered with ZYP were observed in **T3** and **T4**, compared with placebo group.

Metabolic status of ZYP group and placebo group were well clustered at each time point, and permutation test showed that the model is good without any overfitting (**Supplementary Material**).

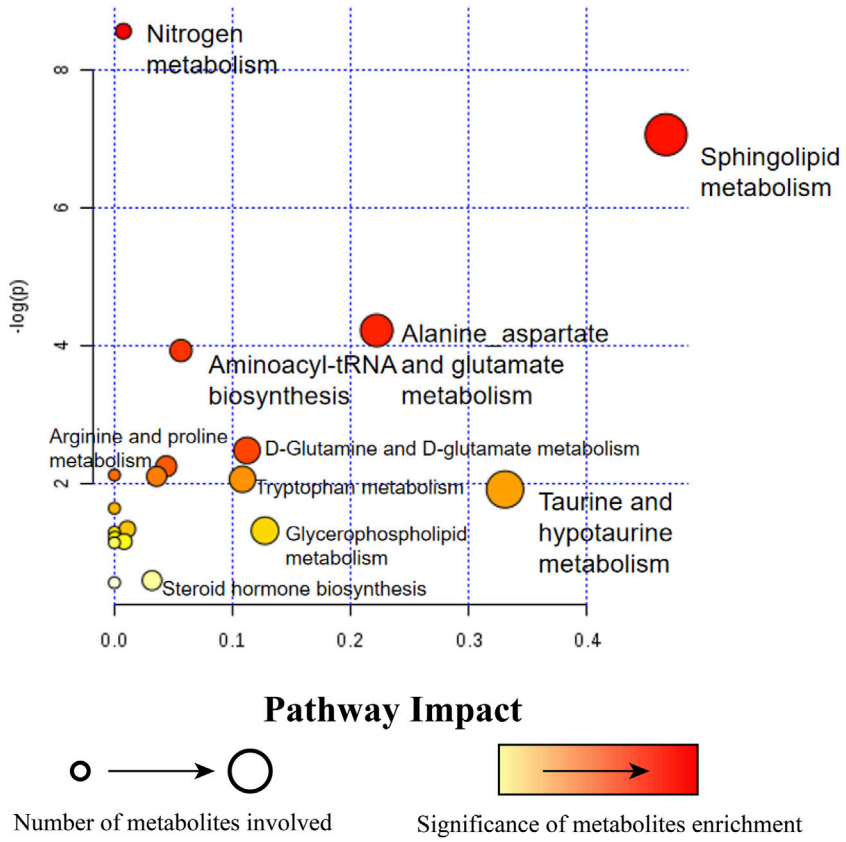

FIGURE 6 | Pathway enrichment analysis of 44 metabolites. A circle with a bigger diagram means more metabolites are in volved in this pathway. Circle in light yellow (■) is regarded as a lower significance than those in red (■).

Changes of Metabolites in the AH Subgroup

A total of 245 plasma samples were selected from all samples in AH subgroup, and the detailed sampling information was reported in **Table 2**. In **Figures 2E,F**, within-group distributions of metabolites were observed using PCA. As can be seen from **Figure 8**, similar changes were observed in AH subgroup as those of AMA subgroup.

Metabolic status of ZYP group and placebo group were well clustered at each time point, and permutation test showed that the model is good without any overfitting (**Supplementary Material**).

Receiver Operating Characteristic Curve

ROC curve analysis is generally considered to one important standard for the assessment of biomarker performance. The results of ROC curve analysis of the 44 differentiated metabolites guaranteed the reliability of potential biomarkers for wide and qualified independent validation. Typical ROC curves and AUC values were shown in **Figures 9A–E**. Multivariate exploratory ROC analysis overview was shown in **Figure 9F**. ROC curves for the AH and AMA subgroup were presented in, **Supplementary Figures S6, S7**. Representative ROC curves presented in **Figure 9**, **Supplementary Figures S6, S7** had AUC values above 0.8.

DISCUSSION

In the present study, metabonomics analysis was conducted with plasma samples of ZYP-RCT, providing information on the mechanisms of ZYP. The MS data of plasma were subjected to multivariate data analysis including PCA, PLS-DA as well as OPLS-DA. All these methods were proved to be reliable, without overfitting. In addition, the conditions of metabolites of ZYP group on **T2** (Gn starting day) and **T3** (HCG trigger day) were closer to **T4** (14 days after ET) compared to that of placebo group. Such findings could be an implication that ZYP is capable of adjusting the body to the state of pre-pregnancy. In addition, the thickness of endometrium on HCG day had shown a trend to increase after ZYP administration (**Table 3**), however without significance ($p > 0.05$). This may be accounted for the limited sample size. Metabolites that cause such differences were subjected to identification and enrichment analysis.

According to statistical analysis, 44 metabolites were found to exhibit significant changes after administration of ZYP compared to those of placebo group. ZYP may exhibited its pharmacological effects, with such metabolites as its characteristics. As can be seen from **Table 4**, detailed information of 44 metabolites were presented. In addition, changes of representative metabolites

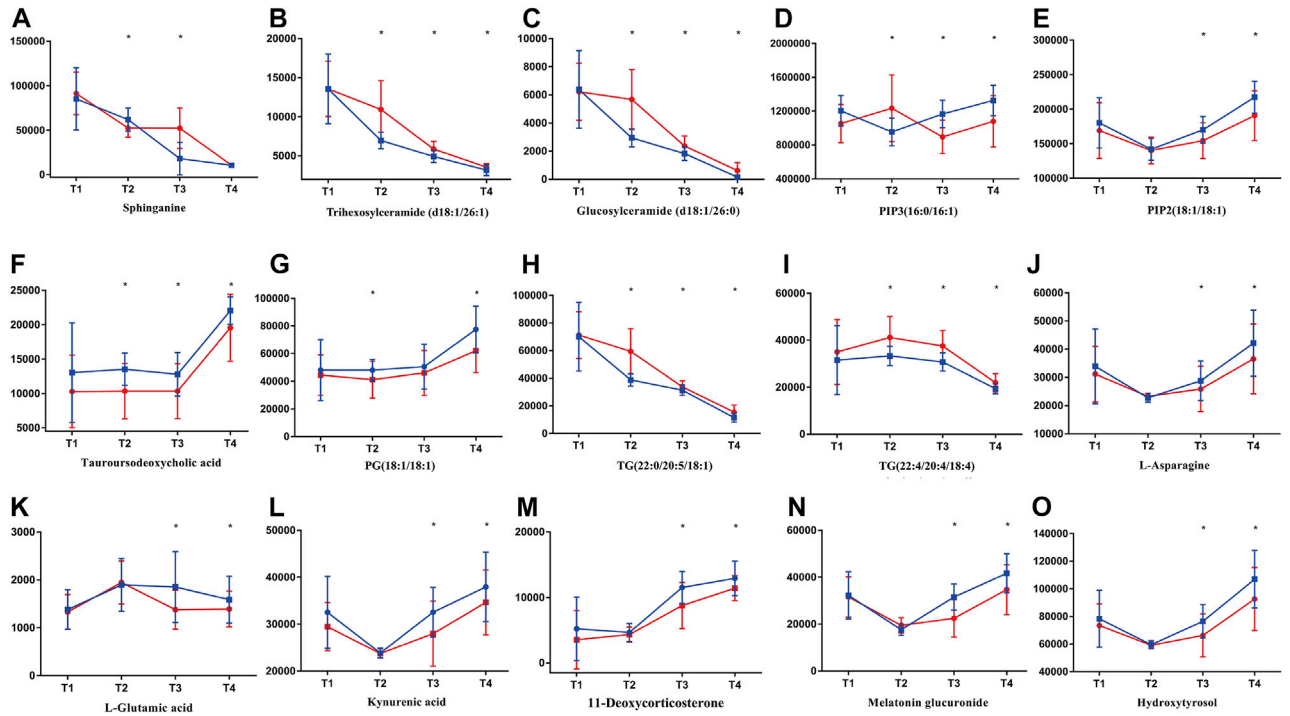

FIGURE 7 | Typical metabolites with significant changes in AMA subgroup. Lines in blue (■) represent ZYP group while in red (■) represent placebo group. Contents of metabolites were assessed with *t* test between ZYP and placebo group. Contents of metabolites are shown in this figure, i.e., **(A)** sphinganine, **(B)** trihexosylceramide (d18:1/26:1), **(C)** glucosylceramide (d18:1/26:0), **(D)** PIP3(16:0/16:1), **(E)** PIP2(18:1/18:1), **(F)** tauroursodeoxycholic acid, **(G)** PG(18:1/18:1), **(H)** TG(22:0/20:5/18:1), **(I)** TG(22:4/20:4/18:4), **(J)** L-asparagine, **(K)** L-glutamic acid, **(L)** kynurenic acid, **(M)** 11-deoxycorticosterone, **(N)** melatonin glucuronide, **(O)** hydroxytyrosol. $^{*}p < 0.05$ were regarded as statistically significant, compared with placebo group.

from the both the AMA and AH subgroup were shown. Forty-four metabolites were found to show significant changes between ZYP and placebo group, including sphinganine, trihexosylceramide (d18:1/26:1), glucosylceramide (d18:1/26:0), PIP3(16:0/16:1), PIP2(18:1/18:1), tauroursodeoxycholic acid, PG(18:1)/(18:1), TGs, L-asparagine, L-glutamic acid, kynurenic acid, 11-deoxycorticosterone, melatonin glucuronide, hydroxytyrosol, providing us with insights into the mechanisms of ZYP.

Subsequently, pathway enrichment was also performed on these metabolites. As can be seen from **Figure 6**, the results of metabonomics in this study showed that among these metabolites, the metabolic pathways were mainly related to amino acid metabolism, lipid metabolism, steroid hormone synthesis and so on. Among those pathways enriched in amino metabolism, the following items were found to be altered after administrations of ZYP, including alanine, aspartate and glutamate metabolism, aminoacyl tRNA biosynthesis, glutamine and glutamate metabolism, arginine and proline metabolism, tryptophan metabolism. Lipid metabolism underwent significant changes as well, with alteration in sphingolipid metabolism, glycerophosphatidylcholine metabolism, etc. It could also be observed that ZYP exerted effects on metabolites relating to steroid hormone biosynthesis as well as taurine and hypotaurine metabolism. Detailed discussion on the pathways and related metabolites were reported as followed.

Lipid Metabolism

Sphingolipid Metabolism

Five metabolites, ceramide (d18:1/16:0), glucosylceramide (d18:1/26:0), ganglioside GM2 (d18:1/16:0), trihexosylceramide (d18:1/26:1) and sphinganine were found enriched in sphingolipids and their metabolic pathways (**Figure 6**).

As shown in **Figures 7**, **8**, most of the metabolites in the ceramide family had undergone a significant decline during the whole IVF-ET process. In addition, after administration of ZYP, this type of metabolites had been decreased compared to those of the placebo group. ROC curve of sphinganine also validated such change (**Figure 9**). It is generally accepted that ceramide and sphingosine are pro-apoptotic and antigrowth factors *via* modulation of key intracellular signaling pathways (Arana et al., 2010). There is also evidence showing that an elevated level of sphingolipid metabolism could be risk factors of pregnancy loss (Mizugishi et al., 2007). Such findings agreed with our previous reports that ZYP could be a promising agent in the prevention of abortion, both in threatened abortion and spontaneous abortion (Li et al., 2015; Zhang et al., 2016).

Glycerophospholipids

Eight glycerophospholipids were found to exhibit significant changes in multivariate analysis, including lysoPE(0:0/24:6), CDP-DG(a-17:0/i-13:0), PIP3(16:0/16:1), PIP2(18:1/18:1), PG(18:1/18:1), lysoPE(0:0/15:0), PE(22:1/20:2) and CL(16:0/16:0/16:0/18:0).

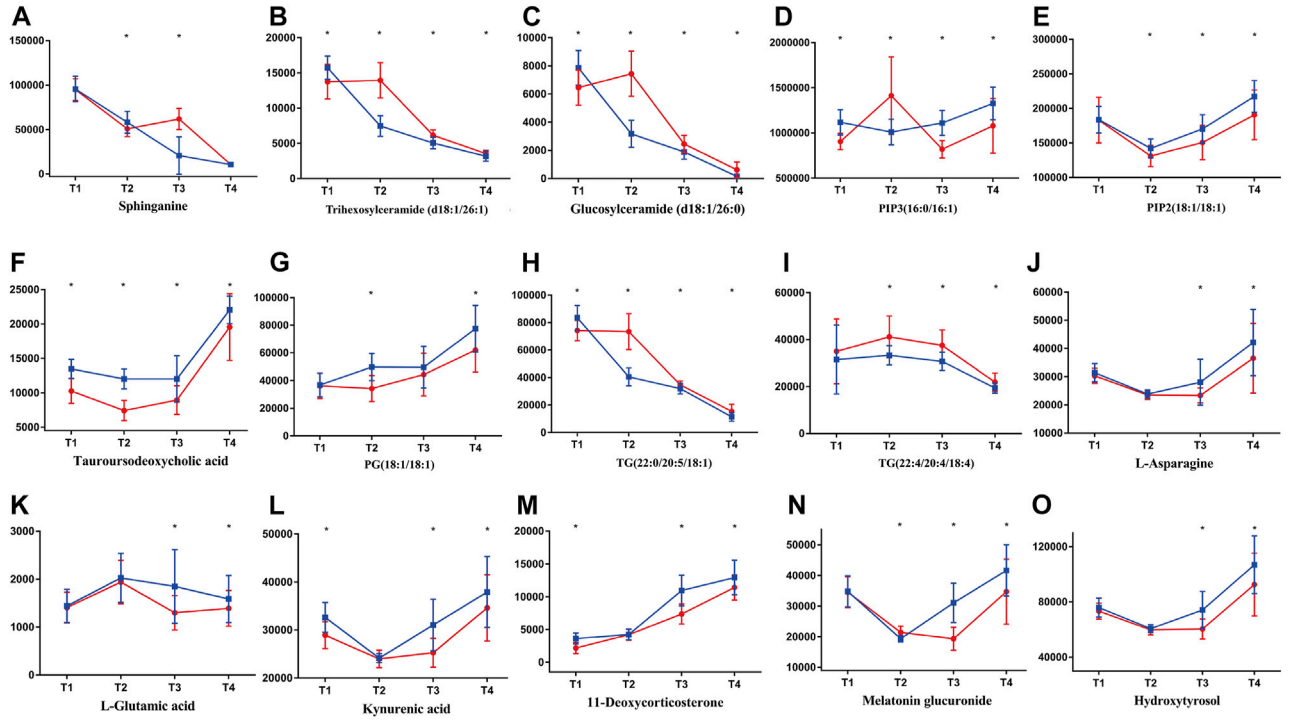

FIGURE 8 | Typical metabolites with significant changes in AH subgroup. Lines in blue (■) represent ZYP group while in red (■) represent placebo group. Contents of metabolites were assessed with *t* test between ZYP and placebo group. Contents of metabolites are shown in this figure, i.e., **(A)** sphinganine, **(B)** trihexosylceramide (d18:1/26:1), **(C)** glucosylceramide (d18:1/26:0), **(D)** PIP3(16:0/16:1), **(E)** PIP2(18:1/18:1), **(F)** tauroursodeoxycholic acid, **(G)** PG(18:1/18:1), **(H)** TG(22:0/20:5/18:1), **(I)** TG(22:4/20:4/18:4), **(J)** L-asparagine, **(K)** L-glutamic acid, **(L)** kynurenic acid, **(M)** 11-deoxycorticosterone, **(N)** melatonin glucuronide, **(O)** hydroxytyrosol. *p < 0.05 were regarded as statistically significant, compared with placebo group.

Glycerophospholipids are the most abundant phospholipids in the body, which could play important roles in many biological processes, including formation of membrane, acting as surfactant, and participating in signal transduction. PIP3(16:0/16:1) and PIP2(18:1/18:1) are typical glycerophospholipids metabolites. After treatment of ZYP, elevated levels of both metabolites were observed as shown in **Figures 7**, **8**. ROC curve of PIP3(16:0/16:1) also validated such change (**Figure 9**). Both metabolites are phosphatidylinositol derivatives, with different fatty acid moiety. The former metabolites possess a phosphatidylinositol triphosphate (PIP3) part, which is capable of activating a wide range of proteins, including protein kinase B (Akt) (Manna and Jain, 2015). Phosphatidylinositol biphosphates (PIP2) could be catalyzed by phosphatidylinositol 3-kinases (PI3K), adding of a phosphate group and resulting in formation of PIP3 (Lee et al., 2020). It is well acknowledged that PI3K/AKT signaling pathway is the regulatory center for many biological functions including protein synthesis, cell survival, differentiation, proliferation, and apoptosis (Maidarti et al., 2020). This signaling pathway is highly related to the proliferation and regeneration of endometrium. Previous studies reported that upregulation of PI3K/AKT pathway could lead to higher expression of HIF and VEGF pathways, and therefore, play important roles in endometrial angiogenesis, and eventually leading to improvements in endometrial receptivity (Gupta et al., 2018). It should also be noted that the development of oval cells is regulated by PI3K/AKT/mTOR pathway (Liu et al., 2018). There may be a potential relationship between elevated levels of both PIP3 and PIP2 and the therapeutic effects of ZYP. However, validation of this hypothesis still requires *in vivo* and *in vitro* experiments.

PG(18:1/18:1) belongs to the class of phosphatidylglycerol (PG), with cardiolipin (CL) as its precursor. Reports have shown that PG is the second most abundant phospholipid in lung surfactant (Tang et al., 2011). Numerous reports have shown that an elevated level of PG could be observed during pregnancy (Chen et al., 2019a).

Glycerides

Several triglycerides were identified in this study, including TG(22:6/15:0/22:6), TG(18:0/14:0/18:0), TG(22:0/20:5/18:1), TG(22:4/20:4/18:4). As can be seen in **Figures 7**, **8**, significant reduction of TG levels was observed in AMA and AH subgroup. ROC curve of TG(22:4/20:4/18:4) also validated such change (**Figure 9**). Numerous reports had proved that an increased level of TG could cause alterations in mitochondrial activity and redox status in oocytes, and may further resulted in poor reproductive outcomes (Igosheva et al., 2010). ZYP may exerts its complementary effects in IVF-ET due to its possible role against lipid dysfunction.

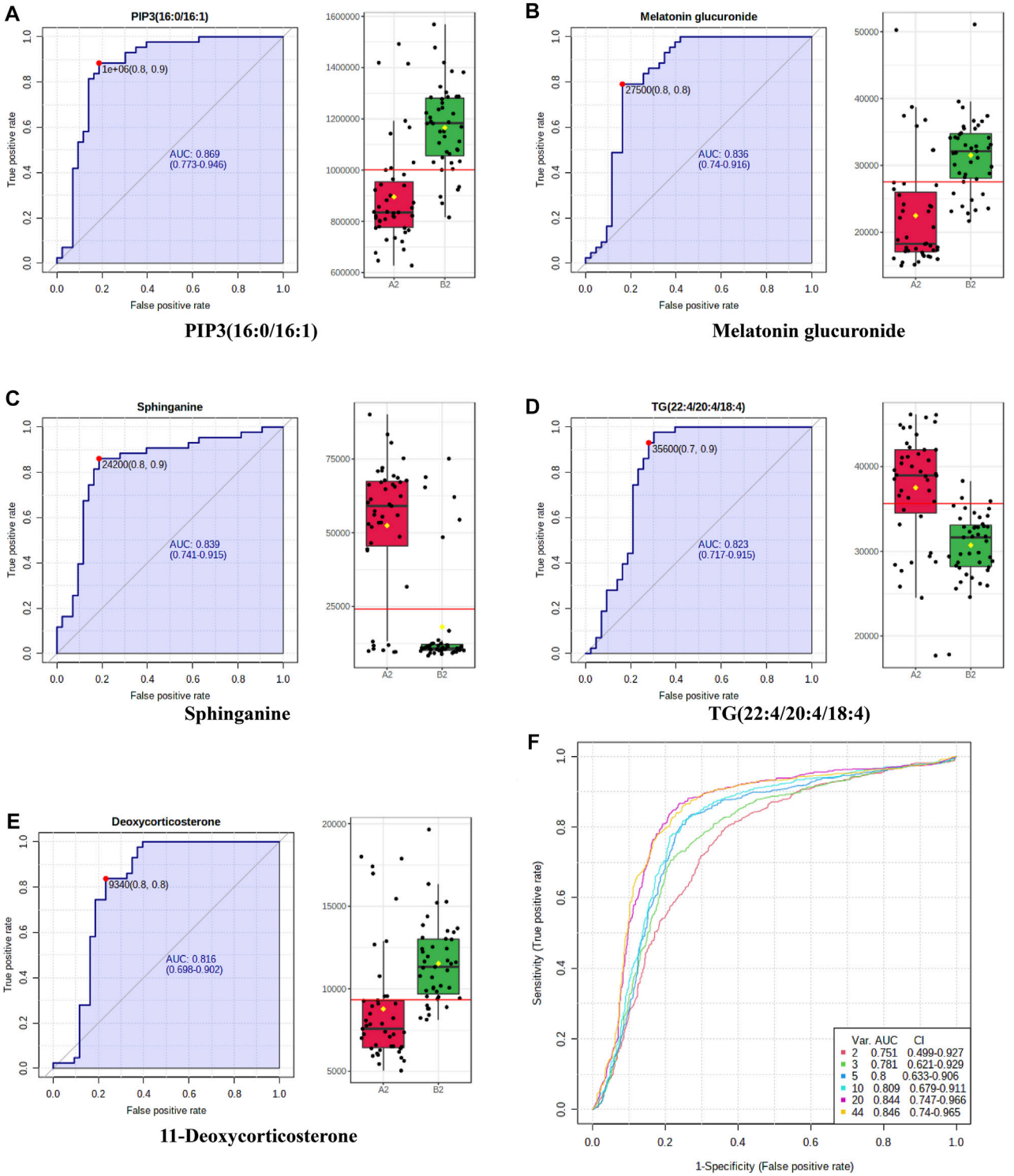

FIGURE 9 | Representative ROC curve plots of potential biomarkers. **(A–E)** Representative ROC curves and AUC values of potential biomarkers with high-performance prediction. Box plots in green (■) represent ZYP group while in red (■) represent placebo group. **(F)** Multivariate exploratory ROC analysis overview.

Fatty Acid Metabolites

This kind of metabolites were fatty acids or derivatives that possess a moiety of fatty acid. Fatty acids are formed after lipolyzation from triglycerides, and exhibit dual roles in the development of oocytes. On one hand, the fatty acid oxidation is an important energy resource for the development of oocytes and early embryos (Prates

TABLE 3 | Thickness of endometrium on baseline (T1) and HCG trigger day (T3)

	Subgroup	Placebo group (mm)	ZYP group (mm)	*p*
Baseline (**T1**)	Whole	7.80 ± 2.20	7.56 ± 2.94	0.672
	AMA	7.95 ± 2.55	7.54 ± 3.24	0.647
	AH	7.87 ± 2.31	7.27 ± 2.75	0.361
HCG trigger day (**T3**)	Whole	11.48 ± 2.61	12.16 ± 2.17	0.168
	AMA	11.50 ± 2.78	11.97 ± 2.51	0.574
	AH	11.10 ± 2.37	11.96 ± 2.20	0.144

p *value was calculated between placebo group and ZYP group with* t *test.*

et al., 2014). On the other hand, excessive fatty acids could also increase levels of reactive oxygen species (ROS), resulting in the dysfunction of mitochondria and endoplasmic reticulum, eventually impairing the subsequent oocyte development (Yang et al., 2012). In this study, some fatty acid metabolites were also found to exhibit significant changes after administration of ZYP, including 7*Z*,10*Z*-hexadecadienoic acid, docosanamide, (*E*)-2-tridecene-4,6,8-triyn-1-ol, heptadecanoic acid, dodecanoyl carnitine. However, due to the dual role of this kind of metabolites, more data, including results from targeted metabolomics and molecular biology experiments, are required to explore the roles of these metabolites in IVF-ET.

Amino Acids Metabolisms

Amino acids are important substances involved in every aspect of cell metabolism, including metabolisms of carbohydrates, maintenance of osmotic pressure and pH value, gene expressions and so on (Wu, 2013). Thus, amino acids play a central role in the biological processes of reproductive tissues. In particular, amino acids are important regulators in the growth of oocytes and embryos. Alterations in the amino acid metabolisms, including types of amino acids, concentrations of different amino acids, could be important factors affecting the outcomes of pregnancy (Brison et al., 2004; Wu, 2013).

Metabolism of Alanine, Aspartic Acid and Glutamic Acid

After administration of ZYP, L-asparagine and L-glutamic acid were significantly up-regulated in **T2**, **T3** and **T4** (**Supplementary Tables S3–S5**). Numerous reports from cell culture and animal studies shows that some of the important members in this pathway, for example, glutamine, glutamic acid, and arginine, play important roles in multiple signaling pathways, resulting in the regulations of gene expressions, intracellular protein turnover, nutrient metabolism, and oxidative defense (Bröer and Bröer, 2017; Mo et al., 2018). It is well acknowledged that the catabolism of L-glutamic acid could be a source of adenosine triphosphate (ATP). And L-glutamic acid is also an important secondary source of carbon and nitrogen for the re-synthesis of pyrimidines and hydrazines (Wu, 2013). Previous report had also proved that L-glutamic acid exhibited potential protective effects on embryos, and this might be related to the activation of mitogen-activated protein kinase (MAPK) pathway and cell proliferation (Boga Pekmezekmek et al., 2020). Another report had also proved that an elevated level of asparagine was significantly correlated with a clinical pregnancy and live birth (Brison et al., 2004).

Tryptophan Metabolism

Tryptophan is one of the essential amino acids for human, and the precursor of 5-hydroxytryptamine. Dietary tryptophan exhibited positive regulation on the size of follicles and fertilization rate (Jiang et al., 2018). In addition, kynurenic acid is an endogenous metabolite which could be synthesized after degradation of tryptophan. In previous reports, kynurenic acid exhibited potential antioxidative activities and may exert protective effects on endometrium and follicles (Lugo-Huitron et al., 2011). Another metabolite presented in this pathway is melatonin. According to metabonomics study, elevated level of melatonin glucuronide was observed after administration of ZYP (**Figures 7**, **8**). ROC curve of melatonin glucuronide also validated such change (**Figure 9**). Reports have shown that melatonin treatment could improve the fertility of aged mice due to reduced ROS level in the oocytes, implying its possible therapeutic effects in the AMA subgroup (Zhang et al., 2019).

Steroid Metabolism

Five metabolites related to steroid metabolism were found in the metabonomics study, i.e., tauroursodeoxycholic acid, taurine, 17a-ethinylestradiol, 5α-tetrahydrocortisol, and 11-deoxycorticosterone.

Chemicals in taurine family are important metabolites, with a wide distribution in different organs and tissues. These metabolites could increase the solubility of lipids and cholesterol, playing an important role in lipid metabolism. *In vivo* and *in vitro* experiment had shown that this type of metabolites could inhibit inflammation mediators, reduce capillary permeability, and thus could exert beneficial effects on follicle growth, oocyte maturation, fertilization (Mu et al., 2019). Thus, the effects of ZYP might be achieved with the up-regulation of this pathway.

In addition, metabolites of the cortisol family, including 17α-ethynylestradiol, 5α-tetrahydrocortisol, and 11-deoxycorticosterone were found to significantly changed after administration of ZYP. ROC curve of 11-deoxycorticosterone also validated such change (**Figure 9**). These metabolites belong to the family of glucocorticoids, and are of great significance in the process of metabolism, which is a potential agent in the response to stress. Glucocorticoids affect the secretion of pituitary hormones and gonadal response to GnRH (Zavala et al., 2020). In addition, glucocorticoid can influence the production as well as the maturation of oocyte. Previous reports suggested that higher cortisol content may related to better pregnancy outcome during IVF-ET, because the cortisol content is an important factor in the

TABLE 4 | Identification of metabolites.

No.	Retention Time	*m/z*	HMDB ID	Compound name	Ion	Formula	Class	Biological Process	KEGG ID
1	1.34	293.0855	HMDB28755	Aspartyl-Histidine	M+Na	$C_{10}H_{14}N_4O_5$	Carboxylic acids and derivatives	NA	NA
2	10.27	133.0649	HMDB0000168	L-Asparagine	M+H	$C_4H_8N_2O_3$	Carboxylic acids and derivatives	Aspartate Metabolism	C00152
3	10.71	308.2198	HMDB13250	Myristoylglycine	M+Na	$C_{16}H_{31}NO_3$	Carboxylic acids and derivatives	NA	NA
4	29.35	228.0076	HMDB0001228	L-Glutamic acid 5-phosphate	M+H	$C_5H_{10}NO_7P$	Carboxylic acids and derivatives	NA	C03287
5	7.00	1,046.5402	HMDB0001035	Angiotensin II	M+H	$C_{50}H_{71}N_{13}O_{12}$	Carboxylic acids and derivatives	Angiotensin Metabolism	C02135
6	1.06	148.1162	HMDB0000148	L-Glutamic acid	M+H	$C_5H_9NO_4$	Carboxylic acids and derivatives	Glutamate Metabolism	C00025
7	3.73	205.0972	HMDB00929	L-Tryptophan	M+H	$C_{11}H_{12}N_2O_2$	Indoles and derivatives	Transcription/Translation;Tryptophan Metabolism	C00078
8	29.40	593.3307	HMDB0001926	17α-Ethynylestradiol	2M+H	$C_{20}H_{24}O_2$	Steroids and steroid derivatives	Lipid metabolism pathway	C07534
9	7.10	367.2692	HMDB0000526	5α-Tetrahydrocortisol	M+H	$C_{21}H_{34}O_5$	Steroids and steroid derivatives	Lipid metabolism pathway	
10	8.48	500.3798	HMDB0000874	Tauroursodeoxycholic acid	M+H	$C_{26}H_{45}NO_6S$	Steroids and steroid derivatives	Lipid metabolism pathway	
11	29.66	331.2091	HMDB0000016	11-Deoxycorticosterone	M+H	$C_{21}H_{30}O_3$	Steroids and steroid derivatives	Steroidogenesis ; Lipid metabolism pathway	C03205
12	6.11	343.2859	HMDB0004666	2-Arachidonylglycerol	M+H-2H$_2$O	$C_{23}H_{38}O_4$	Endocannabinoids	Fatty acid metabolism	C13856
13	12.41	253.2164	HMDB00477	7*Z*,10*Z*-Hexadecadienoic acid	M+H	$C_{16}H_{28}O_2$	Fatty Acyls	Lipid metabolism pathway	
14	21.89	340.3572	HMDB00583	Docosanamide	M+H	$C_{22}H_{45}NO$	Fatty Acyls	Lipid metabolism pathway	
15	10.86	187.1119	HMDB30931	(*E*)-2-Tridecene-4,6,8-triyn-1-ol	M+H	$C_{13}H_{14}O$	Fatty Acyls	Lipid metabolism pathway	
16	0.00	271.2634	HMDB02259	Heptadecanoic acid	M+H	$C_{17}H_{34}O_2$	Fatty Acyls	Lipid metabolism pathway	
17	12.36	308.2796	HMDB0002250	Dodecanoylcarnitine	M+H-2H$_2$O	$C_{19}H_{37}NO_4$	Fatty Acyls	Fatty acid metabolism	
18	25.10	469.3782	HMDB0055895	TG(22:6/15:0/22:6)	M+2H	$C_{62}H_{96}O_6$	Glycerolipids	Lipid metabolism pathway	NA
19	23.68	857.7542	HMDB0010427	TG(18:0/14:0/18:0)	M+Na	$C_{53}H_{102}O_6$	Glycerolipids	Lipid metabolism pathway	
20	28.94	343.9054	HMDB0046910	TG(22:0/20:5/18:1)	M+3H	$C_{63}H_{110}O_6$	Glycerolipids	Lipid metabolism pathway	NA
21	10.71	498.3277	HMDB0054868	TG(22:4/20:4/18:4)	M+Na	$C_{63}H_{98}O_6$	Glycerolipids	Lipid metabolism pathway	NA
22	6.26	576.3234	HMDB0011499	LysoPE(0:0/24:6)	M+Na	$C_{29}H_{48}NO_7P$	Glycerophospholipids	Lipid metabolism pathway	NA
23	26.05	948.4617	HMDB0116105	CDP-DG(a-17:0/i-13:0)	M+Na	$C_{42}H_{77}N_3O_{15}P_2$	Glycerophospholipids	Cardiolipin Biosynthesis	NA
24	23.84	536.1655	HMDB0010148	PIP3(16:0/16:1)	M+H+Na	$C_{41}H_{80}O_{22}P_4$	Glycerophospholipids	Lipid metabolism pathway	C00626
25	21.94	775.5645	HMDB0010618	PG(18:1/18:1)	M+H	$C_{42}H_{79}O_{10}P$	Glycerophospholipids	Lipid metabolism pathway	NA
26	10.07	440.2858	HMDB0011472	LysoPE(0:0/15:0)	M+H	$C_{20}H_{42}NO_7P$	Glycerophospholipids	Lipid metabolism pathway	NA
27	26.20	1,023.5250	HMDB0010089	PIP2(18:1/18:1)	M+H	$C_{45}H_{85}O_{19}P_3$	Glycerophospholipids	Glycerophospholipid metabolism ; Lipid metabolism pathway	C00626
28	21.22	889.6357	HMDB0009528	PE(22:1/20:2)	M+ACN+Na	$C_{47}H_{88}NO_8P$	Glycerophospholipids	Lipid metabolism pathway	C00350
29	11.66	702.4977	HMDB0056388	CL(16:0/16:0/16:0/18:0)	M+H+Na	$C_{75}H_{146}O_{17}P_2$	Glycerophospholipids	Cardiolipin Biosynthesis	NA
30	25.40	520.5090	HMDB0004949	Ceramide (d18:1/16:0)	M+H-H2O	$C_{34}H_{67}NO_3$	Sphingolipids	Lipid metabolism pathway	C00195
31	29.25	302.8993	HMDB04977	Glucosylceramide (d18:1/26:0)	M+3Na	$C_{50}H_{97}NO_8$	Sphingolipids	Lipid metabolism pathway	C01190
32	29.82	700.8428	HMDB04938	Ganglioside GM2 (d18:1/16:0)	M+2Na	$C_{65}H_{117}N_3O_{26}$	Sphingolipids	Lipid metabolism pathway	C04884
33	20.30	410.8958	HMDB0004884	Trihexosylceramide (d18:1/26:1)	M+3Na	$C_{62}H_{117}NO_{18}$	Sphingolipids	Lipid metabolism pathway	C04737
34	1.63	224.0630	HMDB0060830	Melatonin glucuronide	M+H+K	$C_{19}H_{24}N_2O_8$	Nucleoside and nucleotide analogues	NA	NA
35	0.19	445.1205	HMDB33041	1-(1-Propenylthio)propyl propyl disulfide	2M+H	$C_9H_{18}S_3$	Organic disulfides	NA	NA
36	1.15	148.0040	HMDB0000251	Taurine	M+Na	$C_2H_7NO_3S$	Organic sulfonic acids and derivatives	Bile acid biosynthesis ; Taurine and hypotaurine metabolism	C00245
37	28.99	314.9288	HMDB0011625	Dimethylarsinic acid	2M+K	$C_2H_7AsO_2$	Organometalloid compounds	NA	C07308
38	28.58	100.1127	HMDB0013648	Palmitoleoyl ethanolamide	M+3H	$C_{18}H_{35}NO_2$	Organonitrogen compounds	NA	NA
39	10.16	302.3056	HMDB0000269	Sphinganine	M+H	$C_{18}H_{39}NO_2$	Organonitrogen compounds	Sphingolipid Metabolism	C00836
40	7.39	155.0703	HMDB05784	Hydroxytyrosol	M+H	$C_8H_{10}O_3$	Phenols	NA	C01479
41	28.94	205.0173	HMDB0000779	Phenyllactic acid	M+K	$C_9H_{10}O_3$	Phenylpropanoic acids	NA	NA
42	7.10	553.3839	HMDB0033685	α-Tocopherol succinate	M+Na	$C_{33}H_{54}O_5$	Prenol lipids	Lipid metabolism pathway	NA
43	7.20	183.1381	HMDB32050	α-Terpineol formate	M+H	$C_{11}H_{18}O_2$	Prenol lipids	NA	NA
44	12.96	190.0499	HMDB00715	Kynurenic acid	M+H	$C_{10}H_7NO_3$	Quinolines and derivatives	Tryptophan Metabolism	C00152

development of oocytes (Keay et al., 2002). In addition, it was also observed that 17a-ethinylestradiol, as a component of contraceptives, perished in both groups after treatment.

Others

Significant changes were also observed in metabolites, including 2-arachidonylglycerol, hydroxytyrosol, α-tocopherol, α-tocopherol succinate, etc.

2-Arachidonoylglycerol is an endogenous cannabinomimetic lipid derivative. It has been well acknowledged that the exposure to cannabinoids, including 2-arachidonoylglycerol, could lead to adverse effects on reproductive functions, including retarded embryo development, fetal loss, pregnancy failure (Sun and Dey, 2009). The administration of ZYP could significantly reduce the level of 2-arachidonoylglycerol in serum, thus, exhibiting protective effects.

A previous report had shown that hydroxytyrosol was beneficial for pregnant women due to its antioxidative, metabolism-regulatory, anti-inflammatory and immuno-modulatory properties (Menichini et al., 2020).

In the present study, a statistically significant but modest increase was observed in both α-tocopherol succinate and α-terpineol formate. α-Tocopherol, a major composition moiety of vitamin E, is an important compound in the maintenance of reproduction activity and reputed for its antioxidative activity (Rimbach et al., 2010). It could influence the whole process of reproduction, including the health condition of pregnant women, pregnancy outcome, embryotic development, neonatal development, and so on (Cave et al., 2018). However, in the present study, only a modest increase was observed in alpha-tocopherol succinate. But still the alterations in tocopherol are critical in the exploration of mechanisms of ZYP.

A report had proved that α-terpineol possess relaxant effects on rat uterine (Ponce-Monter et al., 2008). In clinical use, ZYP is frequently used as a medicine for the treatment of threatened or spontaneous abortion (Li et al., 2015; Zhang et al., 2016). Thus, the change of this kind of metabolites could also be potential targets in further analysis, particularly in the use of ZYP, in the prevention of pregnancy loss.

CONCLUSION

In brief, this study provided metabonomics analysis between subjects administered with ZYP and placebo. Relating metabolites were identified and pathways were enriched, providing basis on the exploration on the underlying mechanisms of ZYP combined with IVF-ET in the treatment of infertility.

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

Ethics approval has been sought from Ethics Committee at Sun Yat-Sen Memorial Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LL was in charge of metabonomics research, G-aL, LH, and NN supervised the whole experiment. Q-LH, X-fP, and J-bZ participated in the clinical trial and data analysis relating to clinical trial. J-wZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Prescription of Radix *Salvia miltiorrhiza* in Taiwan: A Population-Based Study Using the National Health Insurance Research Database

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Objective: While radix *Salvia miltiorrhiza* (Danshen; RSM) is commonly used in Chinese herbal medicine, its current usage has not yet been analyzed in a large-scale survey. This study aimed to investigate the conditions for which RSM is prescribed and the utilization of RSM in Taiwan.

Methods: 1 million beneficiaries enrolled in the Taiwan National Health Insurance Research Database were sampled to identify patients who were prescribed RSM. Next, the diagnoses of these patients based on the International Classification of Diseases 9th Revision Clinical Modification code were analyzed. Logistic regression analysis was employed to estimate the odds ratio (OR) for RSM utilization.

Results: Patients with disorders of menstruation and abnormal bleeding from the female genital tract due to other causes were the diagnostic group most commonly treated with RSM (9.48%), followed by those with general (9.46%) and cardiovascular symptoms (4.18%). Subjects treated with RSM were mostly aged 35–49 years (30.1%). The most common combination of diseases for which RSM was prescribed (0.17%) included menopausal disorders and general symptoms. Women were more likely to receive RSM than men (OR = 1.75, 95% confidence interval = 1.73–1.78). RSM was frequently combined with Yan-Hu-Suo and Jia-Wei-Xiao-Yao-San for clinical use.

Conclusion: To date, this is the first study to identify the most common conditions for which RSM is used in modern Taiwan. The results indicate RSM as a key medicinal herb for the treatment of gynecological diseases, including menstrual disorders, female genital pain, menopausal disorders, etc. The most common combination for which RSM is prescribed is menopausal disorders and general symptoms. Further research is needed to elucidate the optimal dosage, efficacy, and safety of RSM.

Keywords: radix *Salvia miltiorrhiza*, national health insurance research database, pharmacoepidemiology, danshen (*Salvia miltiorrhiza*), chinese herbal product, traditional chinese medicine

INTRODUCTION

Salvia miltiorrhiza (Danshen) is a deciduous perennial plant and its roots are highly valued in traditional Chinese medicine (TCM) (Zhou et al., 2005). Radix *Salvia miltiorrhiza* (RSM) is one of the most widely used medicinal herbs in China and is now exported to other countries (Hu et al., 2005). It is ranked as a "super grade" medicine in the first official book of Chinese herbal drugs, Shen Nong Materia Medica. RSM is historically known to have beneficial effects on the circulatory system and has been listed in the official Chinese Pharmacopoeia for the treatment of menstrual disorders and blood circulation diseases as well as prevention of inflammation (Matkowski et al., 2008).

Chinese herbal products (CHPs), administered as complementary therapies, have gained widespread popularity in Taiwan. Danshen CHP is indicated for eliminating blood stasis to enhance flow, promoting blood circulation, and regulating menstruation at a daily dose of 1.2–3.6 g in adults. (https://service.mohw.gov.tw/DOCMAP/CusSite/TCMLResultDetail.aspx?LICEWORDID=01&LICENUM=007924#). Danshen was the most commonly used single CHP for ischemic stroke (Hung et al., 2015). However, only a few large-scale pharmacoepidemiological studies have investigated the clinical utilization of RSM. No nationwide population-based surveys have previously been conducted to examine the characteristics of RSM use.

The National Health Insurance (NHI) has provided a universal health insurance program in Taiwan since 1995; this covers both Western medicine and TCM. Almost 98% of all the inhabitants of Taiwan were covered by the NHI program at the end of 2002 (Chen et al., 2014). Therefore, a nationwide population-based study was conducted by analyzing a cohort of one million sampled patients from the NHI Research Database (NHIRD) in Taiwan from 2000 to 2011.

The purpose of this study was to investigate the frequency and characteristics of RSM prescriptions to identify the conditions for which this CHP is prescribed. The results of this study provide valuable information for further pharmacological studies and clinical trials.

METHODS

Data Sources

There are approximately 25.68 million individuals registered in the NHI program in Taiwan (Li and Huang, 2015). This study used data from the Longitudinal Health Insurance Database 2000, a dataset of the NHIRD, which includes all claims data (Huang et al., 2013). The Longitudinal Health Insurance Database 2000 included 1 million randomly selected individuals from the 2000 Registry of Beneficiaries within the NHIRD. The data related to patient identification were encrypted to protect the privacy of all subjects. All outpatient medical information, such as demographic details (gender, date of birth, income status, and urbanization of living area), primary and secondary diagnoses as per the International Classification of Diseases 9th Revision Clinical Modification (ICD-9-CM), procedures, prescriptions, and medical expenditures from 1996 to 2011 are recorded in the NHIRD (Shih et al., 2014). This study was exempted from review by the Internal Review Board of China Medical University and Hospital (CMUH104-REC2-115).

Study Design

In Taiwan, TCM doctors are asked to diagnose a condition based on the ICD-9-CM code (Chien et al., 2013). In this study, ICD-9-CM codes for all patients prescribed RSM were collected. Initially, all 125,566 individuals who received RSM between 1996 and 2011 were selected. Then, all those who received RSM before 2000 were excluded from the study because of diagnosis based on A-code. The case group finally included 104,512 RSM prescriptions, which were recorded for people who used RSM after 2000 for the first time.

A control group of 651,214 subjects was selected from those who visited TCM clinics, but had never used RSM, by randomly selecting subjects with the same TCM clinic visit date as those in the case group, i.e., between 2000 and 2011 (**Figure 1**).

Statistical Analysis

The distribution and comparison of the demographic characteristics of the case and control groups are presented in this study. The odds ratio (OR) and 95% confidence interval (CI) for RSM and RSM-associated risk factors were calculated using multivariable logistic regression, after adjusting for age, gender, urbanization level, occupation, and monthly income. Urbanization was grouped into four levels, with those living in the most urban areas categorized as level 1 and those living in the least urban areas categorized as level 4, as reported by Liu et al. (2006). Occupation was classified as army/education/public sector, farming, fishing, industry, business, or other. Monthly income was grouped into three bands corresponding to a minimum monthly wage of ≤15,840, 15,841–21,900, and >21,900 New Taiwan Dollars (NTD).

The corresponding prescription files were also analyzed and an association rule was applied to evaluate the co-prescription of RSM and other CHPs. The core patterns of disease in RSM users were used as an open-sourced freeware NodeXL (http://nodexl.codeplex.com/) for network analysis. The top-ranked disease and co-disease were used as the widest line, the top 2–5 diseases and co-diseases were used as wide lines, and other combinations were used as thick lines.

Statistical significance was set at $p < 0.05$ for all analyses, and all p-values were two-tailed. SAS version 9.4 (SAS, Cary, NC, United States) was used for statistical analysis.

RESULTS

The detailed demographic characteristics of the RSM user and non-RSM user groups are presented in **Table 1**. The top three age

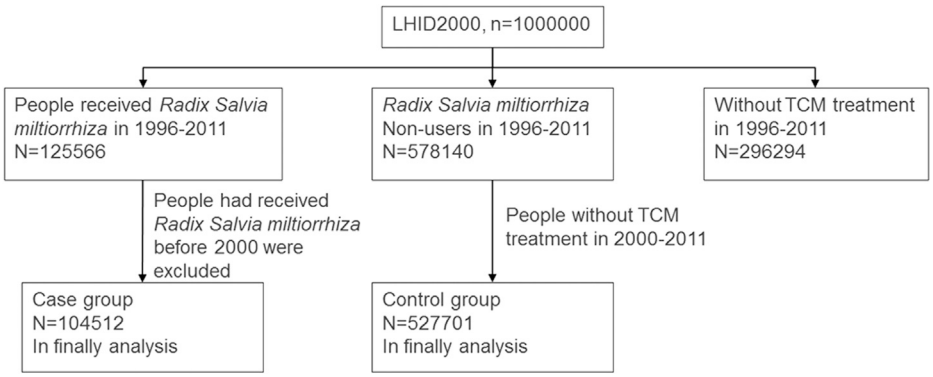

FIGURE 1 | Study flow chart. LHID, Longitudinal Health Insurance Database.

TABLE 1 | Demographic characteristics and multiple logistic regression analysis of radix *Salvia miltiorrhiza* users from 2000 to 2011 among Taiwan Traditional Chinese Medicine users.

	User N = 104,512		Non-user N = 527,701		Adjusted OR	(95% CI)	*p*-value
Age, year							
<20	11,503	11.0	116,098	22.0	Ref.		
20–34	30,973	29.6	149,372	28.3	1.96	(1.91–2.00)	<0.0001
35–49	31,488	30.1	130,042	24.6	2.24	(2.18–2.29)	<0.0001
50–64	19,723	18.9	80,550	15.3	2.27	(2.21–2.33)	<0.0001
65+	10,825	10.4	51,639	9.79	2.12	(2.06–1.81)	<0.0001
Mean (SD)	40.9	(17.2)					
Gender							
Women	68,224	65.3	271,157	51.4	1.75	(1.73–1.78)	<0.0001
Men	36,288	34.7	256,544	48.6	Ref.		
Urbanization							
1 (highest)	34,163	32.7	151,741	28.8	1.26	(1.23–1.28)	<0.0001
2	31,491	30.1	155,752	29.5	1.16	(1.14–1.19)	<0.0001
3	19,124	18.3	100,058	19.0	1.13	(1.11–1.60)	<0.0001
4 (lowest)	19,734	18.9	120,150	22.8	Ref.		
Occupation							
Army/Education/Public	11,812	11.3	52,814	10.0	1.27	(1.22–1.31)	<0.0001
Farmer	10,273	9.83	64,077	12.1	Ref.		
Fisher	1862	1.78	11,612	2.20	1.04	(0.99–1.10)	0.12
Industry	19,435	18.6	93,737	17.8	1.13	(1.09–1.16)	<0.0001
Business	48,880	46.8	241,827	45.8	1.14	(1.11–1.17)	<0.0001
Other	12,250	11.7	63,634	12.1	1.13	(1.09–1.17)	<0.0001
Monthly income, NTD							
≤15,840	41,300	39.5	248,684	47.1	Ref.		
15,841–21,900	39,533	37.8	180,444	34.2	1.04	(1.02–1.05)	0.0001
>21,900	23,679	22.7	98,573	18.7	1.13	(1.11–1.15)	<0.0001

OR, odds ratio; CI, confidence interval; SD, standard deviation; NTD, New Taiwan Dollar.

groups treated with RSM were as follows: patients aged 35–49 years (N = 31,488; 30.1%), patients aged 20–34 years (29.6%), and patients aged 50–64 years (18.9%). Adults were over 1.9-fold more likely to use RSM than subjects aged <20 years. In addition, women were prescribed RSM more frequently than men (women: men = 1.88: l), with an OR of 1.75 (95% CI = 1.73–1.78). The majority of RSM users lived in highly urbanized areas of Taiwan (N = 34,163; 32.7%). Most of the RSM users belonged to the business sector (N = 48,880; 46.8%). Compared with farmers, subjects working in the army/education/public sectors were significantly more likely to be prescribed RSM (OR = 1.27; 95% CI = 1.22–1.31), followed by those who worked in business (OR = 1.14; 95% CI = 1.11–1.17), industry (OR = 1.13; 95% CI = 1.09–1.16), and other sectors (OR = 1.13; 95% CI = 1.09–1.17). The monthly income of subjects who were prescribed RSM was ≤15,840 NTD (N = 41,300; 39.5%). Compared to subjects with this monthly income, those with a high monthly income showed an increase in OR of RSM usage, from 1.04 in those earning 15,841–21,900 NTD to 1.13 in those earning >21,900 NTD. The median daily dose of RSM was 1.5 g, and the most common frequency of administration was three times a day (84.4%).

TABLE 2 | Top 10 diseases (primary code) treated with radix *Salvia miltiorrhiza* from 2000 to 2011 in Taiwan.

Disease (ICD-9-CM)	N (%)	Most commonly combined formulae CHP	N (%)	Most commonly combined single CHP	N (%)
Disorders of menstruation and other abnormal bleeding from female genital tract (626)	36,566 (9.48)	Jia-Wei-Xiao-Yao-San	9,920 (27.1)	Yi-Mu-Cao	12,596 (34.5)
General symptoms (780)	36,497 (9.46)	Jia-Wei-Xiao-Yao-San	6,838 (18.7)	Ye-Jiao-Teng	5,129 (14.1)
Symptoms involving cardiovascular system (785)	16,117 (4.18)	Zhi-Gan-Cao-Tang	6,963 (43.2)	Yu-Jin	2,445 (15.2)
Symptoms involving head and neck (784)	15,940 (4.13)	Chuan-Xiong-Cha-Tiao-San	2,853 (17.9)	Ge-Gen	3,281 (20.6)
Symptoms involving respiratory system and other chest symptoms (786)	15,112 (3.92)	Xue-Fu-Zhu-Yu-Tang	2,794 (18.5)	Yu-Jin	3,513 (23.3)
Disorders of function of stomach (536)	13,122 (3.40)	Ban-Xia-Xie-Xin-Tang	4,841 (36.9)	Yan-Hu-Suo	1,480 (11.3)
Other disorders of soft tissues (729)	11,493 (2.98)	Shu-Jing-Huo-Xue-Tang	2,567 (22.3)	Yan-Hu-Suo	2,408 (21.0)
Chronic liver disease and cirrhosis (571)	11,122 (2.88)	Jia-Wei-Xiao-Yao-San	2,933 (26.4)	Yin-chen-hao	1711 (15.4)
Other and unspecified disorders of back (724)	9,635 (2.50)	Du-Huo-Ji-Sheng-tang	1918 (19.9)	Du-Zhong	2010 (20.9)
Functional digestive disorders, not elsewhere classified (564)	9,618 (2.49)	Mazi-Ren-wan	2,225 (23.1)	Da-Huang	1731 (18.0)

Data shown are out of a total of 385,656 TCM visits.

TABLE 3 | Top 10 combinations of two diseases treated with radix *Salvia miltiorrhiza* from 2000 to 2011 in Taiwan.

Primary disease (ICD-9-CM)	Second disease (ICD-9-CM)	N (%)	Most commonly combined formulae CHP	N (%)	Most commonly combined single CHP	N (%)
Menopausal and postmenopausal disorders (627)	General symptoms (780)	653 (0.17)	Jia-Wei-Xiao-Yao-San	394 (60.3)	Yuan-Zhi	202 (30.9)
Disorders of menstruation and other abnormal bleeding from female genital tract (626)	General symptoms (780)	622 (0.16)	Jia-Wei-Xiao-Yao-San	223 (35.9)	Yi-Mu-Cao	149 (24.0)
Diabetes mellitus (250)	Disorders of lipoid metabolism (272)	556 (0.15)	Liu-Wei-Di-Huang-Wan	132 (23.7)	Ji-Xue-Teng	127 (22.8)
Essential hypertension (401)	General symptoms (780)	530 (0.14)	Jia-Wei-Xiao-Yao-San	114 (21.5)	Ye-Jiao-Teng	95 (17.9)
Other and unspecified disorders of back (724)	Other disorders of female genital organs (729)	434 (0.11)	Du-Huo-Jis-Seng-tang	144 (33.2)	Du-Zhong	204 (47.0)
Chronic liver disease and cirrhosis (571)	General symptoms (780)	417 (0.11)	Jia-Wei-Xiao-Yao-San	182 (43.7)	Huang-Qi	104 (24.9)
Disorders of menstruation and other abnormal bleeding from female genital tract (626)	Functional digestive disorders, not elsewhere classified (564)	410 (0.11)	Jia-Wei-Xiao-Yao-San	94 (22.9)	Yi-Mu-Cao	86 (21.0)
General symptoms (780)	Functional digestive disorders, not elsewhere classified (564)	405 (0.11)	Jia-Wei-Xiao-Yao-San	111 (27.4)	Huang-Qi	82 (20.3)
Other disorders of female genital organs (729)	General symptoms (780)	392 (0.10)	Jia-Wei-Xiao-Yao-San	101 (25.8)	Yuan-Zhi	61 (15.6)
Essential hypertension (401)	Disorders of lipoid metabolism (272)	388 (0.10)	Qi-Ju-Di-Huang-Wan	85 (21.9)	Shan-Cha	81 (20.9)

Data shown are out of a total of 385,656 TCM visits.

Of the 385,656 TCM visits in Taiwan, the top 10 diseases treated using RSM from 2000 to 2011 are presented in **Table 2**. The most common diagnosis for RSM users was "Disorders of menstruation and other abnormal bleeding from the female genital tract (ICD-9-CM: 626)" (N = 36,566; 9.48%), followed by "General symptoms (ICD-9-CM: 780)" (N = 36,497; 9.46%) and "Symptoms involving the cardiovascular system (ICD-9-CM: 785)" (N = 16,117; 4.18%). In disorders of menstruation and other abnormal bleeding from the genital tract of women, the formula and single CHPs most commonly prescribed with RSM were Jia-Wei-Xiao-Yao-San (JWXYS) (27.1%) and Yi-Mu-Cao (34.5%), respectively. In patients with general symptoms, the most commonly prescribed single and formula CHPs with RSM were JWXYS (18.7%) and Ye-Jiao-Teng (14.1%). In patients with symptoms involving the cardiovascular system, the most commonly prescribed single and formula CHPs with RSM were Zhi-Gan-Cao-Tang (43.2%) and Yu-Jin (15.2%).

RSM was also prescribed to treat combinations of two conditions (**Table 3**). Among these, "Menopausal and postmenopausal disorders (ICD-9-CM: 627)" and "General symptoms (ICD-9-CM: 780)" were the most common reasons for RSM use (N = 653; 0.17%). This was followed by "Disorders of menstruation and other abnormal bleeding from the female genital tract (ICD-9-CM:

TABLE 4 | Top 10 combinations of three diseases treated with radix *Salvia miltiorrhiza* from 2000 to 2011 in Taiwan.

Primary disease (ICD-9-CM)	Second disease (ICD-9-CM)	Second disease (ICD-9-CM)	N	%
Anxiety, dissociative and somatoform disorders (300)	Menopausal and postmenopausal disorders (627)	General symptoms (780)	159	0.04
Anxiety, dissociative and somatoform disorders (300)	Erythematosquamous dermatosis (690)	Diseases of hair and hair follicles (704)	105	0.03
Disorders of function of stomach (536)	Cardiac dysrhythmias (427)	Functional digestive disorders, not elsewhere classified (564)	79	0.02
Menopausal and postmenopausal disorders (627)	Anxiety, dissociative and somatoform disorders (300)	General symptoms (780)	72	0.02
Diabetes mellitus (250)	Malignant neoplasm of colon (153)	Hyperplasia of prostate (600)	65	0.02
Diabetes mellitus (250)	Disorders of lipoid metabolism (272)	Essential hypertension (401)	57	0.01
Dementias (290)	Disorders of lipoid metabolism (272)	Essential hypertension (401)	57	0.01
Secondary malignant neoplasm of other specified sites (198)	Malignant neoplasm of trachea, bronchus, and lung (162)	Malignant neoplasm of other and ill-defined sites (195)	53	0.01
Ill-defined descriptions and complications of heart disease (429)	Disorders of function of stomach (536)	Functional digestive disorders, not elsewhere classified (564)	53	0.01
Essential hypertension (401)	Menopausal and postmenopausal disorders (627)	Osteoarthrosis and allied disorders (715)	50	0.01

TABLE 5 | Top 10 single and formula CHPs prescribed with radix *Salvia miltiorrhiza*.

Single CHPs	N (%)	Most common disease (ICD-9-CM)	N (%)	Formula CHPs	N (%)	Most common disease (ICD-9-CM)	N (%)
Yan-Hu-Suo (*Corydalis*, Rhizoma)	35,904 (9.36)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	3,758 (10.5)	Jia-Wei-Xiao-Yao-San	50,689 (13.21)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	11,075 (21.9)
Yu-Jin (*Curcumae*, Tuber)	30,556 (7.96)	General symptoms (780)	4,496 (14.7)	Xue-Fu-Zhu-Yu-Tang	31,043 (8.09)	General symptoms (780)	3,156 (10.2)
Ge-Gen (*Puerariae Lobatae*, Radix)	28,569 (7.45)	General symptoms (780)	4,102 (14.4)	Zhi-Gan-Cao-Tang	22,886 (5.96)	Symptoms involving cardiovascular system (785)	7,579 (33.1)
Xiang-Fu (*Cyperi*, Rhizoma)	26,299 (6.85)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	8,612 (32.8)	Tian-Wang-Bu-Xin-Dan	18,247 (4.76)	General symptoms (780)	5,229 (28.7)
Huang-Qi (*Astragali*, Radix)	25,225 (6.57)	General symptoms (780)	2,968 (11.8)	Shu-Jing-Huo-Xue-Tang	16,570 (4.32)	Other disorders of soft tissues (729)	3,333 (20.1)
Yi-Mu-Cao (*Leonuri,* Herba)	24,877 (6.48)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	13,245 (53.2)	Liu-Wei-Di-Huang-Wan	15,775 (4.11)	General symptoms (780)	1865 (11.8)
San-Qi (*Notoginseng*, Radix)	21,327 (5.56)	Symptoms involving cardiovascular system (785)	2,324 (10.9)	Gan-Lu-Yin	14,973 (3.90)	General symptoms (780)	1826 (12.2)
Ji-Xue-Teng (*Caulis Spatholobi*)	19,553 (5.10)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	2,610 (13.4)	Shao-Yao-Gan-Cao-Tang	14,243 (3.71)	Other disorders of soft tissues (729)	2031 (14.3)
Du-Zhong (*Eucommiae*, Cortex)	17,812 (4.64)	Other and unspecified disorders of back (724)	2,451 (13.8)	Ji-Sheng-Shen-Qi-Wan	13,923 (3.63)	General symptoms (780)	1,358 (9.75)
Da-Huang (Radix *et Rhizoma Rhei*)	16,733 (4.36)	Functional digestive disorders, not elsewhere classified (564)	2,405 (14.4)	Gui-Zhi-Fu-Ling-Wan	13,676 (3.56)	Disorders of menstruation and other abnormal bleeding from female genital tract (626)	5,158 (37.7)

Data shown are out of a total of 383,731 outpatient visits; CHP, Chinese herbal product.

626)" and "General symptoms (ICD-9-CM: 780)" (N = 622; 0.16%) and "Diabetes mellitus (ICD-9-CM: 250)" and "Disorders of lipid metabolism (ICD-9-CM: 272)" (N = 556; 0.15%). The most commonly prescribed formula and single CHPs with RSM for "Menopausal and postmenopausal disorders" and "General symptoms" were JWXYS (60.3%) and Yuan-Zhi (30.9%), respectively; for "Disorders of menstruation and other abnormal bleeding from the female genital tract" and "General symptoms," JWXYS (35.9%) and Yi-Mu-Cao (24.0%), respectively; and for "Diabetes mellitus" and "Disorders of lipid metabolism," Liu-Wei-Di-Huang-Wan (23.7%) and Ji-Xue-Teng (22.8%), respectively.

RSM was also prescribed for combinations of three conditions (**Table 4**). The most frequent combination was "Anxiety, dissociative, and somatoform disorders (ICD-9-CM: 300)," "Menopausal and postmenopausal disorders (ICD-9-CM: 627)," and "General symptoms (ICD-9-CM: 780)" (N = 159; 0.04%). The second most

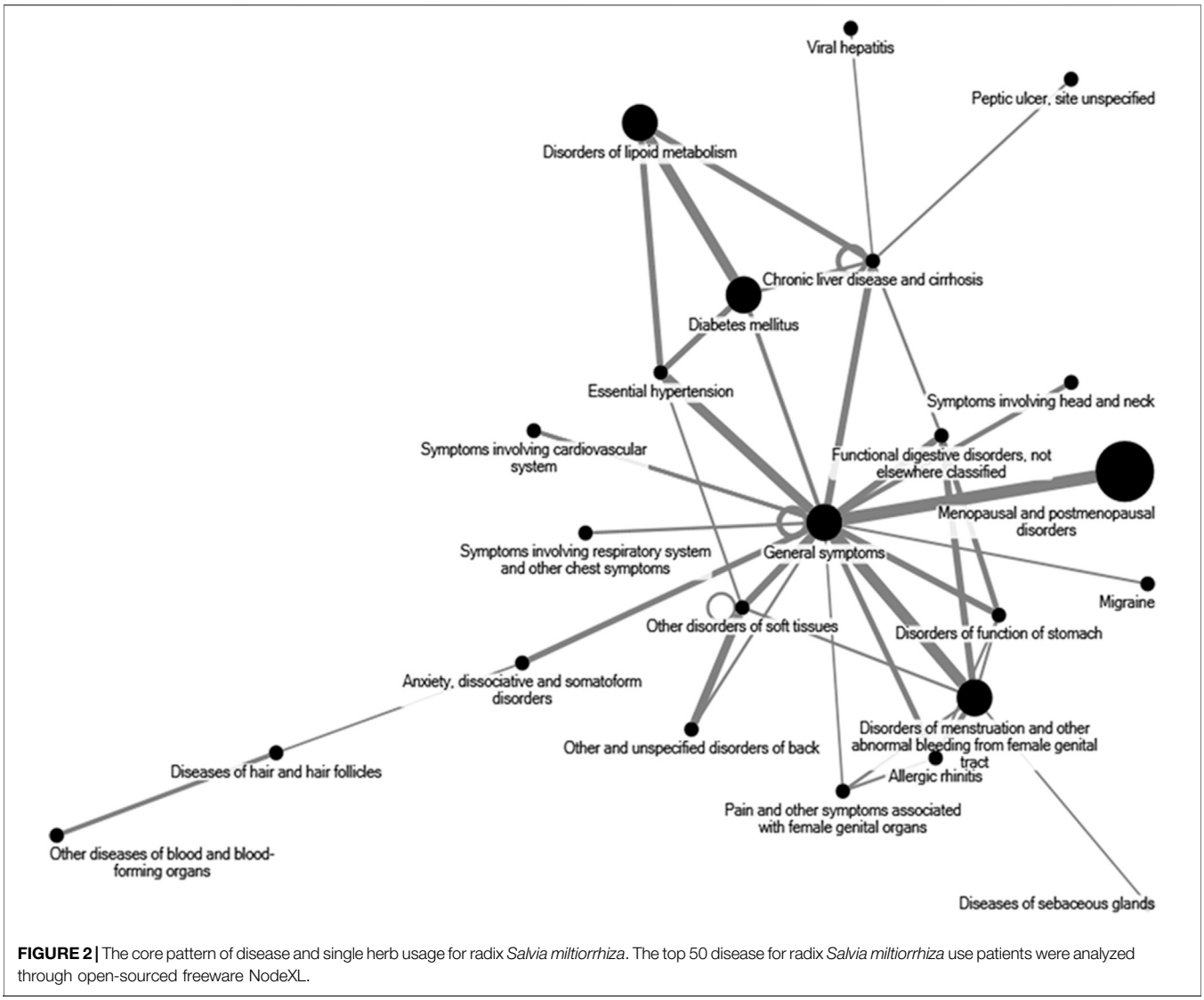

FIGURE 2 | The core pattern of disease and single herb usage for radix *Salvia miltiorrhiza*. The top 50 disease for radix *Salvia miltiorrhiza* use patients were analyzed through open-sourced freeware NodeXL.

frequent combination was "Anxiety, dissociative, and somatoform disorders (ICD-9-CM: 300)," "Erythematosquamous dermatosis (ICD-9-CM: 690)," and "Diseases of hair and hair follicles (ICD-9-CM: 704)" (N = 105; 0.03%). The third most frequent combination was "Disorders of stomach function (ICD-9-CM: 536)," "Cardiac dysrhythmias (ICD-9-CM: 427)," and "Functional digestive disorders, not elsewhere classified (ICD-9-CM: 564)" (N = 79; 0.02%).

Between 2000 and 2011 in Taiwan, 383,731 outpatient visits involved RSM prescriptions by TCM physicians. **Table 5** presents the most commonly prescribed single and formula CHPs with RSM, and the frequency of these prescriptions. Yan Hu Suo (N = 35,904; 9.36%) was the most commonly prescribed single CHP, followed by Yu-Jin (N = 30,556; 7.96%) and Ge-Gen (N = 28,569; 7.96%). JWXYS (N = 50,689; 13.21%) was the most commonly prescribed formula CHP with RSM, followed by Xue-Fu-Zhu-Yu-Tang (N = 31,043; 8.09%) and Zhi-Gan-Cao-Tang (N = 22,886; 5.96%). The most common conditions for which the top three single CHPs were prescribed with RSM included "Disorders of menstruation and other abnormal bleeding from female genital tract" (10.5%, Yan-Hu-Suo), "General symptoms" (14.7%, Yu-Jin), and "General symptoms" (14.4%, Ge-Gen). The most common conditions for which the top three formula CHPs were prescribed included "Disorders of menstruation and other abnormal bleeding from female genital tract" (21.9%), "General symptoms" (10.2%), and "Symptoms involving cardiovascular system" (33.1%).

Figure 2 shows the core pattern of disease and RSM use. The core patterns of disease in RSM users were "Menopausal and postmenopausal disorders," "General symptoms," "Disorders of menstruation and other abnormal bleeding from female genital tract," "Diabetes mellitus," and "Disorders of lipid metabolism."

DISCUSSION

This nationwide population-based study was designed to investigate the conditions for which RSM is commonly

prescribed by licensed TCM doctors. The present study showed that RSM was most frequently prescribed for patients with disorders of menstruation and other abnormal bleeding from the female genital tract (ICD-9-CM: 626) in Taiwan. This may be because TCM doctors considered the function of RSM to be similar to that of Si-Wu-Tang. In ancient times, it was believed that the function of RSM was similar to that of Si-Wu-Tang, which has been used as a classical formula to treat menstruation disorders. From this perspective, it is easy to understand why RSM has been widely used in the treatment of gynecological diseases (Zheng et al., 2015). RSM was traditionally used to remove stasis and relieve pain, activate blood to promote menstruation, clear heart fire, and cause tranquilization (Yuan et al., 2015).

A previous study has revealed that RSM is the most frequently prescribed single CHP for menopausal syndrome (Chen et al., 2011). The results of this study showed that "Menopausal and postmenopausal disorders" and "General symptoms" was the most common combination of two diseases for which RSM is prescribed (**Table 3**). Tanshinone IIA (one of the main constituents of RSM) exerts several beneficial effects for the treatment of postmenopausal symptoms, including cardiovascular protection, prevention of bone loss, prevention of skeletal muscle loss, and anti-carcinogenicity; these involve the binding of tanshinone IIA to estrogen receptors (Zhao et al., 2015). RSM also exerts estrogenic effects by stimulating the biosynthesis of estrogen in circulation, increasing the expression of estrogen receptors in target tissues, and activating estrogen receptor-estrogen response element-dependent pathways (Xu et al., 2016). An ethanol extract of RSM has been reported to suppress trabecular bone loss by inhibiting bone resorption and osteoclast differentiation in menopausal mouse models; therefore, it is thought to be a potential agent for the treatment of osteoporosis (Lee et al., 2020). The main water-soluble compounds in RSM, salvianic acid A and salvianolic acid B, may play a role in the RSM-mediated treatment of infertility by ameliorating oxidative stress-induced damage in H_2O_2-exposed human granulosa cells by inhibiting the overexpression of cleaved caspase-3, cleaved caspase-9, and tumor necrosis factor-α (Liang et al., 2021). Real-world data from the Taiwan NHIRD revealed that RSM exerted protective effects on patients with breast cancer. Additionally, dihydroisotanshinone I, a chemical constituent of RSM, has been reported to suppress the proliferation of breast cancer cells through apoptosis and ferroptosis (Lin et al., 2019). Therefore, RSM is a key medicinal herb for gynecological diseases. The top three gynecological conditions that are treated with radix *Salvia miltiorrhiza* include menstrual disorders and abnormal bleeding from the female genital tract (66.6%); pain and other symptoms associated with female genital organs (15.6%); and menopausal and postmenopausal symptoms (7.82%). (**Table 6**).

The second most frequent diagnosis in patients prescribed RSM in Taiwan was "General symptoms (ICD-9-CM: 780)." The most common conditions in RSM users with "General symptoms" was "Sleep disturbances (ICD-9-CM: 780.5)" (N = 25,249; 69.18%), followed by "Dizziness and giddiness (ICD-9-CM: 780.4)" (N = 7,675; 21.03%). Lee et al. reported that 10 diterpenoids isolated from RSM displaced the binding of [^{3}H] flunitrazepam with gamma-aminobutyric acid-benzodiazepine receptors. Among these compounds, miltirone had the highest binding activity (IC_{50} = 0.3 μM) and was orally active in animal models as a tranquillizer (Lee et al., 1991). Fang et al. reported that administration of an ether extract (600 mg/kg) of RSM significantly decreased sleep latency and increased sleep duration in mice treated with pentobarbital (Fang et al., 2010). Tanshinone IIA showed neuroprotective activity against cerebral ischemia via the inhibition of macrophage migration inhibitory factor (Chen et al., 2012).

The third most frequent diagnosis in patients prescribed RSM in Taiwan was "Symptoms involving the cardiovascular system (ICD-9-CM: 785)." It was in the 1930s that modern chemical and medical methods were first used for studying the active constituents of RSM and its pharmacological actions. RSM exerts its effects on the cardiovascular system and is used mainly to treat coronary artery disease. TanshinoneⅡA is the major compound that yields the most notable results in coronary artery disease treatment (Zheng et al., 2015). According to modern pharmacological studies, RSM and its main components exert protective effects on the cardiovascular and cerebrovascular systems (Yuan et al., 2015). RSM was used to treat "Essential hypertension (ICD-9-CM: 401)" and "Disorders of lipid metabolism (ICD-9-CM: 272; **Table 3**)." It is the most frequently prescribed single herb for hypertension. Multiple pharmacological effects of RSM on the cardiovascular system have been reported, including anti-hypertensive effects (Kang et al., 2002). RSM is the most commonly prescribed single CHP for atrial fibrillation treatment in Taiwan. Patients with atrial fibrillation using TCM have a reduced risk of new-onset ischemic stroke (Hung et al., 2016a). RSM exerts anti-atherosclerotic, anti-cardiac hypertrophic, anti-oxidant, and anti-arrhythmic effects by promoting blood circulation, and it provides relief from blood stasis (Chen et al., 2015). It improves microcirculation, causes coronary vasodilatation, suppresses the formation of thromboxane, inhibits platelet adhesion and aggregation, and protects against myocardial ischemia (Cheng, 2005). RSM protects endothelial cells, exerts anti-inflammatory effects, reduces lipid peroxidation, and prevents calcium overload. RSM has been frequently used to treat hyperlipidemia, chronic hepatitis, hepatic fibrosis, chronic renal failure, and gynecological conditions, including dysmenorrhea, amenorrhea, and lochioschesis, without any serious adverse effects (Peng et al., 2001; Chen et al., 2013). This explains why RSM is commonly prescribed by TCM doctors for the treatment of "Symptoms involving the cardiovascular system."

RSM was also prescribed to patients in Taiwan with "Chronic liver disease and cirrhosis (ICD-9-CM: 571)" and "Functional digestive disorders, not elsewhere classified (ICD-9-CM: 564; **Table 2**)." Recent studies have shown that RSM and its main constituents demonstrate protective effects in models of liver injury induced by carbon tetrachloride, D-galactosamine, acetaminophen, and alcohol administration. Several active ingredients that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage have

TABLE 6 | Top 10 gyncological diseases (primary code) treated with radix *Salvia miltiorrhiza*.

Disease (ICD-9-CM)	N (%)	Most commonly combined formula CHP	N (%)	Most commonly combined single CHP	N (%)
Disorders of menstruation and other abnormal bleeding from female genital tract (626)	106,102 (66.6)	Jia-Wei-Xiao-Yao-San	9,920 (12.3)	Yi-Mu-Cao (Leonuri, Herba)	12,596 (10.0)
Pain and other symptoms associated with female genital organs (625)	24,895 (15.6)	Dang-Gui Shao-Yao-San	2092 (11.5)	Yi-Mu-Cao (Leonuri, Herba)	2,493 (8.90)
Menopausal and postmenopausal disorders (627)	12,456 (7.82)	Jia-Wei-Xiao-Yao-San	2,211 (19.3)	Di-Gu-Pi (Lycium barbarum L.)	478 (2.82)
Noninflammatory disorders of vagina (623)	4,269 (2.68)	Wan-Dai-Tang	513 (14.0)	Yi-Mu-Cao (Leonuri, Herba)	255 (5.58)
Infertility, female (628)	4,134 (2.59)	Jia-Wei-Xiao-Yao-San	322 (12.1)	Tu-Si-Zi (Cuscuta chinensis Lam.)	369 (7.55)
Endometriosis (617)	3,937 (2.47)	Gui-Zhi-Fu-Ling-Wan	409 (18.6)	Yi-Mu-Cao (Leonuri, Herba)	219 (5.69)
Other current conditions in the mother classifiable elsewhere, but complicating pregnancy, childbirth, or the puerperium (648)	705 (0.44)	Gui-Pi-Tang	112 (11.4)	Huang-Qi (Astragali, Radix)	80 (5.54)
Disorders of uterus, not elsewhere classified (621)	673 (0.42)	Dang-Gui Shao-Yao-San	48 (16.8)	Xiang-Fu (Cyperi, Rhizoma)	46 (7.29)
Inflammatory disease of ovary, fallopian tube, pelvic cellular tissue, and peritoneum (614)	581 (0.36)	Gui-Zhi-Fu-Ling-Wan	109 (17.4)	Xiang-Fu (Cyperi, Rhizoma)	112 (12.1)
Noninflammatory disorders of ovary, fallopian tube, and broad ligament (620)	397 (0.25)	Gui-Zhi-Fu-Ling-Wan	56 (17.1)	Xiang-Fu (Cyperi, Rhizoma)	29 (4.92)

Data shown are from a total of 159,363 outpatient visits; CHP, Chinese herbal product.

been identified (Peng et al., 2001). Some animal studies have shown that RSM exerts protective effects on the intestinal mucosa of rats with severe acute pancreatitis and obstructive jaundice, perhaps by inhibiting apoptosis and downregulating the expression of nuclear factor-κB at the protein level (Kim et al., 2005; Zhang et al., 2010). Previous studies have shown that RSM can exert protective effects on the intestinal mucosa in animal models of acute pancreatitis (Kim et al., 2005) and obstructive jaundice by reducing the translocation of intestinal bacteria in patients (Chen et al., 2013).

This study showed that RSM was prescribed for patients with "Secondary malignant neoplasm of other specified sites (ICD-9-CM: 198)," "Malignant neoplasm of trachea, bronchus, and lungs (ICD-9-CM: 162)," and "Malignant neoplasm of other and ill-defined sites (ICD-9-CM: 195; **Table 4**). Tanshinone IIA is a derivative of phenanthrene-quinone that shows cytotoxic activity against many human carcinoma cell lines, induces differentiation and apoptosis and inhibits invasion and metastasis of cancer cells. It is thought to function by inhibiting DNA synthesis and proliferation in cancer cells, regulating the expression of genes associated with proliferation, differentiation, and apoptosis, inhibiting the telomerase activity of cancer cells, and altering the expression of cell surface antigens (Yuan et al., 2003; Shan et al., 2009). The specific components responsible for the antitumor activity of RSM may be a group of diterpenoids with furano-1,2- or furano-1,4-naphthoquinone skeletons (tanshinones); however, the mechanism of action of these compounds is yet to be elucidated. In addition, salvinal, isolated from RSM, has been shown to inhibit proliferation and induce apoptosis of various human cancer cells (Peng et al., 2001). Therefore, salvinal may be useful for the treatment of human cancers, particularly in patients with drug resistance (Chang et al., 2004). Salvinal exhibits no cross-resistance with current microtubule inhibitors, including vinca alkaloids and taxanes, in cells overexpressing P-glycoprotein or multidrug resistance-related proteins (Chang et al., 2004). Moreover, the anti-tumor effects of tanshinone IIA include enhancing the apoptosis of advanced cervix carcinoma CaSki cells (Shan et al., 2009), inhibiting the invasion and metastasis of human colon carcinoma cells (Pan et al., 2013), suppressing angiogenesis in human colorectal cancer (Zhou et al., 2012), downregulating the expression of epidermal growth factor receptors in hepatocellular carcinoma cells (Zhai et al., 2009), and reducing Stat3 expression in breast cancer stem cells (Lin et al., 2013; Chen, 2014). The aqueous extracts of RSM have long been used in TCM for the treatment of cancer. Cryptotanshinone has been reported to be a potential anticancer agent (Peng et al., 2001). RSM may inhibit cancer cell proliferation through its anti-oxidant activity against tumor initiation and induce apoptosis or autophagy through reactive oxygen species generation, which inhibits tumor progression, development, and metastasis (Hung et al., 2016b).

In the present study, RSM was prescribed for patients with "Dementia (ICD-9-CM: 290)," "Disorders of lipid metabolism (ICD-9-CM: 272)," and "Essential hypertension (ICD-9-CM: 401; **Table 4**)." The three combined diagnosis groups indicated that dementia had some relationship with circulation and metabolic diseases. Some animal studies have strongly indicated that compound Danshen tablet could help ameliorate learning and memory deficits in mice by rescuing the imbalance between the levels of cytokines and neurotrophins (Teng et al., 2014). In addition to RSM, compound Danshen tablet contains *Panax notoginseng* and borneol. Several active ingredients of compound Danshen tablet have been shown to

exert therapeutic effects in animal models of Alzheimer's disease (Yin et al., 2008; Lee et al., 2013). Furthermore, clinical trials have indicated that RSM is an effective agent for the prevention and treatment of Alzheimer's disease (Chang et al., 2004).

The most common diagnosis for RSM users was "Disorders of menstruation and other abnormal bleeding from the female genital tract", followed by "General symptoms" and "Symptoms involving the cardiovascular system". (**Table 2**). These conditions are associated with stress and lifestyle, explaining why most of the RSM users are in the business profession and live in higher urbanized areas. The reason why lower income is associated with RSM use is unclear. Nevertheless, we have presented the data here and hope that sparks discussion. A previous study, which enrolled 2,380 participants from the Stanford Five-City Project in the United States, examined the independent contribution of education, income, and occupation to a set of cardiovascular disease risk factors, such as cigarette smoking, high blood pressure, and high cholesterol (Winkleby et al., 1992). Education was the only factor that was significantly associated with the cardiovascular risk factors; higher education results in better socioeconomic status and thus predicts good health. Although lower income may not directly correlate with lower education status, it may still contribute to the results. The Prospective Urban Rural Epidemiology (PURE) study demonstrates that there are a greater number of cases of cardiovascular diseases and stroke in urban areas than in rural areas (Teo et al., 2013). The urban areas have more business sectors than the rural areas. Farmers living in rural areas exhibit lower cases of cardiovascular diseases than other occupations living in urban areas and thereby had less need to take Danshen in this study. We also note that those with a higher monthly income are more capable of affording medical expenses and visiting the TCM outpatient clinic for Danshen medication.

This study has several limitations. First, a definitive conclusion could not be made about the effectiveness of RSM. The data were collected retrospectively from databases and the choice of herbal medicine was at the discretion of Chinese Medicine practitioners who were trained how to apply RSM in clinical practice. The prescription of RSM is largely dependent on the subjective judgment of TCM doctors. Their educational background, years of experience, and site of practice were not available from the NHIRD. Second, although TCM physicians in Taiwan use ICD-9-CM for diagnosis in clinical practice, no reliable and suitable disease-coding system exists for TCM (Yang et al., 2015b). Consistent with the therapeutic principles of RSM, several variations were observed in the prescription characteristics used in this study. Therefore, the development of a TCM diagnostic coding system in the future will considerably improve TCM research. Third, the data file used in this study was provided by Taiwan NHRI, which had been authorized by the Ministry of Health and Welfare to manage the claims data of the NHI. The latest, updated version of the database by the NHRI is not currently available. Finally, the NHI only provided reimbursement for finished herbal products prescribed by TCM physicians. This did not include decoctions and other herbal preparations provided by pharmacies, and this may have resulted in the underestimation of diagnoses and frequency of RSM utilization. However, this underestimation is likely to be small because most Chinese herbal medicines are reimbursed (Yang et al., 2015a).

CONCLUSION

This is the first large-scale investigation of RSM usage in patients with different conditions. Using claims data from the NHIRD, a nationwide, population-based, cross-sectional, descriptive study was conducted to investigate the conditions and characteristics of RSM use. The largest group of patients prescribed RSM had menstrual disorders, followed by general symptoms, and cardiovascular symptoms. The most common combination of diseases for which RSM was prescribed included Menopausal disorders and General symptoms. These results indicate that RSM is a key medicinal herb for the treatment of gynecological disorders, including menstrual disorders, female genital pain, and menopausal disorders. Women aged 35–49 years, living in the urban areas were the main RSM users. For clinical purposes, RSM was most frequently combined with Yan-Hu-Suo and JWXYS. Further research is needed to strengthen the available clinical evidence regarding the efficacy and safety of RSM, either alone or in combination with other CHPs, in these conditions.

DATA AVAILABILITY STATEMENT

The dataset used in this study is held by the Taiwan Ministry of Health and Welfare (MOHW). The Ministry of Health and Welfare must approve our application to access this data. Any researcher interested in accessing this dataset can submit an application form to the Ministry of Health and Welfare requesting access. Please contact the staff of MOHW (Email: stcarolwu@mohw.gov.tw) for further assistance. Taiwan Ministry of Health and Welfare Address: No.488, Sec. 6, Zhongxiao E. Rd., Nangang Dist., Taipei City 115, Taiwan (R.O.C.). Phone: +886-2-8590-6848.

AUTHOR CONTRIBUTIONS

W-LH and Y-CH conceived and designed the study. W-LH, CH, and C-HM acquired and interpreted the data. Y-JT, C-JC, C-EK, and W-LH drafted the manuscript. C-HM analyzed the data. S-FH critically revised the manuscript. All authors reviewed the manuscript.

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Fractions of Shen-Sui-Tong-Zhi Formula Enhance Osteogenesis *Via* Activation of β-Catenin Signaling in Growth Plate Chondrocytes

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Background: Shen-sui-tong-zhi formula (SSTZF) has been used to treat osteoporosis for decades and shows excellent clinical efficacy. This article aims to explore the optimal anti-osteoporotic ingredient and its precise mechanisms in mice models.

Methods: In this study, we first screened the optimal anti-osteoporosis fraction of SSTZF extract *in vivo*, and then further explored the mechanism of its effects both *in vivo* and *in vitro*. Ten-week-old female C57BL/6J mice were administrated with each fraction of SSTZF. At 10 weeks after ovariectomy (OVX), femurs were collected for tissue analyses, including histology, micro-CT, biomechanical tests, and immunohistochemistry for ALP, FABP4, and β-catenin. Additionally, we also evaluated the mRNA expression level of ALP and FABP4 and the protein expression level of β-catenin after being treated with SSTZF extract in $C_3H_{10}T1/2$ cells. Moreover, we investigated the anti-osteoporosis effect of SSTZF extract on mice with *β-catenin* conditional knockout in growth plate chondrocytes (*β-catenin*^{Gli1ER} mice) through μCT, histology, and immunohistochemistry analyzes.

Results: At 10 weeks after treatment, osteoporosis-like phenotype were significantly ameliorated in SSTZF n-butanol extract (SSTZF-NB) group mice, as indicated by increased trabecular bone area and ALP content, and decreased lipid droplet area and FABP4 content. No such improvements were observed after being treated with other extracts, demonstrating that SSTZF-NB is the optimal anti-osteoporosis fraction. Additionally, the elevated β-catenin was revealed in both OVX mice and $C_3H_{10}T1/2$ cells with SSTZF-NB administered. Furthermore, a significant osteoporosis-like phenotype was observed in *β-catenin*^{Gli1ER} mice as expected. However, SSTZF-NB failed to rescue the deterioration in *β-catenin*^{Gli1ER} mice, no significant re-upregulated ALP and downregulated FABP4 were observed after being treated with SSTZF-NB, demonstrating that SSTZF-NB prevents bone loss mainly via β-catenin signaling.

Conclusion: SSTZF-NB enhances osteogenesis mainly via activation of β-catenin signaling in growth plate chondrocytes. SSTZF-NB is the optimal anti-osteoporosis fraction of SSTZF and it can be considered a salutary alternative therapeutic option for osteoporosis.

Keywords: Shensuitongzhi formula, osteoporosis, osteogenesis, β-catenin signaling, transgenic mice

1 INTRODUCTION

Osteoporosis (OP) is the most common bone disorder around the world. It is characterized by fragile bone fracture, which results from reduced bone mass and deteriorated bone microstructure (Force et al., 2018). Studies have shown that there are 10 million people with osteoporosis in the United States alone, and nearly 34 million people with low bone mass, which means they suffer from an increased risk of osteoporosis (Wu et al., 2019). With the speeding tendency of an aging society, the number of potential OP patients will continuously rise. Currently, the drug for treating OP is still limited due to unexpected side effects, like the impact on the uterus and breast (Cheng et al., 2021). At the moment when Covid-19 is rampant all over the world, it will undoubtedly bring a heavy burden and pressure to the social economy and medical systems (Mattioli et al., 2020). Thus, it is essential to explore and create more alternative therapies for OP treatment (Vandenbroucke et al., 2017). Botanical drugs and their natural extracts attract more and more attention due to the potential anti-osteoporosis effects and the fact that they are relatively safe (Zhang et al., 2016; Zhu et al., 2018).

Traditional Chinese Medicine (TCM) has been widely used for various medical purposes for centuries in East Asia. According to the "kidney dominates bone" theory in TCM, OP is the result of kidney deficiency and decreased marrow. Therefore, the therapeutic strategy should emphasize tonifying kidney and regulating the marrow. Based on the theory mentioned above, the Shen-sui-tong-zhi formula (SSTZF) was devised and has been used for treating bone-related disorders for dozens of years. SSTZF is an experiential effective recipe devised by well-known doctors of the Zhejiang School and is modified from Yougui Pills, which was first recorded in *"jin yue quan shu,"* a medical classic text written by Jingyue Zhang in the Ming Dynasty. It encompasses *Carthamus tinctorius L.* (CTL), *Rehmannia glutinosa (Gaertn.) DC.* (RG), *Eucommia ulmoides oliver* (EUO), *Aconitum carmichaelii debeaux* (ACD), *Lycium barbarum L.* (LBL), *Cornus officinalis Siebold & Zucc.* (COS), *Dioscorea oppositifolia L.* (DOL) *Glycyrrhiza glabra L.* (GGL), and *Prunus davidiana (CarriŠre) Franch* (PD), *Cinnamomum cassia (L.). J.Presl* (CCL). In the context of Yougui Pills, the combination of RG, EUO, ACD, LBL, COS, DOL, GGL, and CCL has proven efficacy for bone protection and reveals the potential of activating β-catenin signaling (Yan et al., 2018; Zhang et al., 2019). In addition, several studies have indicated that CTL and its active ingredients could promote BMSCs and differentiate into osteoblasts and show a capacity for bone protection (Kim et al., 2002; Alam et al., 2006; Cui et al., 2019). PD is included to inhibit adipogenesis (Choi et al., 1991a; Choi et al., 1991b; Jung et al., 2017). Combining osteogenic botanical drugs and anti-adipogenic botanical drugs with bone-friendly components has offered a promising alternative therapy for bone related disease, especially for OP. Our previous study indicated that SSTZF drug serum promotes osteoblast proliferation and mineralization in the β-catenin related pathway *in vitro*. However, the precise underlying mechanisms and optimal fractions remain unclear, which limits its further exploitation and promotion.

Bone is a living organ in vertebrates. In clinical practice, bone loss and fat accumulation in bone marrow were found in age-related OP (Pei and Tontonoz, 2004). Even though the mechanism of OP remains unclear, more and more evidence indicates that the unbalance between osteogenesis and adipogenesis plays an important role during the progression (Colaianni et al., 2015; Zou et al., 2020). As is well-known, mesenchymal stem cells (MSCs) are a type of pluripotent stem cell that can differentiate into mesenchymal tissue lineage including osteoblast and adipocyte, the promotion of osteogenesis inhibits adipogenesis and vice versa (Lorthongpanich et al., 2019). During the OP process, the balance between osteogenesis adipogenesis is disturbed and the latter occupies an advantage. The ratio of fat in bone marrow leads to the reduction of osteoblasts, osteoclasts are activated afterward and finally causes the loss of bone mass (Uezumi et al., 2010; Chen et al., 2016; Hu et al., 2018; Liu et al., 2020). There are multitudinous signaling pathways and factors involved in this complex process, there into, the canonical Wnt/β-catenin signaling deserves attention. Research has indicated that β-catenin signaling regulates the differentiation of MSCs as a switch directly (Qiang et al., 2012). To be specific, once the β-catenin was inhibited, the osteogenesis of MSCs was restricted subsequently and the adipogenesis was enhanced on the contrary (de Winter and Nusse, 2021). Therefore, targeting β-catenin could be a potential strategy for OP treatment.

In the current study, we screened the optimal fraction of SSTZF in mice, furthermore, the mechanism of the anti-osteoporotic effect of SSTZF extract was elucidated by transgenic mice and cell experiments, *in vivo* and *in vitro* respectively.

2 METHODS AND MATERIALS

2.1 Preparation of SSTZF Extract

All ten botanical drugs in SSTZF (**Table 1**) were provided by the First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou China). The process of SSTZF extract preparation includes two parts: a concentrate of SSTZF preparation and secondary extraction with the organic solvent. The specific steps are as follows:

TABLE 1 | Composition of SSTZ formula.

Chinese name	Botanical name	Family	Parts used	Weight (kg)
Hong hua	Carthamus tinctorius L.	Compositae	Flower	1.5
Di huang	Rehmannia glutinosa (Gaertn.) DC.	Plantaginaceae	Root	4.5
Du Zhong	Eucommia ulmoides Oliver.	Eucommiaceae	Bark	3
Fu zi	Aconitum carmichaelii Debeaux	Ranunculaceae	Root	3
Gou qi	Lycium barbarum L.	Solanaceae	Bark	3
Shan zhu yu	Cornus officinalis Siebold & Zucc.	Cornaceae	Fruit	1.5
Shan yao	Dioscorea oppositifolia L.	Dioscoreaceae	Fruit	3
Gan cao	Glycyrrhiza glabra L.	Leguminosae	Root	1.5
Tao ren	Prunus davidiana (CarriŠre) Franch.	Rosaceae	Seed	3
Rou gui	Cinnamomum cassia (L.) J.Presl	Lauraceae	Bark	1.5

TABLE 2 | Fractions of SSTZF and yield.

Fraction	Isolated solvent	Isolated content (g)	Percentage of total crude extract (%)
SSTZF-PE	Petroleum ether	2.8	2.9
SSTZF-EA	Ethyl acetate	10.3	10.8
SSTZF-NB	Normal butanol	15.4	16.2

After soaking in 6 volumes of distilled water for 1 h, CTL, RG, EUO, ACD, LBL, COS, DOL, GGL were mixed (the total dry weight was 21 kg) and in the ratio of 1:3:2:2:2:1:2:1(w/w) for reflux extraction (three times,1.5 h/time). PD and CCL were soaked in 5 volumes 60% EtOH for 1 h and were mixed in 2: 1(w/w) (the total dry weight was 4.5 kg) for reflux extraction (three times, 1.5 h/time). Then the two portions of extracts were completely mixed into 7.5 L solution and then concentrated into the form of concentrated solution (3.4 g crude drug/mL). The procedures mentioned above are the preparation method of SSTZF concentrates. To extract its optimal anti-osteoporosis fraction, organic solvent including petroleum ether, ethyl acetate, and n-butanol solutions were used for further separation and extraction according to molecular polarity. 95 ml of the extract was diluted in 300 ml of distilled water, then, 300 ml of petroleum ether, 300 ml of ethyl acetate, and 300 ml of n-butanol solution were added and mixed in equal proportions respectively. The mixed solution was poured into the separating funnel and allowed to stand at room temperature for 12 h. The precipitation of the emulsion layer was discarded and the supernatant was collected. Each mixed solution was extracted three times in this mode. A rotary evaporator was used to volatilize the organic solvent completely, then the refined extract powders (2.8 g for petroleum ether extract, 10.3 g for ethyl acetate extract, 15.4 g for normal butanol extract) were obtained (**Table 2**). The powders were redissolved in 300 ml distilled water respectively and stored at −20°C. Each extract fraction was named SSTZF- petroleum ether extract (SSTZF-PE), SSTZF-ethyl acetate extract (SSTZF-EA), and SSTZF-n-butanol extract (SSTZF-NB).

2.2 Experimental Animals

To construct the OVX mice model, 10 week-old female C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang Chinese Medical University (Hangzhou, China). For the sake of constructing growth plate chondrocytes -specific *β-catenin* conditional knockout mice, $\beta\text{-catenin}^{flox/flox}$ mice were crossed with $Gli1\text{-}CreER^{T2}$ transgenic mice to generate $Gli1\text{-}CreER^{T2}$; $\beta\text{-catenin}^{flox/flox}$ mice hereinafter referred to as $\beta\text{-catenin}^{Gli1ER}$ mice (**Table 3**). All original mice were purchased from Jackson Lab (Bar Harbor, ME, United States). To avoid gender-dependent differences, only females were selected for further experiments. To induce conditional gene knockout, tamoxifen was injected for three consecutive days (1 mg/10 g body weight, one time a day, intra-peritonelly) in 1-month-old mice. All studies were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (LZ12H27001).

2.3 Experimental Groups and Drug Administration

All C57BL/6 mice were arranged into six groups (n = 6 in each group) randomly: the sham group, OVX group, SSTZF-PE group, SSTZF-EA group, SSTZF-NB group, and SSTZF group. According to the screening results, the transgenic mice were divided into three groups (n = 6 in each group): Cre-negative group, $\beta\text{-catenin}^{Gli1ER}$ group, and $\beta\text{-catenin}^{Gli1ER}$ + SSTZF-NB group. Both ovaries were removed in all C56BL/6 mice groups except the sham group. Instead, a sham operation that only excised the surrounding fat tissues equally and preserved bilateral ovaries intact was performed. SSTZF-PE, SSTZF-EA, SSTZF-NB were orally administrated to each corresponding group respectively for 10 consecutive weeks (0.2 ml/10 g body weight, once a day) from the day after OVX surgery. After tamoxifen inducement, SSTZF-NB were orally administrated to mice in the $\beta\text{-catenin}^{Gli1ER}$ + SSTZF-NB group. Other groups were treated with an identical dosage of PBS.

2.4 μCT Analyses

Samples of the femur from each group were collected and scanned with micro-CT (μCT). The cross-section of the distal femur metaphysis was reconstructed in three dimensions. Bone mineral density (BMD, g/mm^3), bone volume fraction (BV/TV, %), average trabecular thickness (Tb.Th, mm), the average number of trabecular (1/mm), and average trabecular separation (Tb.Sp, mm) were collected for morphometry quantitative analysis.

TABLE 3 | Breeding of *Gli1-CreER; β-catenin$^{fx/fx}$* mice.

Breeding	Desired progeny
(a) Gli1-CreER × β-catenin$^{fx/fx}$	(a) Gli1-CreER; β-catenin$^{fx/wt}$
(b) Gli1-CreER; β-cateninfx/wt ×β-catenin$^{fx/fx}$	(b) Gli1-CreER; β-catenin$^{fx/fx}$
(c) Gli1-CreER; β-cateninfx/fx ×β-catenin$^{fx/fx}$	(c) Gli1-CreER; β-cateninfx/fx andβ-catenin$^{fx/fx}$

2.5 Biomechanical Testing

To test the modulus of elasticity (MOE) and maximum loading of femurs in six groups of wild type mice, three-point bending test was performed with the use of the Axial-Torsion Fatigue Testing System (Instron, 5569R1412, United States).

2.6 Histology, Histomorphometry, and Immunohistochemistry

The preparation for the production of sections was processed as described previously (Xu et al., 2020). Then 3 μm sections at the site of femur metaphysis were cut coronally for Alcian Blue Hematoxylin/Orange G (ABH) staining. The indexes of histomorphometry including the area of lipid droplets and area of trabecular bone were detected with the use of OsteoMetrics software (Decatur, GA). IHC was performed using anti-alkaline phosphatase (ALP, ARIGO, ARG57422 1: 300), anti-fatty acid-binding protein (FABP4, Abcam, ab92501, 1:200), anti-β-catenin (HuaBio, ER0805, 1:200). The quantitative analyses for positive staining area were performed by the software of image-pro plus6.0 (Media Cybernetics, Silver Spring, United States).

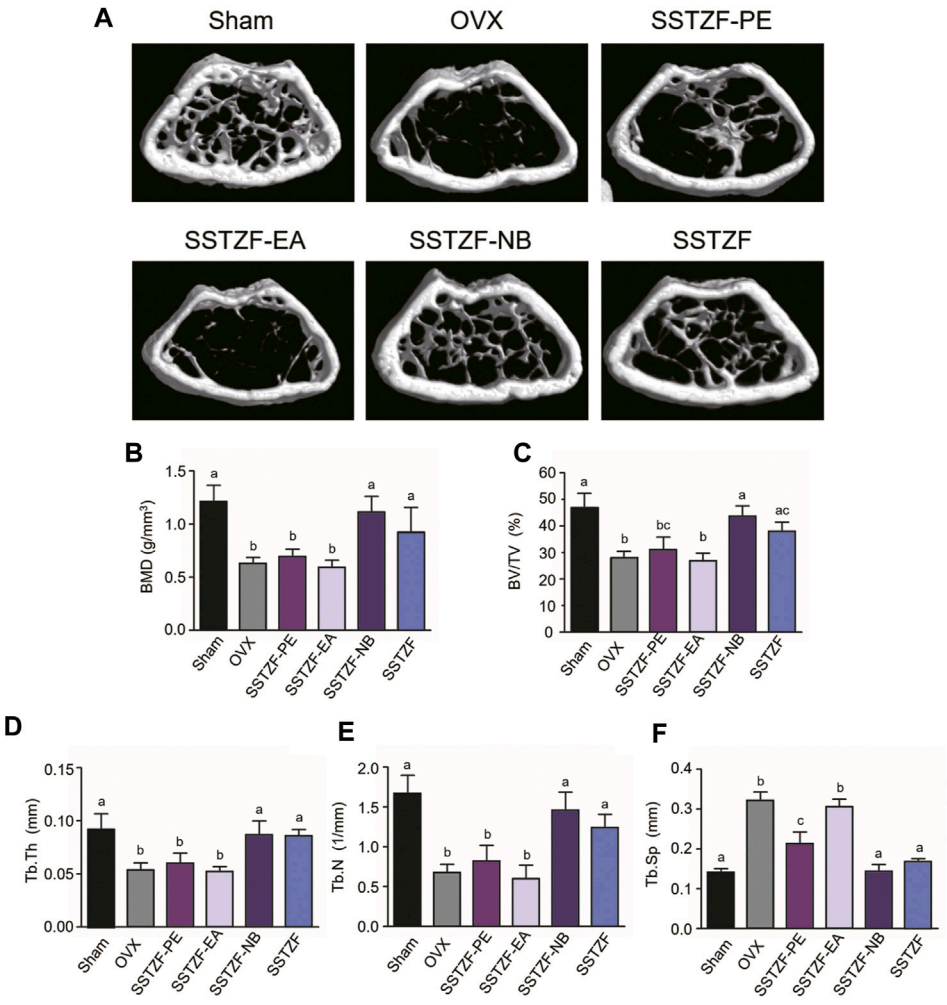

FIGURE 1 | SSTZF-NB prevents bone loss in OVX mice. **(A)** Representative μCT images of each group. Quantification of microstructural data including BMD **(B)**, BV/TV **(C)**, Tb.Th **(D)**, Tb.N **(E)**, and Tb.Sp **(F)**. a–d: The means not sharing a common letter are significantly different among the groups at $p < 0.05$ by one-way ANOVA with Duncan's multiple-range test.

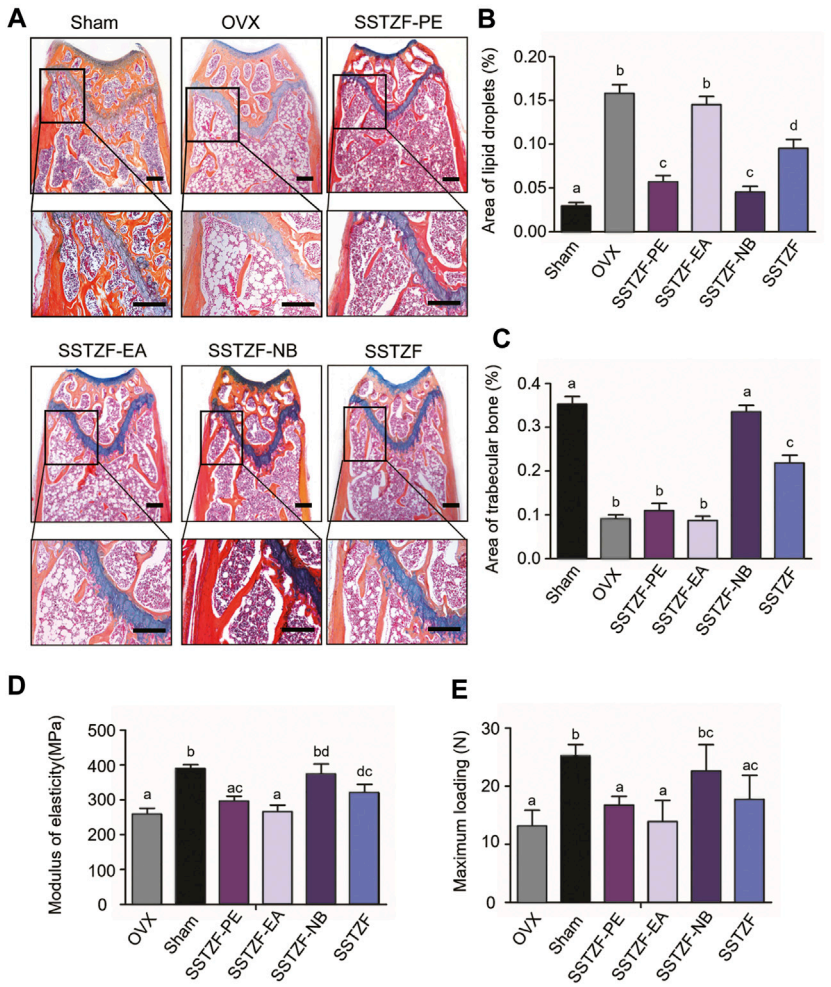

FIGURE 2 | SSTZF-NB increases trabecular bone formation and decreases fat accumulation in the area of the chondro-osseous junction. **(A)** Alcian Blue Hematoxylin/Orange G staining of the distal femur. **(B)** The area of lipid droplets. **(C)** The area of trabecular bone. SSTZF-NB enhances bone strength. **(D)** The modulus of elasticity. **(E)** The max-loading. Scale bars: 1000 μm **A–D**: The means not sharing a common letter are significantly different among the groups at $p < 0.05$ by one-way ANOVA with Duncan's multiple-range test.

2.7 Cell Culture

Mesenchymal stem cells line C3H10T1/2 cells (ATCC, Manassas, VA, United States) were cultured in Alpha modified Eagle's medium (Gibco, MD, United States) containing 10% (v/v) fetal bovine serum (FBS) (Sigma, MO, United States) and 1% penicillin and streptomycin (Gibco) at 37°C in 5% CO_2 atmosphere.10%FBS, FBS containing 10 μg/ml SSTZF-NB and FBS containing 50 μg/ml SSTZF-NB were added to corresponding wells separately. With treatment for 72 h, cells were measured for qRT-PCR and western blot analyzes.

2.8 Real-Time Quantitative PCR

Total RNA was extracted from $C_3H_{10}T1/2$ cells with the use of TRIzol reagent (Invitrogen, CA, United States). Subsequently, 2 μg of total RNA was sucked out and used for cDNA synthesis by RevertAid First Strand cDNA Synthesis Kit (Invitrogen, CA, United States) following the manufacturer's instructions. Then, the expression level of *ALP* and *FABP4* was determined by real-time quantitative PCR [relative to β-actin control with a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Scientific, MA, United States)]. Forward and reverse sequence of the target gene are as follows: primer sequences of ALP, forward: 5′-TCCTGACCAAAAACCTCAAAGG-3′, reverse: 5′-TCGTTCATGCAGAGCCTGC-3′; sequences of FABP4, forward: 5′-AAATCACCGCAGACGACAGG-3′, reverse: 5′-GGCTCATGCCCTTTCATAAAC-3′; sequence of β-actin, forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′, reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′.

2.9 Western Blot

Total proteins were extracted respectively from $C_3H_{10}T1/2$ cells, which were cultured in control serum and serum containing SSTZF-NB, by using lysis buffer containing protease and phosphatase inhibitors, then incubated on ice for 30 min and isolated on a 12% SDS-PAGE gel. Samples were transferred on polyvinylidene fluoride membranes and blocked in 5% milk for

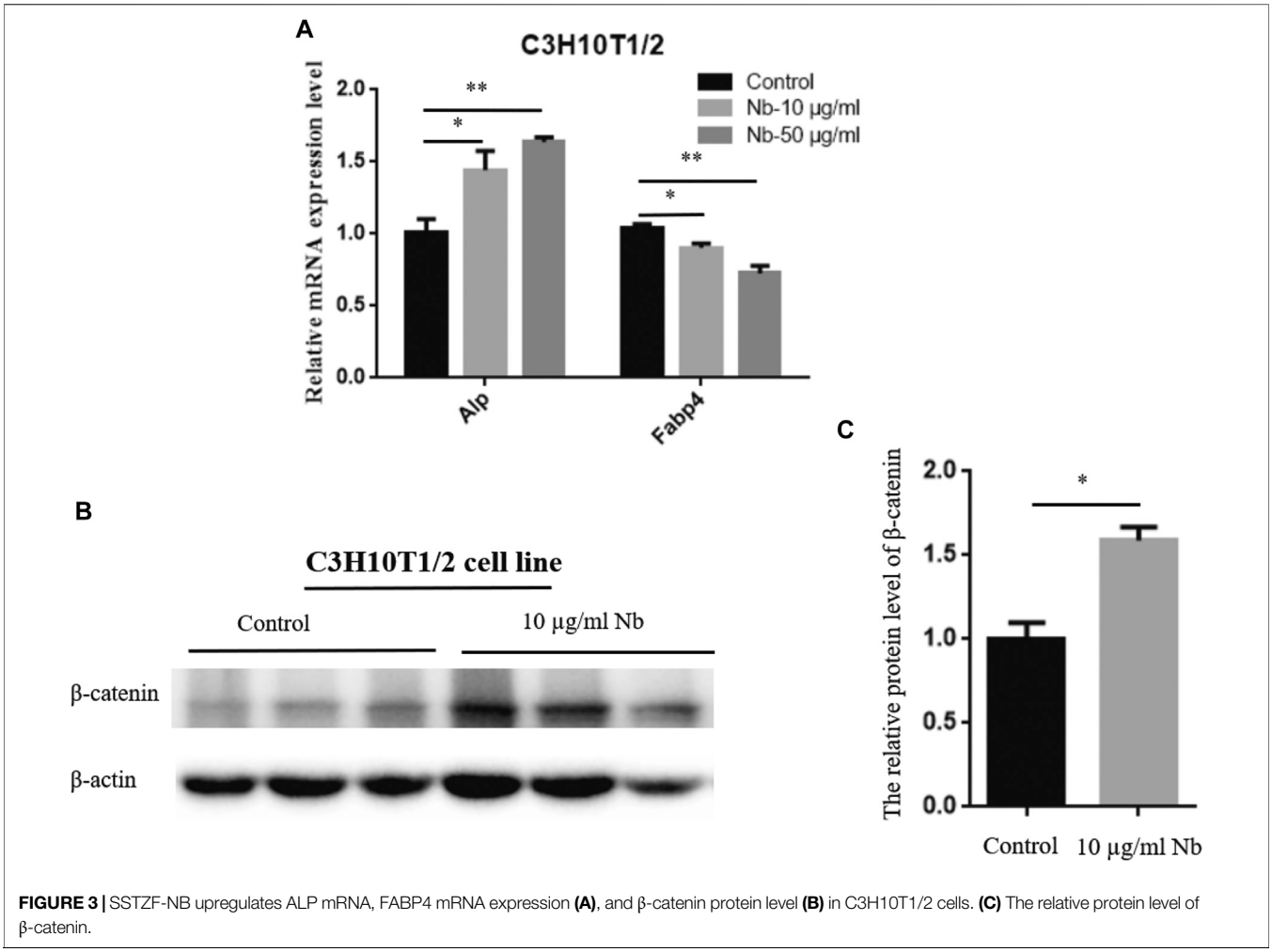

FIGURE 3 | SSTZF-NB upregulates ALP mRNA, FABP4 mRNA expression **(A)**, and β-catenin protein level **(B)** in C3H10T1/2 cells. **(C)** The relative protein level of β-catenin.

1.5 h. The membranes were incubated overnight at 4°C with anti-β-actin (1:5,000, Abcam, United Kingdom) and anti-β-catenin (1: 1,000 HuaBio, CN). TBS-T was used to wash the membranes, and then membranes were incubated for 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000, Abcam, MA, United States). The protein bands were visualized with Image Quant LAS 4000 (EG, United States). Finally, ImageJ was used to calculate the densitometry of each band for quantification.

2.10 UPLC/MS Analysis of SSTZF

Sample preparation for the Ultra Performance Liquid Chromatography (UPLC) analysis was as follows: 1 mg of SSTZF-NB was diluted in methanol/water (50/50) solution. Then, 1 ml of the previous liquid was diluted with methanol to 1/10 concentration. The test sample was harvested after being filtered with 0.22 μm membrane. The conditions of UPLC were as follows: the type of ACQUITY UPLC™ HSS T3 (100 mm × 2.1 mm, 1.8 μm) chromatographic column was selected. The sample size for injection, rate of flow, and column temperature were 1 μl, 0.3 ml/min, and 30°C respectively. The mobile phase was acetonitrile (A)—0.1% formic acid (B) and the elution gradients were as below: 00 min ~ 7 min, 15%A–85%B; 7 ~ 15 min, 35%A–65%B; 15–18 min, 60%A–40%B; 18 ~ 23 min, 90%A–10%B; 23 ~ 26 min, 90%A–10%B; 27–29 min, 5% A–95%B.

For Mass Spectrometry (MS), MS^E continuum mode was selected and the following parameter settings were used: ESI + mode: capillary voltage 3.0 kV; sample cone 40 V; source offset 80 V; source temperature 120°C; desolvation temperature 400°C; cone gas 50 L/h; desolvation gas 800 L/h; nebulizer 6.0Bar. TOF MS and TOF MS/MS were scanned with the mass range of m/z 50–1,500 and 50–1,500, respectively. 100 ng/ml leucine enkephalin solution was used as the calibration standard solution for quality control, and sodium-formate was used to calibrate the instrument. Accurate mass and composition for the precursor and fragment ions were analyzed by using UNIFI software integrated with the instrument. Base peak ion chromatogram of SSTZF-NB is shown in **Supplementary File S1**. The detected compounds are listed in **Supplementary File S2**.

2.11 Statistical Analysis

All data were presented as mean ± standard deviation. Student's t-test and one-way ANOVA test followed by the Tukey-Kramer

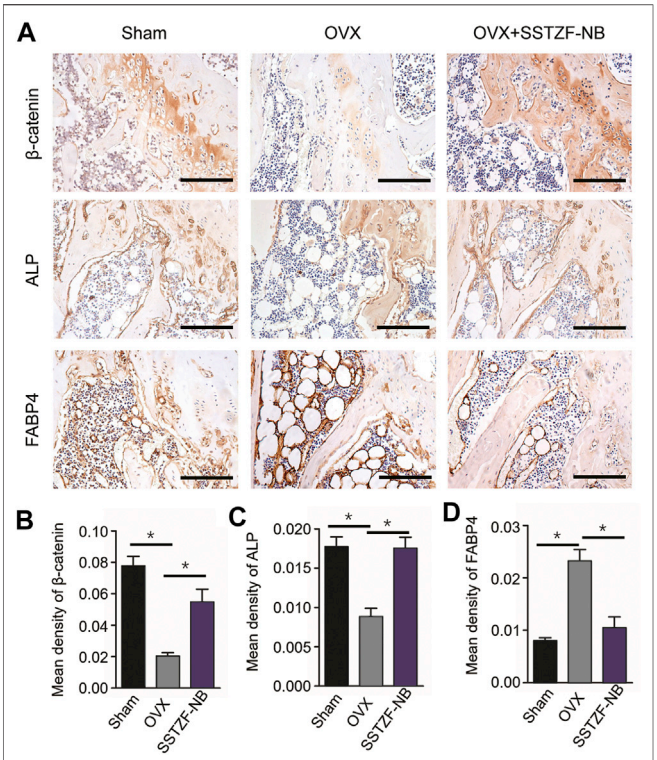

FIGURE 4 | SSTZF-NB enhances osteogenesis and inhibits adipogenesis via activating β-catenin in mice. **(A)** Representative IHC staining images of β-catenin in growth plate chondrocytes, ALP, and FABP4 in the area of the chondro-osseous junction. Semi-quantitative analysis of positive expression of β-catenin **(B)**, ALP **(C)** and FABP4. **(D)** Scale bars: 1,000 μm *$p < 0.001$.

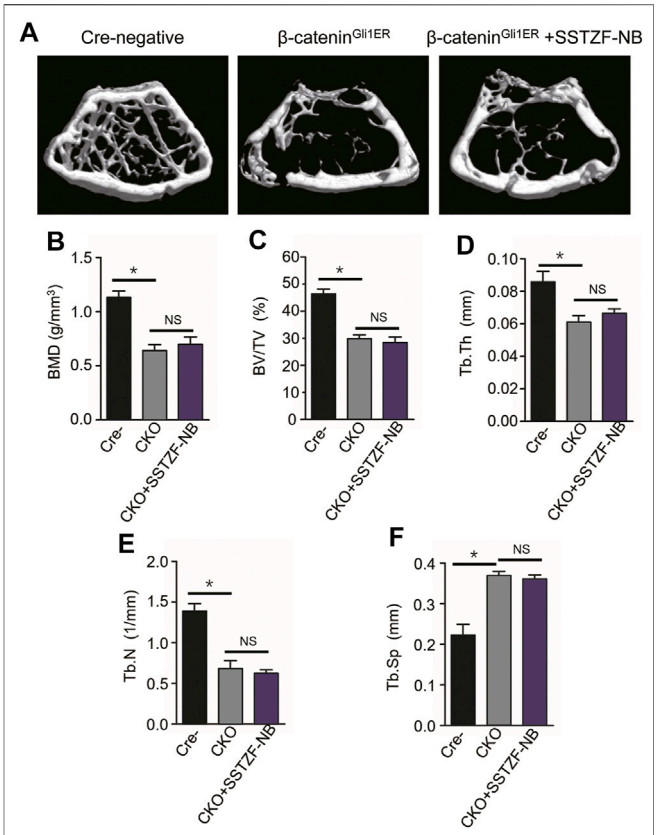

FIGURE 5 | The positive effect of SSTZF-NB preventing bone loss is blocked in *β-catenin^{Gli1ER}* mice. **(A)** Representative μCT image of each group. Quantification of microstructural data including BMD **(B)**, BV/TV **(C)**, Tb.Th **(D)**, Tb.N **(E)**, and Tb.Sp **(F)**. Cre-: Cre-negative mice group, CKO: *β-catenin^{Gli1ER}* mice group, CKO + SSTZF-NB: *β-catenin^{Gli1ER}* mice treated with SSTZF-NB group. NS: no significant difference. *$p < 0.001$.

test were performed using SPSS 24.0 software. *$p < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Screening the Optimal Anti-osteoporotic Fraction of SSTZF Extract

To determine the optimal anti-osteoporotic fraction of SSTZF extract, three kinds of extracts and original SSTZF were orally administrated to the OVX mice respectively. No adverse events happened during the experiment. The 3D reconstruction images revealed that OVX could induce obvious bone loss of the distal femur metaphysis area when compared to sham group mice. SSTZF-PE and the original SSTZF could partially restrain deterioration, however, SSTZF-EA hardly works. Interestingly, SSTZF-NB showed a remarkable inhibitory effect on bone loss when compared to each intervention group (**Figure 1A**). The μCT analysis showed a similar trend. The indexes of BMD, BV/TV, Tb.Th, Tb.N, and Tb.Sp were significantly deteriorated after OVX inducement and significantly ameliorated by SSTZF-NB treatment (**Figures 1B–F**).

Results of ABH staining and histomorphometry analyses showed that OVX surgery could cause serious trabeculae deterioration and fat droplet accumulation in the area of the femoral metaphysis, compared to the sham group. However, the lesion could be alleviated with the treatment of SSTZF-NB, but not with other extract fractions. SSTZF could partially improve the deterioration (**Figures 2A–C**).

Outcomes of biomechanical experiments indicated that OVX could lead to a decreased MOE and reduced max-loading when compared with the sham group, and these indexes were improved only in the SSTZF-NB group (**Figures 2D–E**). All data mentioned above testify that SSTZF-NB is the optimal anti-bone-loss fraction of SSTZF extract.

3.2 SSTZF-NB Fraction Upregulated the Expression of β-Catenin

To determine the possible molecular mechanism of SSTZF-NB anti-osteoporosis, qRT-PCR was performed to examine the effect of SSTZF-NB on the regulation of ALP and FABP4 mRNA expression in $C_3H_{10}T1/2$ cells. Surprisingly, we found that ALP was highly expressed while FABP4 was downregulated in $C_3H_{10T}1/2$ cells with any dose of SSTZF-NB treatment (**Figure 3A**). To provide more evidence of SSTZF-NB

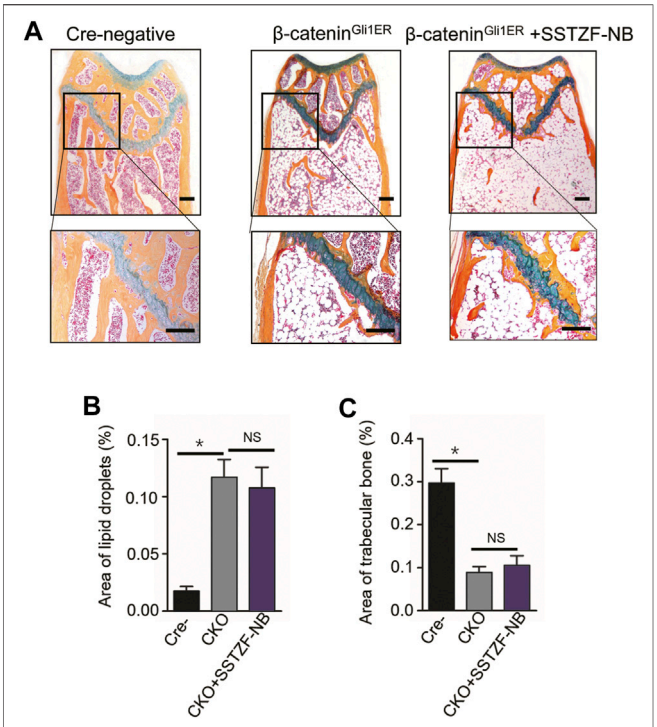

FIGURE 6 | Influence of SSTZF-NB on increases of trabecular bone formation and decreases of fat accumulation in the area of the chondro-osseous junction inhibited in *β-catenin*^{Gli1ER} mice. **(A)** ABH staining of each group. Scale bar: 1000 μm. Morphologic quantitative analyses of **(B)** lipid droplet area and **(C)** trabecular bone area. NS: no significant difference. *$p < 0.001$.

preventing bone loss, western bolt was performed to examine the protein expression level of *β-catenin* after being treated with SSTZF-NB. As expected, *β-catenin* was significantly upregulated in the low dose SSTZF-NB treatment group (**Figures 3B,C**). These data indicate SSTZF-NB anti-osteoporosis possibly via enhancing osteogenesis and inhibiting adipogenesis and are related to the activation of β-catenin.

3.3 SSTZF-NB Fraction Enhanced Osteogenesis and Inhibited Adipogenesis in OVX Mice

To further determine the molecular mechanism of the SSTZF-NB anti-osteoporotic IHC of ALP (an osteogenic specific matrix protein), FABP4 (a fatty acid-binding protein that specifically labels adipocytes) and *β-catenin* were used. Compared to the sham group mice, the expression of ALP was significantly downregulated while FABP4 was upregulated in the area of the chondro-osseous junction in the OVX mice. These phenomena were significantly reversed and ameliorated in the SSTZF-NB treatment group (**Figures 4A–D**). Furthermore, the expression level of *β-catenin* in growth plate chondrocytes was decreased in OVX mice and the reduction was elevated in SSTZF-NB group mice (**Figures 4A,B**).

3.4 SSTZF-NB Fraction Failed to Ameliorate Bone Loss and Lipid Drops Accumulation in *β-catenin*^{Gli1ER} Mice

To provide conclusive evidence about the role of *β-catenin* signaling in SSTZF-NB preventing bone loss, growth plate-specific *β-catenin* conditional KO mice (*β-catenin*^{Gli1ER}) were generated. To examine the anti-osteoporotic effect of SSTZF-NB, μCT analyzes were first performed. Significantly bone loss was observed in *β-catenin*^{Gli1ER} group mice as expected through the 3D constructed images when compared with Cre-negative group mice. Contrary to previous results, SSTZF-NB failed to alleviate the abnormality (**Figure 5A**). The indexes of BMD, BV/TV, Tb.Th, Tb.N, and Tb.Sp were not improved significantly in the *β-catenin*^{Gli1ER} + SSTZF-NB group (**Figures 5B–F**). Additionally, according to the results of ABH staining, significant bone loss and lipid droplet accumulation at the area of femoral metaphysis were found in *β-catenin*^{Gli1ER} group mice compared to cre-negative mice, especially in the chondro-osseous junction area. These pathological changes were also observed in *β-catenin*^{Gli1ER} + SSTZF-NB group mice according to histologic and histomorphometric analyses (**Figures 6A–C**). These data indicate that SSTZF-NB could not ameliorate the osteoporosis-like changes caused by *β-catenin-deficiency*.

IHC assay showed that the protein expression of *β-catenin* decreased significantly in the area of the growth plate chondrocytes in *β-catenin*^{Gli1ER} group mice when contrasted with cre-negative group mice. Furthermore, a decreased protein expression of ALP and an increased protein expression of FABP4, which could lead to osteoporotic-like changes, were also observed in the area of the chondro-osseous junction in *β-catenin*^{Gli1ER} group mice. By contrast, these aberrant osteogeneses (decreased ALP) and adipogenesis (increased FABP4) induced by the inhibition of *β-catenin* signaling in growth-plate-chondrocytes were not restored by SSTZF-NB. No significant difference in the protein expression of ALP and FABP4 was found in *β-catenin*^{Gli1ER} + SSTZF-NB group mice when compared with *β-catenin*^{Gli1ER} group mice (**Figures 7A–D**).

4 DISCUSSION

In this study, we confirmed that SSTZF-NB could ameliorate the osteoporotic-like phenotype and analyzed its potential mechanisms in mice models. Both μCT and histological results demonstrated that SSTZF-NB could prevent bone loss and alleviate fat accumulation. The fact that the positive effect of SSTZF-NB on anti-osteoporotic could be blocked when *β-catenin* was specifically deleted in growth plate chondrocyte which has been reported to have multi-differentiation potential, indicating that the mechanism of SSTZF-NB in preventing bone loss may largely depend on its contribution to the activation of *β-catenin* signaling in growth plate chondrocyte.

TCM is a popular and effective therapeutic method for treating osteoporosis. Shen-sui-tong-zhi formula is a classical anti-osteoporosis TCM that has a favorable curative effect in

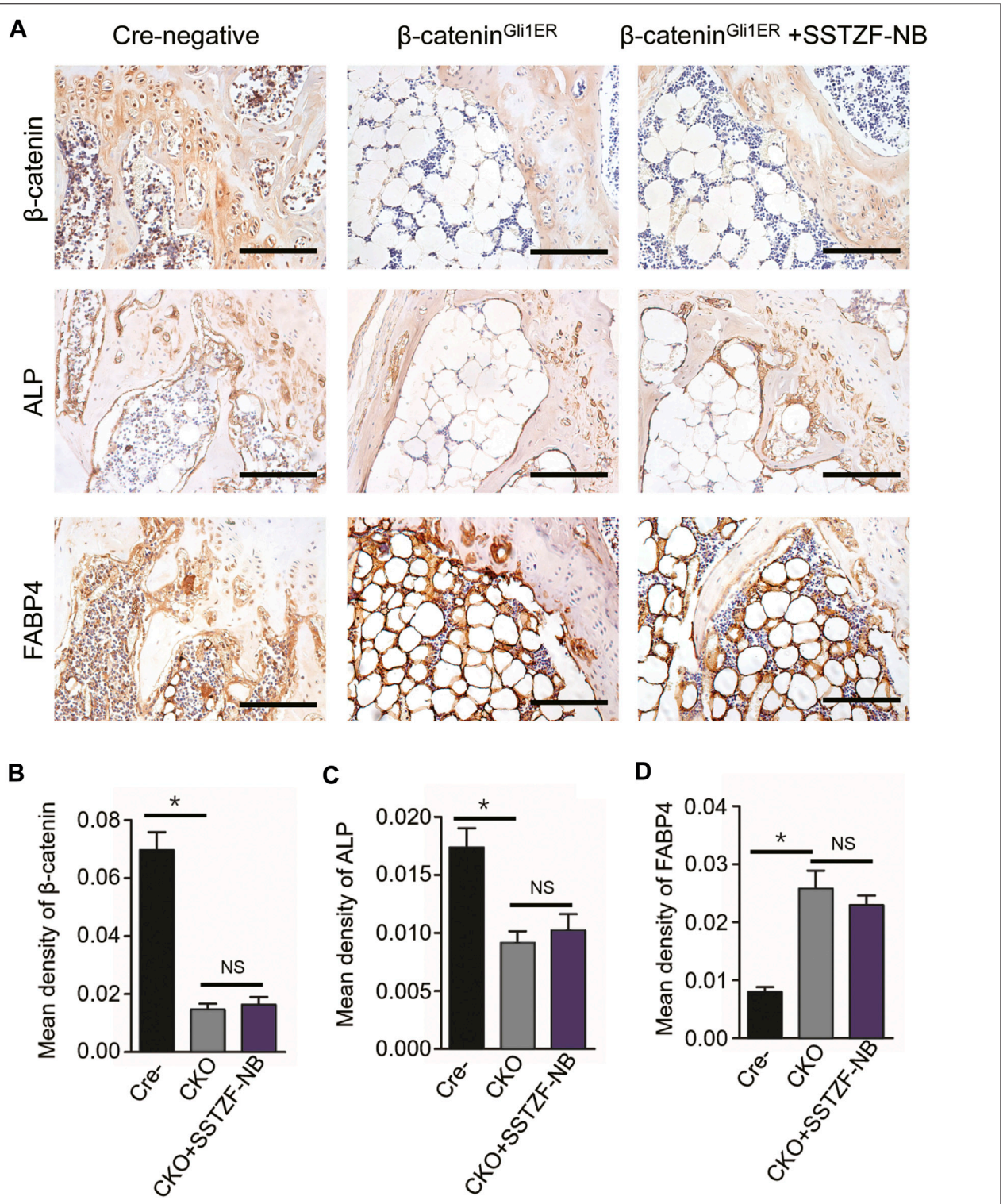

FIGURE 7 | SSTZF-NB failure to restore the down-regulation of ALP and the up-regulation of FABP4 in *β-catenin*^{Gli1ER} mice. **(A)** Representative IHC staining images of β-catenin in growth plate chondrocytes, ALP, and FABP4 in the area of chondro-osseous junction. Scale bars: 1,000 μm. Semi-quantitative analysis of positive expression of β-catenin **(B)**, ALP **(C)** and FABP4 **(D)**. *$p < 0.001$.

China. Although there have been many studies for the identification of the ten botanical drugs contained in SSTZ formula (Qu et al., 2016; Liang et al., 2019; Zheng et al., 2019; Wang et al., 2020), many phytochemicals exist in formula at the same time. Therefore, choosing the appropriate extraction method and combining the active ingredients is important for

enhancing the curative effect and avoiding excessive intake of unnecessary chemical compounds (Wang et al., 2008). In this study, according to molecular polarity, petroleum ether, ethyl acetate, and n-butanol were selected as the extraction media for different fractions of the SSTZ formula. μCT analysis, biomechanical tests, and histomorphometry analysis consistently indicated that SSTZF-NB is the optimal anti-osteoporosis fraction. Then, UPLC/MS was used to identify its material basis. Unsurprisingly, a considerable number of the detected chemical components were reported to have the potential of promoting osteogenesis and anti-osteoporosis, like liquiritigenin, formononetin, etc. (Uchino et al., 2015; Mansoori et al., 2016; Gautam et al., 2017; Carnovali et al., 2020). In our study, the combination of these active components presented a satisfactory effect of preventing bone loss and restraining fat accumulation.

It is well known that *β-catenin* plays a key role in regulating osteogenic differentiation and adipogenic differentiation from MSCs. Moreover, the abnormality of osteogenesis and adipogenesis is a crucial part of the pathological progress of osteoporosis (Wu et al., 2018; Cao et al., 2019). Our research is consistent with previous studies, in which excessive fat accumulation and serious bone loss were observed in the area of chondro-osseous junction, additionally combined with a decreased protein expression of ALP and an increased expression of FABP4. Meanwhile, the expression of *β-catenin* was also downregulated in growth plate chondrocytes. Recent lineage tracing research indicates that Gli1-expressed growth plate chondrocytes have the properties of progenitor cells, which can differentiate into osteoblasts for osteogenesis, not only chondrogenesis (Shi et al., 2017; Haraguchi et al., 2018). Therefore, to further confirm whether SSTZF-NB could cause anti-osteoporosis when *β-catenin* signaling was inhibited in growth plate chondrocytes, $\beta\text{-catenin}^{Gli1ER}$ mice were generated and utilized. However, the positive effect of SSTZF-NB on anti-osteoporosis was almost blocked, neither osteogenesis nor adipogenesis, two of the most important aspects of osteoporosis, were ameliorated following SSTZF-NB administration in $\beta\text{-catenin}^{Gli1ER}$ mice. These results demonstrated that SSTZF-NB enhances osteogenesis and then acts as an anti-osteoporotic function mainly via activation of β-catenin signaling.

5 CONCLUSION

The data of this study illuminated the potentiation and molecular mechanism of SSTZF-NB in treating osteoporosis. Based on the aforementioned findings, we conclude that SSTZF-NB enhances osteogenesis mainly via activation of β-catenin signaling in growth plate chondrocytes. Our findings provide a salutary alternative preventive option for osteoporosis.

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 Animal Ethics Committee of Zhejiang Chinese Medical University.

AUTHOR CONTRIBUTIONS

Study design: RX and HJ. Data collection: RX, QZ, CX, JC, PW, SZ, TX, and WY. Data analysis and interpretation: RX, HL, HX, SL, and ZL. Drafting the article or revising it critically for important intellectual content: RX. Final approval of the article: PT, MG, and HJ.

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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.2021.711004/full#supplementary-material

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Use of Herbal Medicines for the Treatment of Mild Mental Disorders and/or Symptoms During Pregnancy: A Cross-Sectional Survey

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Little is known about the treatment of mild mental disorders and/or symptoms (MDS) during pregnancy. Our main purpose was to compare the use of herbal medicines during pregnancy in women with and without MDS. A questionnaire consisting of 21 multiple-choice questions was distributed in the participating obstetrics clinics or birth centers in the Canton of Zurich, in Switzerland, from August 2018 to March 2019; 398 questionnaires were considered in the analysis. The use of any type of herbal medicines–including pharmaceutical herbal products as well as teas–during pregnancy was reported by 358 women (out of 398, 89.9%). Of these, 272 participants used pharmaceutical herbal products, whereby ginger (49.2%), raspberry leaf (42.7%), bryophyllum (37.8%), chamomile (27.2%), lavender (22%) and iron-rich herbs (12.3%) were the ones most commonly mentioned. More than half (207/398, 52.0%) of all participants reported suffering from MDS during pregnancy; only a few took (synthetic) psychoactive medications (5/398, 1.3%). The percentage of use of pharmaceutical herbal medicines was higher among women reporting MDS than among the remaining women (90.0 vs 75.9%; $p < 0.001$). At the same time, the prevalence of MDS was higher among users of pharmaceutical herbal products than among non-users (59.6 vs 34.0%; $p = 0.001$). Specific questions on candidate herbal medicines for the treatment of mild MDS revealed that bryophyllum (mentioned by 107 women), lavender (56 women) and valerian (20 women) were used to reduce stress, restlessness, sleep disorders and others, in part with perceived good to very good effectiveness and tolerability. The large majority of the pregnant women participating in the survey make use of herbal medicines. The particularly high prevalence of MDS among herbal medicine-users and the very rare use of synthetic psychoactive medications suggest that pregnant women rely on herbal medicines for treatment of mild MDS. The reported good effectiveness and tolerability of a few candidate herbal medicines deserve particular attention.

Keywords: pregnancy, herbal medicines, phytopharmacy, mental health disorders, survey, bryophyllum, lavender, valerian

INTRODUCTION

Little is known about the treatment of mild mental disorders and/or symptoms (MDS) during pregnancy. Most medications for MDS may not only cause side-effects in the mother, but also easily cross the placental barrier and reach the foetus. Concerns on tolerability, teratogenicity and impact on neonatal outcomes exist (Sivojelezova et al., 2005; Rahimi et al., 2006; Grigoriadis et al., 2014; Yonkers et al., 2017; Gao et al., 2018). Pregnant women in need of such medications therefore face a dilemma between using and refraining from synthetic medications.

It is therefore understandable that a considerable proportion of women suffering from mild MDS opt for treatment with herbal medicines, which tend to be perceived as safe (Kalder et al., 2011; Pallivalappila et al., 2013). For instance in Germany, approximately one-fifth of the pregnant women who take herbal medications do it for psychological problems (Munstedt et al., 2013). Even though the toxicity of herbal medications taken during pregnancy has not in most cases been thoroughly investigated, a considerable proportion of health care professionals who deal with pregnant women–midwives, obstetricians, anaesthetists, and especially those with their own experiences–recommend herbal medicine (Stewart et al., 2014). In Switzerland, 40.6% of pregnant women reported using herbal medicine during pregnancy, which was higher than the average proportion detected in a multinational study [average 28.0% (Kennedy et al., 2013)].

Our main purpose was to compare the use of herbal medicines in women with and without MDS. Further goals were to characterise the use, perceived effectiveness and tolerability of a few candidate herbal medicines for mild MDS treatment.

METHODS

Study Design

The present analysis is based on self-reported data from obstetric patients participating in a cross-sectional survey undertaken between August 2018 and March 2019.

Ethics Statement

The study was conducted in accordance with the Helsinki Declaration and with Swiss laws and regulations. In compliance with Swiss Federal Law on data protection (Human Research Act, Article 2), since the data were anonymously collected, no special authorisation was needed. This was confirmed by jurisdictional declaration of the ethics committee of Zurich (Enquire BASEC-Nr. Req-2017-00966; letter from December 14, 2017).

Selection and Description of Participants

The survey took place in the Canton of Zurich–often considered to be representative of the Swiss population as a whole–whose inhabitants correspond to one-sixth of the entire Swiss population. Eight obstetric clinics and birth centres agreed to participate. In these institutions, pregnant women (at or after 28 weeks of pregnancy) or women in the puerperium were invited to participate in the survey if they had not previously completed the questionnaire, could read German, English, French or Italian, and were not emergency patients.

The aim of our survey was to compare the use of herbal medicines during pregnancy in women with and without MDS. Assuming that twice as many pregnant women take herbal medicines for symptoms related to physical conditions than for symptoms related to mental diseases [40 and 20%, respectively, compare with Munstedt et al. (2013)], a sample size of 106 in each group would allow the detection of a difference

TABLE 1 | Herbs mentioned in the questionnaire by common names and corresponding full taxonomical names.

Common names	Full taxonomic names
Mentioned under pharmaceutic products	
Ginger	*Zingiber officinale* Roscoe
Raspberry leaf	*Rubus idaeus* L
Bryophyllum/Goethe plant[a]	*Kalanchoe pinnata* (Lam.) Pers
Chamomile	*Matricaria chamomilla* L[b]
Lavender	*Lavandula angustifolia* Mill
Iron-rich herbs (Floradix®)[c]	-
Echinacea	*Echinacea angustifolia* DC
Lemon balm	*Melissa officinalis* L
Valerian	*Valeriana officinalis* L
St. John's wort	*Hypericum perforatum* L
Horsetail	*Equisetum arvense* L
Passionflower	*Passiflora incarnata* L
Hops	*Humulus lupulus* L
Horse chestnut	*Aesculus hippocastanum* L
Ginseng	*Panax ginseng* C.A. Mey[d]
Golden root (Vitango®)[e]	*Rhodiola rosea* L
California poppy	*Escholzia californica* Cham
Winter cherry	*Withania somnifera* (L) Dunal
Kava	*Piper methysticum* G. Forst
Mentioned under teas	
Fennel	*Foeniculum vulgare* Mill
Chamomile	*Matricaria chamomilla* L[b]
Raspberry leaf	*Rubus idaeus* L
Herbal mixture	-
Peppermint	*Mentha* × *piperita* L
Fruit mixture	-
Lime blossom	*Tilia* × *europaea* L
Verveine/verbena	*Verbena officinalis* L
Rosehip	*Rosa canina* L
Stinging nettle	*Urtica dioica* L
Lady's mantle	*Alchemilla alpina* L
Lemon balm	*Melissa officinalis* L
Orange blossom	*Citrus* × *aurantium* L
Aniseed	*Pimpinella anisum* L
Sage	*Salvia officinalis* L
Elderflower	*Sambucus nigra* L
Cumin	*Cuminum cyminum* L
St. John's wort	*Hypericum perforatum* L
Ginger	*Zingiber officinale* Roscoe
Horsetail	*Equisetum arvense* L
Valerian	*Valeriana officinalis* L

[a] *Bryophyllum was referred to also by an earlier genus name (that is also a trade name) and one common name, as common names (Goethe plant, life plant, air plant, love plant, and Cathedral bells) are relatively unknown in Switzerland.*
[b] *And/or* Chamaemelum nobile *(L.) All.*
[c] *Floradix® is a vitamin- and iron-containing food supplement with natural herbal extracts.*
[d] *And/or* Panax quinquefolius *L.*
[e] *Vitango® is a product based on Golden root (roots and rootstock).*

between the percentage of women taking herbal medicines in the groups with and without MDS. The survey was pursued until 106 women taking (pharmaceutical) herbal medicines in each of the groups with and without MDS had participated.

Questionnaire

The questionnaire consisted of 21 multiple-choice questions and was available in German, English, French and Italian. Piloting of the questionnaire–distributed to 14 women, at least three per language–was conducted to ensure readability and clarity of the questions.

Four of the 21 (complex) questions were related to the intake of herbal medicines during pregnancy and were inspired by a previous survey (Zuzak et al., 2009). The questionnaire distinguished between herbal infusions/teas and pharmaceutical herbal medicines, but did not specify the type of preparation. For a selection of six herbs, detailed information on perceived effectiveness and tolerability was collected. In the questionnaire, the common names of the herbs were used, with a few exceptions (see **Table 1**, also for the correspondence between the used names and full taxonomic names).

To contextualise our results, data on sociodemographic characteristics, acute/chronic disorders and symptoms and synthetic/conventional medications were collected [in part published elsewhere (Randecker et al., 2020)]. To avoid counting missing answers as "non-use" answers, the questions on medicinal herbal medicines included the option "never used"; in these cases, the number of total answers differs from question to question.

We defined an existing MDS on the basis of specific questions. If the women reported suffering from acute or chronic mental disorder, or related symptoms, or reported taking psychotropic drugs, they were considered to have MDS. No MDS severity assessment was performed; psychotic diseases were not addressed in the questionnaire.

Data Collection

A total of 1,653 envelopes–each one containing an information sheet, the questionnaire and a post-paid envelope addressed to the Department of Obstetrics, University Hospital Zurich–were handed out to potential participants in obstetric clinics and birth centres in the Canton of Zurich. Professionals were instructed by the study team to distribute the envelopes to patients during prenatal check-ups or during hospitalisation in the early puerperium. Sealing of the envelopes after insertion of the completed questionnaires was emphasised to the patients to ensure anonymity. Data were entered manually into a Microsoft Excel file.

Statistical Data Analysis

Descriptive statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), Version 25.0. for Windows (IBM® SPSS® Statistics). Pearson's chi-square test was used to compare use of herbal medicines between participants with and without MDS, and MDS prevalence between herbal medicines users and non-users. A two-sided p-value smaller than 0.05 was considered statistically significant; no correction for multiple testing was applied. The number of missing answers is depicted either in the tables or corresponding legends.

RESULTS

Number and Sociodemographic and Health-Related Characteristics of Participants

From a total of 1,653 questionnaires distributed, 398 were completed at or after 28 week's gestation or shortly after birth and returned either by post or through collection boxes placed in the various institutions (overall response rate: 398/1,653, 24.1%). The 398 participants were treated at the following institutions: University Hospital Zurich (n = 164), City Hospital Triemli (n = 61), Hospital Zollikerberg (n = 50), Hospital Bülach (n = 40), Paracelsus-Hospital Richterswil (n = 31), Hospital Limmattal (n = 21) and Delphys Birthing Center (n = 8); in some cases, other institutions (n = 10), more than one institution (n = 10) or none were named (n = 3).

Most women completed the questionnaires in German (362/398, 91.0%), followed by English (28/398, 7.0%), Italian (5/398, 1.3%) and French (3/398, 0.8%). The survey population was comprised mainly of women between 18 and 43 years, with a medium to high level of education (high school to university). The majority of women were from Switzerland (202/398, 50.8%), followed by Germany (64/398, 16.1%) and other European countries (86/398, 21.6%), America (19/398, 4.8%), Asia (16/398, 4.0%), Africa (2/398, 0.5%) and Australia (1/398, 0.3%), others (6/398, 1.5%). The majority of participants had delivered in the days before receiving the questionnaire (221/371, 59.6%); the remaining women were either in pregnancy weeks 28–37 (79/371, 21.3%) or 38-42 (71/371, 19.1%). Women were slightly more often primiparous (193/372, 51.9%) than multiparous. For detailed data, see **Supplementary Table S1**.

About a quarter of women reported chronic disorders (101/373, 27.1%), of which allergies (29/373, 7.8%), thyroid disorders (24/373, 6.4%), and headaches/migraines (20/373, 5.4%) were the most common (**Supplementary Table S2**). The following pregnancy-related acute disorders were the most common: 18.6% of women (69/371) reported suffering from gastroesophageal reflux, 17.0% (63/371) from iron deficiency/anemia, and 14.3% (53/371) from morning sickness. The most commonly reported symptoms during pregnancy were fatigue (294/370, 79.5%), nausea (248/365, 67.9%), heartburn (211/359, 58.8%) and shortness of breath (191/359, 53.2%). For additional information on disorders and symptoms during pregnancy, see **Supplementary Tables S2, S3**, respectively.

MDS During Pregnancy

Four percent of the women (15/372) reported one of the following chronic mental disorders: minor depression (n = 5); mood disorder (n = 5); anxiety disorder (n = 3); major depression (n = 1) and sleeping disorder (n = 1). Moreover, a prevalence of acute mental disorders during pregnancy of 13.2% (48/371) was observed, which

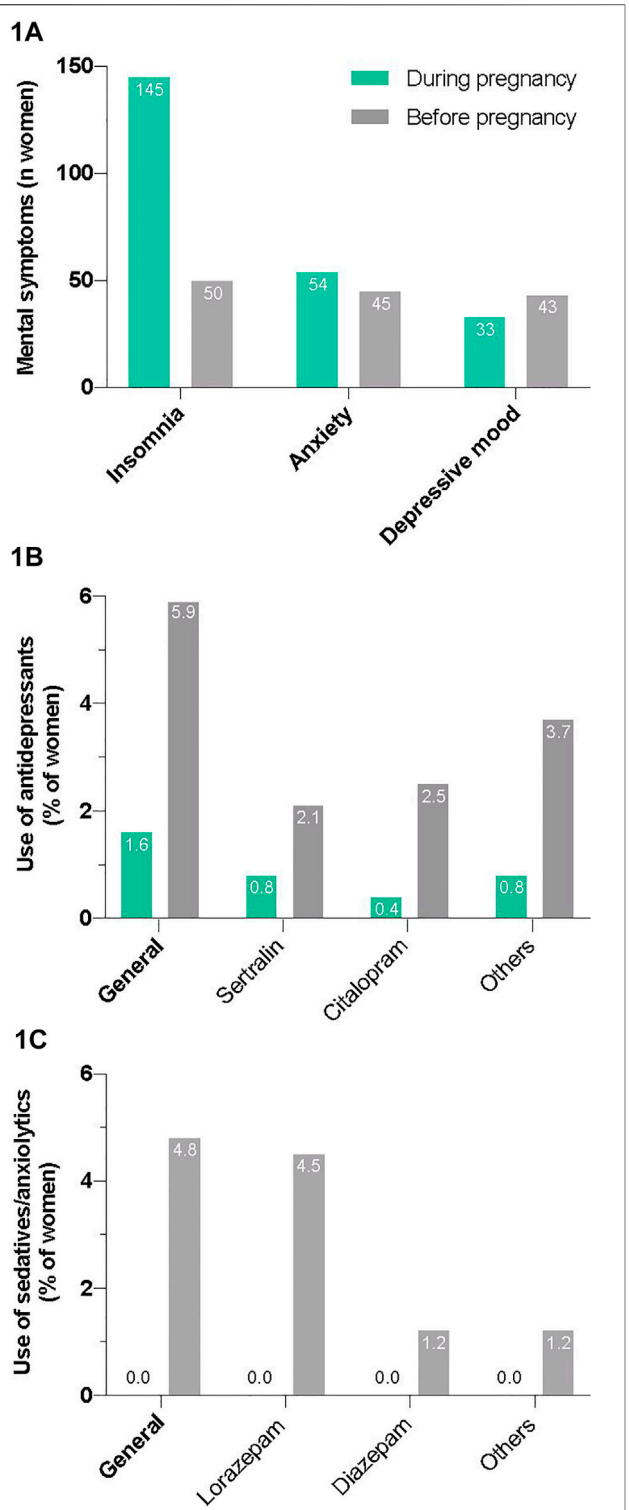

FIGURE 1 | Perceived mental symptoms **(A)** and reported use of antidepressants **(B)** and sedatives/anxiolytics **(C)** during pregnancy and in the time before.

included sleeping disorder ($n = 27$); mood disorder ($n = 13$); minor depression ($n = 5$) and anxiety disorder ($n = 3$) (**Supplementary Table S2**). More than half (51.3%) of the participants reported having suffered from a mental symptom during pregnancy (204/398), namely: insomnia (145/338, 42.9%), anxiety (57/320, 17.8%), and depressive mood (33/335, 9.9%). Compared with the pre-pregnancy period, the prevalence of anxiety and insomnia was higher, whereas the prevalence of depressive mood was slightly lower during pregnancy (**Supplementary Table S3**). **Figure 1A** provides an overview of the prevalence of mental symptoms during and before pregnancy.

Only a few participants used antidepressants (5/315, 1.6%) and none used sedatives/anxiolytics during pregnancy (**Figures 1B,C**; **Supplementary Table S4**). The number of participants taking antidepressants and sedatives/anxiolytics before becoming pregnant was markedly higher (19/321 or 5.9%; and 15/315 or 4.8%, respectively). Two women (2/240; 0.8%) answered that they were taking an antidepressant not mentioned in the questionnaire during pregnancy, namely escitalopram ($n = 1$) and paroxetine ($n = 1$), both selective serotonin reuptake inhibitors (SSRIs); additional other sedatives/anxiolytics were not mentioned.

Use of Herbal Medicines in General and Versus MDS

The vast majority of women reported using some type of herbal medications, either as teas or as pharmaceutic/clinical medicines, during pregnancy (358/398, 89.9%). Of these, 272 participants (272/325, 83.7%) used pharmaceutical herbal products, most frequently ginger (49.2%), raspberry leaf (42.7%), bryophyllum (37.8%), chamomile (27.2%), lavender (22.0%) and iron-rich herbs (12.3%); see **Table 2**. The majority of participants (263/358, 86.5%) who reported using herbal medicines during pregnancy, also used herbal medicines before the current pregnancy. The consumption of teas was very widespread in our study population (**Table 3**).

The percentage of use of pharmaceutical herbal medicines was higher among women reporting MDS than among the remaining women (teas not considered; 162/180, 90.0% vs 110/145, 75.9%; $p < 0.001$). Almost 60% of the women who used pharmaceutical herbal medicines suffered from MDS (162/272, 59.6%), which is significantly higher than the corresponding value for non-users (18/53, 34.0%; $p = 0.001$). More than half of the women who used any type of herbal preparation, teas and pharmaceutical herbal medicines, reported MDS (192/358, 53.6%). **Figure 2** shows the most frequently used herbal medicines, pharmaceutical herbal medicines and teas counted together. Several differences between the groups with and without MDS are apparent. Pregnant women with MDS also more often reported using medicines from integrative and complementary medicine than women without (**Table 4**).

Effectiveness and Tolerability of Candidate Herbal Medicines for MDS Treatment

Some questions on the questionnaire specifically targeted the use of well-known plants for the treatment of mild MDS, namely St. John's wort, hops, valerian, lavender, and bryophyllum.

St. John's wort was taken by 3.5% of women who answered the corresponding questions (12/341), in half of the cases during

TABLE 2 | Use of pharmaceutical herbal medicines during pregnancy by participants with and without mental disorders and/or symptoms (MDS).

Medicine	Total users (*n* = 272)	With MDS (*n* = 162)	Without MDS (*n* = 110)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Ginger	121/246 (49.2)	71/147 (48.3)	50/99 (50.5)	0.734
Raspberry leaf	106/248 (42.7)	58/148 (39.2)	48/100 (48.0)	0.169
Bryophyllum	93/246 (37.8)	75/148 (50.7)	18/98 (18.4)	<0.001
Chamomile	66/243 (27.2)	40/144 (27.8)	26/99 (26.3)	0.794
Lavender	52/236 (22.0)	44/141 (31.2)	8/95 (8.4)	<0.001
Iron-rich herbs	30/243 (12.3)	19/144 (13.2)	11/99 (11.1)	0.628
Echinacea	19/232 (8.2)	13/138 (9.4)	6/94 (6.4)	0.408
Lemon balm	19/232 (8.2)	9/137 (6.6)	10/95 (10.5)	0.280
Valerian	11/234 (4.7)	10/140 (7.1)	1/94 (1.1)	0.031
St. John's wort	7/231 (3.0)	6/138 (4.3)	1/93 (1.1)	0.155
Horsetail	7/235 (3.0)	5/141 (3.5)	2/94 (2.1)	0.531
Passionflower	5/235 (2.1)	5/140 (3.6)	0/95 (0.0)	0.063
Hops	4/231 (1.7)	4/137 (2.9)	0/94 (0.0)	0.095
Horse chestnut	4/229 (1.7)	3/135 (2.2)	1/94 (1.1)	0.510
Ginseng	4/231 (1.7)	1/137 (0.7)	3/94 (3.2)	0.159
Golden root	3/233 (1.3)	3/139 (2.2)	0/94 (0.0)	0.152
Others	23/113 (20.4)	13/72 (18.1)	10/41 (24.4)	0.421

[a]*Percentage values without considering missing data.*

Data are sorted by frequency of total herbal medicine users. The questionnaire also contained items on California poppy, winter cherry and kava; no participant reported their use.

TABLE 3 | The most commonly used teas during pregnancy considering mental disorders and/or symptoms (MDS).

Tea	Total users (*n* = 329)	With MDS (*n* = 172)	Without MDS (*n* = 157)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Fennel	157/329 (47.7)	87/172 (50.6)	70/157 (44.6)	0.301
Chamomile	153/329 (46.5)	82/172 (47.7)	71/157 (45.2)	0.621
Raspberry leaf	150/329 (45.6)	76/172 (44.2)	74/157 (47.1)	0.552
Herbal mixture	130/329 (39.5)	69/172 (40.1)	61/157 (38.9)	0.782
Peppermint	122/329 (37.1)	70/172 (40.7)	52/157 (33.1)	0.175
Fruit mixture	104/329 (31.6)	56/172 (32.6)	48/157 (30.6)	0.672
Lime blossom	68/329 (20.7)	42/172 (24.4)	26/157 (16.6)	0.096
Verveine/verbena	62/329 (18.8)	31/172 (18.0)	31/157 (19.7)	0.609
Rosehip	58/329 (17.6)	36/172 (20.9)	22/157 (14.0)	0.095
Stinging nettle	55/329 (16.7)	25/172 (14.5)	30/157 (19.1)	0.216
Lady's mantle	38/329 (11.6)	24/172 (14.0)	14/157 (8.9)	0.148
Lemon balm	37/329 (11.2)	17/172 (9.9)	20/157 (12.7)	0.328
Orange blossom	36/329 (10.9)	20/172 (11.6)	16/157 (10.2)	0.663
Aniseed	28/329 (8.5)	17/172 (9.9)	11/157 (7.0)	0.439
Sage	26/329 (7.9)	17/172 (9.9)	9/157 (5.7)	0.159
Elderflower	23/329 (7.0)	12/172 (7.0)	11/157 (7.0)	0.997
Cumin	23/329 (7.0)	15/172 (8.7)	8/157 (5.1)	0.193
St. John's wort	14/329 (4.3)	7/172 (4.1)	7/157 (4.5)	0.870
Ginger	11/329 (3.3)	4/172 (2.3)	7/157 (4.5)	0.287
Horsetail	8/329 (2.4)	6/172 (3.5)	2/157 (1.3)	0.190
Valerian	3/329 (0.9)	2/172 (1.2)	1/157 (0.6)	0.613
Others	30/329 (9.1)	18/172 (10.5)	12/157 (7.6)	0.366

[a]*Percentage values without considering missing data.*

Data are sorted by frequency of use of teas by total population.

pregnancy weeks 28–42. Three women reported good to very good tolerability (**Figure 3**) and moderate to good effectiveness. St. John's wort was mostly taken for the treatment of mood disorders (4/12, 33.3%).

Hops was used by 2.3% of women (8/343), in all phases of pregnancy. Hops was tolerated well: three of eight women reported very good tolerability and one of eight reported poor tolerability. Seven of eight women evaluated its effectiveness as very good. The two most frequently reported indications were stress and restlessness (both 3/8, 37.5%), followed by sleep disorders (2/8, 25.0%).

Valerian was used most frequently in the last trimester (7/20, 35.0%). Overall, 20 women reported using valerian (20/344,

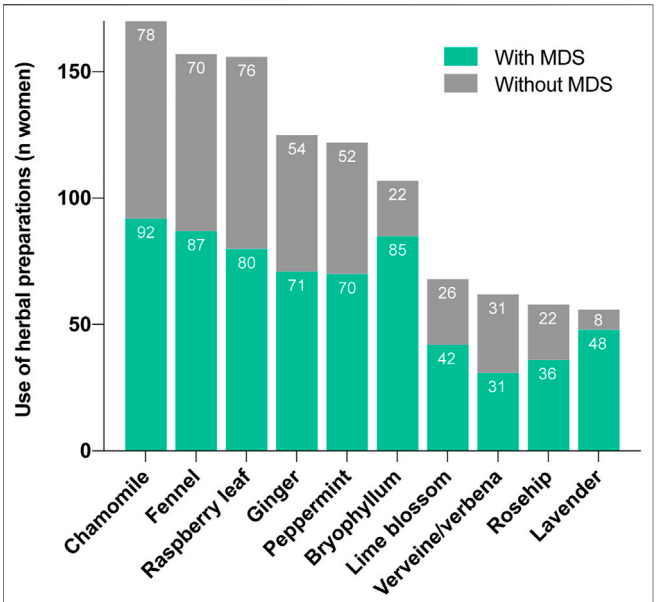

FIGURE 2 | Comparison of the most frequently used herbal medicines (pharmaceutical products and teas) in women with and without mental disorders and/or symptoms (MDS).

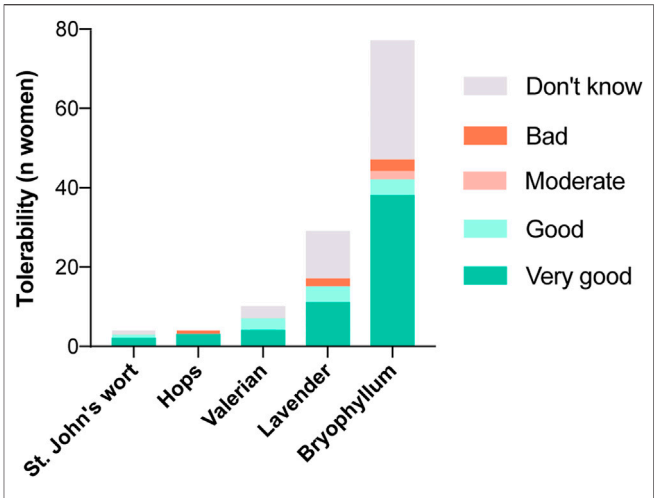

FIGURE 3 | Experiences with candidate herbal medicines for the treatment of mental disorders and/or symptoms (MDS) during pregnancy regarding their tolerability.

5.8%), none of the participants reported poor tolerability and the majority tolerated the herb well ("very good" 4/20, 20.0%; "good" 3/20, 15.0%). As shown in **Figure 4**, half of the participants rated its effectiveness as very good (10/20, 50.0%). Valerian was used most commonly to overcome sleep disorders (9/20, 45.0%), and restlessness (8/20, 40.0%).

The use of lavender was also increased toward the end of pregnancy. Lavender was used by 16.2% of women (56/345) and 11 of 56 women rated its tolerability as very good. Its effectiveness was described as good to very good by almost half of the women who used it (26/56, 46.4%), but bad by 35.7% of women (20/56). Lavender was used mostly to combat restlessness (28/56, 50.0%), sleep disorders (19/56, 33.9%) and stress (15/56, 26.8%).

Bryophyllum was used during pregnancy by almost one-third of the participants (107/360, 29.7%). Of these, more than three-quarters used it during pregnancy weeks 28–42 (84/107, 78.5%). Only a few women (3/107) reported poor tolerability. Regarding the effectiveness of bryophyllum, 58.8% of women rated it good to very good (63/107, all indications together; comparable percentages in the main single indications; see **Figure 4**). Restlessness was mentioned as a main indication by 27.1% of women (29/107) and 22.4% (24/107) used bryophyllum for stress relief. Another 10.3% (11/107) reported using bryophyllum for sleep disorders. Sixty-six percent of the women who had used

TABLE 4 | Use of medicines from integrative and complementary medicine during pregnancy.

	Total users (*n* = 116)	With MDS (*n* = 78)	Without MDS (*n* = 38)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Anthroposophic medicine	73/326 (22.4)	51/78 (65.4)	22/38 (57.9)	0.759
Herbal medicines	72/336 (21.4)	51/78 (65.4)	21/38 (55.3)	0.600
Others	17/303 (5.6)	11/78 (14.1)	6/38 (15.8)	0.778
Not sure about intake	30/364 (8.2)	4/78 (5.1)	5/38 (13.2)	0.131
Homeopathy	61/330 (18.5)	40/78 (51.3)	21/38 (55.3)	0.418
Mother tinctures	19/352 (5.4)	14/78 (17.9)	5/38 (13.2)	0.579
Diluted herbal components	33/351 (9.4)	24/78 (30.8)	9/38 (23.7)	0.572
Others	28/320 (8.8)	16/78 (20.5)	12/38 (31.6)	0.259
Not sure about intake	18/370 (4.9)	13/78 (7.7)	2/38 (5.3)	0.684
Traditional Chinese medicine	24/336 (7.1)	17/78 (21.8)	7/38 (18.4)	0.735
Herbal medicines	21/353 (5.9)	16/78 (20.5)	5/38 (13.2)	0.331
Others	5/329 (1.5)	2/78 (2.6)	3/38 (7.9)	0.193
Not sure about intake	14/367 (3.8)	5/78 (6.4)	1/38 (2.6)	0.380
Ayurvedic medicine	12/337 (3.6)	8/78 (10.3)	4/38 (10.5)	0.910
Herbal medicines	12/351 (3.4)	8/78 (10.3)	4/38 (10.5)	0.959
Others	1/328 (0.3)	1/78 (1.3)	0/38 (0.0)	0.493
Not sure about intake	17/367 (4.6)	6/78 (7.7)	1/38 (2.6)	0.313

[a]*Percentage values without considering missing data.*

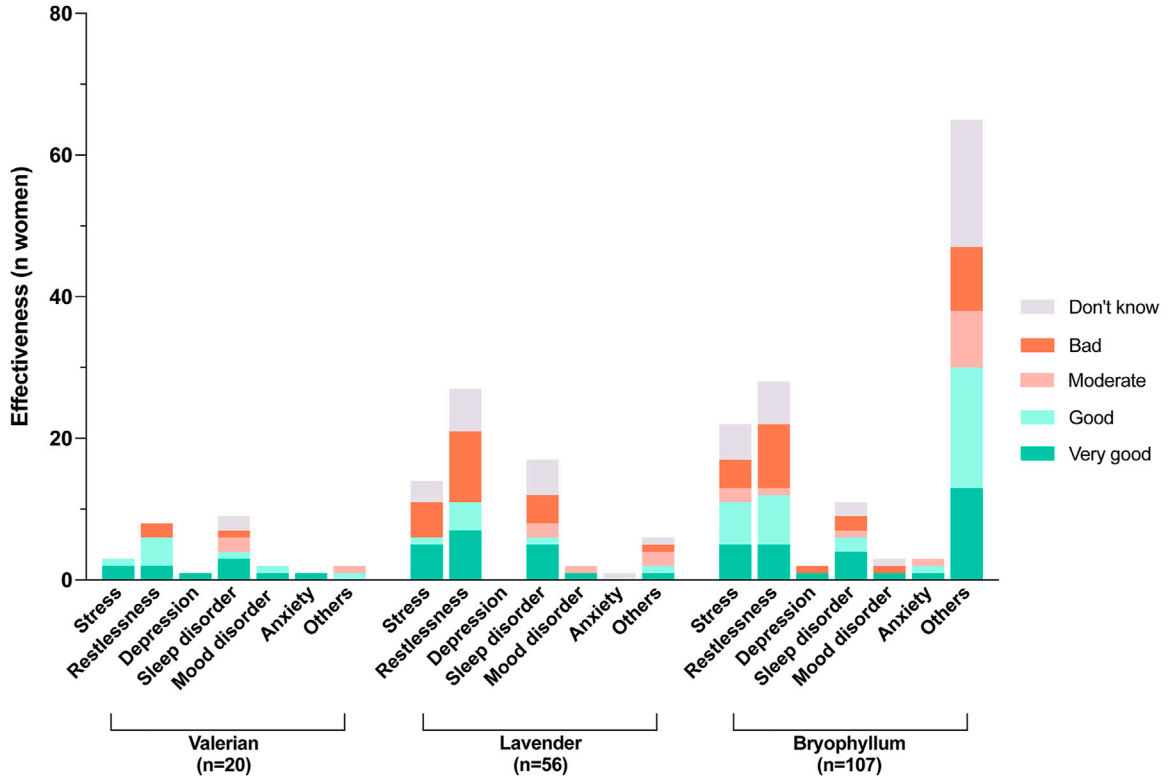

FIGURE 4 | Experiences with candidate herbal medicines for the treatment of mental disorders and/or symptoms (MDS) during pregnancy regarding their effectiveness.

bryophyllum (71/107, 66.4%) reported taking the herbal medicine for other reasons than those listed, and the majority wrote that the indications were any type of contractions (44/107, 41.1%). In addition, some women reported using bryophyllum for uterus soothing (n = 3), against abdominal cramps/pain (n = 3), against general pain (n = 1) and against cervix shortening (n = 1).

DISCUSSION

Main Findings

In our study population, the majority of women made use of herbal medicines, including pharmaceutical herbal products and teas. A wide variety of herbal products were mentioned by the survey participants, but ginger, raspberry leaf, bryophyllum, chamomile, lavender and iron-rich herbs were most commonly used. Our data further show that more than half (52.0%) of all participants had MDS during pregnancy, although few made use of (synthetic) psychoactive medications. The prevalence of MDS was higher among users of pharmaceutical herbal medicines than among non-users. Focusing on the most commonly used herbal medicines, users of bryophyllum and lavender reported suffering from MDS particularly often. Specific questions about candidate herbal medicines for the treatment of MDS revealed that bryophyllum (mentioned by 107 women), lavender (56 women) and valerian (20 women) were used to reduce stress, restlessness, sleep disorders and others, frequently with perceived good to very good effectiveness and tolerability.

Strengths and Limitations

All data were self-reported. While the number of pregnant participants (n = 398) can be considered a strength of the present survey, the moderate response rate of 24.1% is a limitation of the study. To avoid selections biases, following measures were taken: 1) all hospitals from the Canton of Zurich with an obstetrics ward were invited to participate; 2) the institutions that agreed to participate were regularly reminded of the survey; 3) the questionnaires were available in four languages (the three main country languages and English); 4) mental conditions were not mentioned in the title or cover letter of the questionnaire; 5) it was clearly stated that the survey was fully anonymous. Nevertheless, some selection bias due to interest in herbal medicines (mentioned in the title and cover letter) or the limited number of available questionnaire languages cannot be excluded. In the case of patients from the University Hospital Zurich (41.2% of survey participants), an internal analysis of the main patient demographic data revealed comparable characteristics to those of the survey participants (own unpublished observations). Finally, most questionnaires were handed over to women after delivery. While this can be

considered a strength–as postpartum women are able to describe their use of herbal medicines throughout the whole pregnancy–it is conceivable that some participants may not have remembered the medicines taken at the beginning of pregnancy.

Use of Pharmaceutic Herbal Medicines During Pregnancy

The high use of herbal medicines observed in the present survey is consistent with findings of a previous pilot survey conducted in a comparable population in the late 1990s (Gut et al., 2004). Studies from other countries reported lower use, and a multinational study of the use of herbal medications in pregnancy in 23 countries, and involving 9,459 women, revealed a markedly lower rate [28.9% of the women (Kennedy et al., 2013)]. This could be related to different perceptions of "herbal medicines" among participants in the different studies. In general, women in our study seemed to be more likely to use herbal medicines if they were primiparous and had used herbal medicines in the past/before pregnancy (data not shown). This could also be seen in an Australian study (Low Dog, 2009), where the most commonly used herbals were ginger, cranberry, valerian, raspberry, chamomile and peppermint (Kennedy et al., 2013), which is similar to our results.

In the following, we will focus on the herbal medicines used by at least 20% of the participants, first with respect to frequency of use during pregnancy and effectiveness, then summarising what is known on their safety. Our finding that ginger was the most commonly used herbal medicine confirms the results of previous surveys, and corresponds well with the high prevalence of nausea during pregnancy found in our survey (67.9%). Ginger can be considered a possibly effective treatment for nausea and vomiting during pregnancy (Viljoen et al., 2014). Raspberry leaf was the second most commonly used herbal medicine among pregnant women in our study, in accordance with studies showing that it is often recommended by midwives (Allaire et al., 2000; Holst et al., 2009). It is used to strengthen or prepare the uterus, to soften the cervix, and to induce and shorten labor (Briggs et al., 2022). In a placebo-controlled randomized trial, raspberry leaf tablets did not shorten the first stage of labor, but resulted in a small shortening of the second stage and less forceps deliveries (Simpson et al., 2001). Bryophyllum was the third most commonly used herbal medicine. Because this plant is recommended in several perinatal centres in Switzerland for the treatment of anxiety states, restlessness, and sleep disturbances (Schenkel et al., 2018), the questionnaire contained additional questions about its use (see below). Nevertheless, most women used bryophyllum for the treatment of other disorders, often related to the attenuation of uterine contractions. In anthroposophic medicine, bryophyllum was introduced in the 1970s as a well-tolerated agent for the treatment of preterm labor (Fürer et al., 2016; Hamburger et al., 2017); in Switzerland it is recommended for this indication (Schenkel et al., 2018) and commonly used in the main perinatal institutions (Fürer et al., 2015). Chamomile, the fourth most commonly used medicine has been also widely used and is known as a treatment for nausea and vomiting during pregnancy (Sanaati et al., 2016). Finally, lavender was frequently used (see below for information on its anxiolytic effects).

The Committee on Herbal Medicinal Products (CHMP) of the European Medicine Agency (EMA) does not recommend the use of ginger (European Medicines Agency, 2012b), raspberry leaf (European Medicines Agency, 2014), chamomile (European Medicines Agency, 2015) and lavender (European Medicines Agency, 2012a) during pregnancy because of insufficient safety data; so far, no community herbal monograph was published on bryophyllum. According to a reference work on drugs in pregnancy (Briggs et al., 2022), ginger and raspberry leaf are classified as compatible with pregnancy, whereas about chamomile it is considered that human data are limited and no relevant human data are available. According to a systematic review and meta-analysis from 2014, ginger can be considered a harmless treatment for nausea and vomiting during pregnancy: here no significant differences were found between the ginger and placebo treated groups for all reported adverse effects in various studies (Viljoen et al., 2014). A systematic review come to comparable conclusions (Stanisiere et al., 2018). With respect to raspberry leaf use, an unclear association with caesarean sections (Nordeng et al., 2011) and an interaction with a conventional medicine (hypoglycaemia when used with insulin) have been reported (Cheang et al., 2016). A randomized trial, however, revealed no adverse effects for mother or child (Simpson et al., 2001). Several retrospective and prospective studies on the use of bryophyllum during pregnancy are indicative of a good safety profile (Lambrigger-Steiner et al., 2014; Fürer et al., 2015; Fürer et al., 2016; Hamburger et al., 2017; Simões-Wüst et al., 2018). A qualitative study pointed towards an association between regular use of chamomile during pregnancy and a higher incidence of threatening miscarriages and preterm labor [without correction for possible confounders; Cuzzolin et al. (2010)].

Herbal Medicines and MDS Treatment

Of the herbal medicines specifically addressed in the present questionnaire, there were several that–irrespective of pregnancy–are used in the treatment of mild MDS. Evidence for their use during pregnancy is still scarce, therefore the existing studies are briefly discussed below (on the herbal medicines used by at least 20 participants).

Valerian was mostly used to treat restlessness and sleep disorders. As a sleep-aid, its benefits and side effect profile have been shown in several studies (Dorn, 2000; Andreatini et al., 2002; Hattesohl et al., 2008). In a small survey conducted in southern Italy, no influence of valerian use on pregnancy and neonatal outcomes was found ($n = 9$) (Low Dog, 2009). Data from the Swedish Birth Register from 1995 to 2004 also suggest good safety, as no abnormalities were found in the infants of mothers who had taken phytotherapeutics–often valerian–during pregnancy ($n = 787$, 0.9% of all mothers in the register).

In our study, women reported using **lavender** more often during the last trimester of pregnancy, mainly against

restlessness, sleep disorders and stress. Several clinical trials revealed good efficacy and tolerability of a medicine prepared from lavender flowers in the treatment of anxiety (Kasper et al., 2010; Woelk and Schlafke, 2010; Kasper et al., 2014). Furthermore, lavender tea has been shown to enhance the effects of the antidepressant citalopram (Nikfarjam et al., 2013). We are aware of only one previous study during pregnancy: in a randomised placebo-controlled trial, lavender cream was shown to reduce anxiety, stress and depression (Effati-Daryani et al., 2015).

Although most participants used **bryophyllum** to treat indications other than MDS, this plant was also used to treat restlessness, stress, sleep disorders, mood disorders, anxiety, and depression. In anthroposophic medicine, the use of bryophyllum medicines for mental disorders is well documented (Simões-Wüst et al., 2012). Prospective observational studies revealed improvements in sleep quality after treatment with bryophyllum in pregnant women (Lambrigger-Steiner et al., 2014) [and in cancer patients (Simões-Wüst et al., 2015)].

The particularly high prevalence of MDS among herbal medicine-users and the rare use of synthetic psychoactive medications suggest that pregnant women avoid them and prefer recurring to herbal medicines for mild MDS treatment. The reported positive experiences with some candidate herbal medicines for mild MDS treatment and their well-perceived tolerability deserve particular attention.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GG and APS-W designed the study. GG developed the questionnaire, contacted the obstetric clinics and birth centres in the Canton of Zurich, performed the analyses of data on herbal medicines and wrote the first version of the manuscript. ER analysed the data on conventional medications and contributed to survey realization. DS and APS-W verified the analyses. All authors were involved in the interpretation of data, provided critical revision of the manuscript, then read and approved the submitted version. GG contribution constitutes her MD thesis, ER contribution constituted her Master's thesis in pharmacy; APS-W supervised both theses.

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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.2021.729724/full#supplementary-material

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Efficacy and Safety of Ejiao (Asini Corii Colla) in Women With Blood Deficient Symptoms: A Randomized, Double-Blind, and Placebo-Controlled Clinical Trial

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Equus asinus L [Equidae; Asini Corii Colla] (donkey-hide gelatin, Ejiao), a well-known traditional Chinese medicine, has been widely used to nourish the blood, especially for women. The aim of this study was to assess the efficacy and safety of Ejiao in blood-deficient patients. A total of 210 participants were recruited and randomly allocated into the placebo control group and Ejiao-treated group (6 g/day). The primary outcomes on the efficacy of Ejiao included traditional Chinese medicine symptom scores, blood indicators, and SF-36. The secondary outcomes were changes in fireness and safety evaluation. Results showed that Ejiao treatment for 8 weeks had significantly improved dizziness symptoms. Among the tested 24 blood biochemical parameters, the hematocrit and red blood cell numbers decreased in the placebo control group, but decreased significantly less in the Ejiao treatment group. The white blood cell and neutrophil counts increased in the Ejiao group but were within the normal range. In addition, the quality of life improved as the scores in SF-36 domains were significantly higher in the Ejiao group. At the same time, there was no significant change in the fire–heat symptoms score or other safety parameters. Considering all these, our study showed that Ejiao has a promising effect in women suffering from blood deficiency without obvious adverse effects.

Keywords: asini corii colla (Ejiao), blood deficient symptoms, randomized controlled trial, anemia, TCM syndromes

INTRODUCTION

Blood deficiency syndrome (BDS) is one of the common clinical syndromes of traditional Chinese medicine (TCM), which refers to the pathological state of insufficient blood. The main clinical symptoms are dizziness, pale complexion, and reduced menorrhagia in women. BDS in TCM is not exactly the same as in anemia in modern medicine, although both of them refer to the reduction of blood cells or hemoglobin. BDS is often associated with impaired hematopoietic function, peripheral blood pancytopenia, hypovisceral dysfunction, malnutrition, and even myelosuppression (Zhang

et al., 2014; Ji et al., 2018; Zhang et al., 2020). Therefore, BDS is related to but different from anemia in modern medicine.

It has been reported that many traditional Chinese medicine showed curative effects on BDS (Wu J. et al., 2007; Zhang et al., 2014; Ji et al., 2018). Ejiao is an ancient traditional Chinese medicine, which is prepared by stewing and concentrating from *Equus asinus* L. donkey hide. Ejiao has been used for more than 2000 years in China, and the main components have been collagen and amino acids (Du et al., 2019). According to the ancient books, the major functions of Ejiao were tonifying blood and nourishing Yin, which have been considered as the first choice for the treatment of BDS. Nowadays, Ejiao has been widely used in the clinic for its biological activities of anti-fatigue, immunity improvement, tumor suppression, and specifically anti-anemia effect (Wu H. et al., 2007; Chen et al., 2012; Wang et al., 2012; Liu S. et al., 2014; Zhang et al., 2018). The hematopoietic effect of Ejiao has also been approved by modern biological studies, and it was reported that the fractions from Ejiao promoted hematopoiesis on mice with 5-fluorouracil–induced anemia (Wu H. et al., 2007). RNA-sequencing studies have indicated that the molecular mechanisms of Ejiao might be related to the extracellular matrix–receptor interaction, Wnt signaling, and PI3K-Akt signaling pathway (Zhang Y. et al., 2019). However, most clinical studies are focused on the formula that comprises Ejiao, such as the *Fufang E'jiao Jiang* that has been reported to improve the hematopoietic functions and increase the Hb level of postpartum anemic women rapidly and significantly (Li et al., 2018). However, the clinical efficacy of Ejiao alone has not been studied systemically.

Ejiao has been used for thousands of years, and the demand for it is increasing every year. However, there have always been concerns about "fireness" induced by improper use of Ejiao. Fireness is a traditional syndrome of Chinese medicine without specific physiological indicators (Chong and Oberholzer, 1988; Zhang M. et al., 2019). According to the TCM theory, fireness refers to the hot symptoms in the human body which are caused by an imbalance of Yin and Yang. The specific symptoms include red and swollen eyes, erosion at the corners of mouth, yellow urine, toothache, sore throat, etc. (Xiao and DeFranco, 1997; Wu J. et al., 2007). In the clinic, fireness is mainly diagnosed according to the feelings of patients. Although many people thought Ejiao might cause fireness, there has been no systematic study on it. In our study here, the double blind and randomized clinical trial was carried out to evaluate the effect of Ejiao on patients with BDS. The aim of the present study was to evaluate the safety, particularly the side effects of "fireness," and the anti-fatigue effect of Ejiao on patients with deficiency syndrome.

MATERIALS AND METHODS

Participants

This study was designed as a randomized, double-blind, placebo-controlled study according to the CONSORT 2017 statement (Cheng et al., 2017). The participants were recruited from March 2019 to December 2020 at Guanghua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

In this study, the inclusion criteria were as follows: 1) the syndrome differentiation of TCM is BDS; 2) males or females aged 18–60 years; 3) no anti-anemia foods or drugs used within 3 months; and 4) without any severe disease of the heart, liver, kidneys, or blood system.

The exclusion criteria included the following: 1) patients with severe anemia (Hb < 90 g/L); 2) acute hemorrhage and hemolysis; 3) acute infection, acute stage of a chronic disease; 4) lactating or pregnant women; 5) not suitable for Ejiao treatment according to their physician; 6) unable to follow dietary or drug restrictions; and 7) participation in another clinical trial within the past 3 months.

The sample size was calculated using PASS 15. A total sample size of 177 provided a test power of 0.8 to detect the difference by using a two-sided t-test when the significance level was 0.05. We utilized a parallel-controlled design with an equal number of participants in each group. However, considering a 11 and 15% withdrawal rate in the control and Ejiao groups, respectively, 210 participants were recruited, and 181 participants completed the study (Chow et al., 2008).

All participants submitted an informed consent before the start of the study. This study was approved by the Institutional Review Board of Guanghua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (No. AF13v2-2). The clinical trial has been registered at the Chinese Clinical Trial Registry with the registration number: ChiCTR-TRC-1900021651.

Study Design

Those patients who meet the inclusion criteria were randomly assigned to receive either Ejiao or placebo at a 2:1 ratio. Randomization was performed by a third party responsible for sequentially assigning random numbers and distributing trial samples to the patients. The patients received capsules either with Ejiao or placebo, 6 g each day. Both the Ejiao capsules were manufactured by the company Dong-E-E-Jiao Co. Ltd. These capsules are identical in size, weight, color, and taste. The researchers and patients did not know which group the patients are allocated to from the appearance of the medication given.

Outcome Measurements

The primary outcome included the change in the total score of the Blood Deficiency Symptoms Grading and Quantifying scale (BDS scale). Based on the Guideline of Clinical Research of TCM New Drugs (Zheng, 2002), the BDS scale consists of the following seven items categorized as major and secondary symptoms: 1) pale complexion; 2) dizziness; 3) palpitations; 4) pale tongue; 5) thready pulse; 6) limb numbness; and 7) insomnia. The items from one to five are the main symptoms; the other items are the secondary symptoms. Each symptom is directly transformed into a 0–6 scale based on the severity of the disease. The instrument yields a summed total score ranging between 0 and 30 (0 = no symptoms).

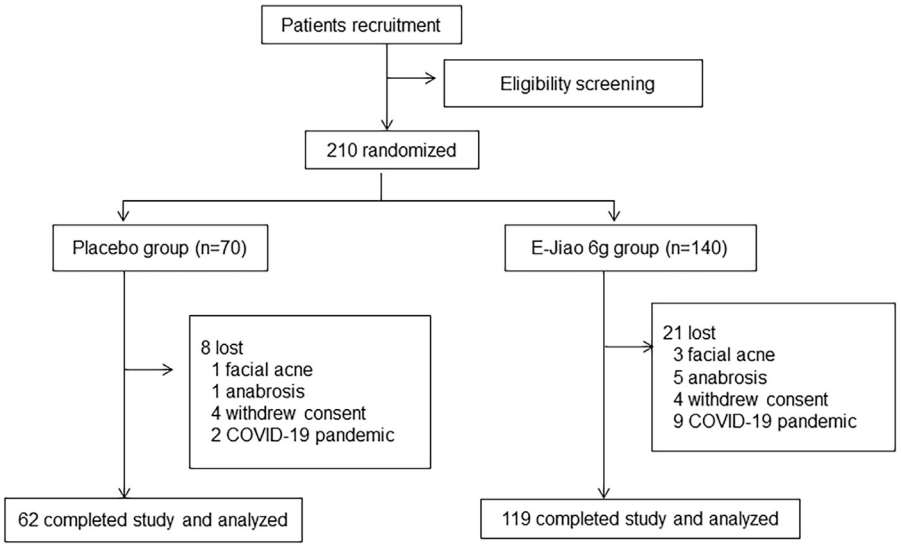

FIGURE 1 | Trial flow chart primary recruitment identified 210 volunteers, the authors randomly allocated the patients to groups as follows: placebo (n = 70), Ejiao 3 g twice a day (BID) (n = 140).

TABLE 1 | Baseline characteristics of the study participants.

Characteristics	Ejiao	Placebo	*p*-values
Age (years)	37.99±13.50	39.63±13.21	0.436
Height (cm)	161.65±4.60	162.18±4.26	0.474
Weight (kg)	56.37±7.14	55.36±6.63	0.384
Body temperature (°C)	36.56±0.36	36.63±0.29	0.234
BMI (kg/m^2)	21.61±2.42	21.27±2.53	0.569
Systolic blood pressure (mmHg)	110.84±9.33	108.47±10.16	0.674
Diastolic blood pressure (mmHg)	73.76±6.88	69.35±7.46	0.532
Heart rate	80.33±4.61	83.18±5.76	0.546

165 participants were randomly assigned to the placebo; Ejiao 3 g (BID). Data are expressed as mean ± standard deviation. p-*values were calculated by independent samples t-test.*
BID, twice a day; BMI, body mass index.

Secondary Outcomes

The secondary outcomes included the changes from the baseline in blood cells; the quality of life that is assessed by a change in the Short Form 36 scale score (Ware and Sherbourne, 1992; Steinhaus et al., 2019; Chen et al., 2020).

Safety Assessments

Safety assessments included the fire–heat symptom scale, routine examination, and adverse events (AEs).

The fire–heat symptom scale was used to determine fireness in this study as had been described in our previous study (Zhang M. et al., 2019), which includes excess fire–heat (29 items) and deficiency fire heat (14 items) (Lin et al., 2012; Liu M. et al., 2014). Routine examination (blood and urine routine examination, liver and renal functions, and electrocardiogram) were carried out at the baseline and at the end of the study. AEs were monitored throughout the study to assess the safety of Ejiao.

Statistical Methods

Analyses of efficacy were performed on the intent-to-treat population (full analysis set). The study used the EpiData database for data entry and management, and the data entry was carried out using a double-entry verification. All statistical analyses were performed with SPSS software, version 26.0. For the non-normality of the data, a nonparametric signed-rank test was performed. The numerical variables were presented as the mean ± SD, and intragroup and intergroup comparisons were analyzed by the means of paired samples t-test and independent samples t-test, respectively. The categorical variables were described using the number of cases (%) and the Chi-square test was carried out. p values < 0.05 were considered statistically significant, and all tests were two-tailed.

RESULTS

Characteristics of Patients

According to the inclusion and exclusion criteria, 210 patients (Ejiao, n = 120; placebo, n = 70) were enrolled in the hospital. During the trial, 29 of the patients were lost. Of the 29 lost, 11 discontinued due to the COVID-19 pandemic and 18 for AEs such as facial acne and anabrosis. Finally, 181 patients completed the trial, 119 in the treatment group and 62 in the control group. The flow diagram of the trial is shown in **Figure 1**. Among the 210 recruited patients, 187 of them were women, while among completed patients, 163 out of the 181 patients were women. Therefore, we focused our analysis on the effect and safety of Ejiao in women with blood deficient symptoms. The general characteristics of the participants are shown in **Table 1**. The groups were well matched about baseline characteristics, including sex, age, body mass index, blood pressure, and heart rate ($p > 0.05$).

TABLE 2 | The symptom of dizziness after administration of Ejiao for 8 weeks.

Time Point	Group	Mild	Moderate	Severe	*P*
0 days	Ejiao	58 (53.7%)	45 (41.7%)	5 (4.6%)	0.647
	Placebo	31 (56.4%)	23 (41.8%)	1 (1.8%)	
28 days	Ejiao	91 (84.3%)	17 (15.7%)	0 (0%)	0.918
	Placebo	46 (83.6%)	9 (16.4%)	0 (0%)	
56 days	Ejiao	101 (93.5%)	7 (6.5%)	0 (0%)	0.043
	Placebo	46 (83.6%)	8 (14.5%)	1 (1.8%)	

Data are expressed as the number of cases (%). p-*values were analyzed by Chi-square test.*

Impact of Ejiao on TCM Syndromes

The severity of the symptoms were classified as "mild, moderate, and severe" according to the Guideline of Clinical Research of TCM New Drugs (Zheng, 2002). Our results indicated that after the treatment with Ejiao, the symptoms of dizziness were greatly alleviated. At enrollment, the majority of the patients were in the "mild" and "moderate group" and the remaining patients were in the "severe" (less than 5%) group. The baseline severity classification was similar between the two groups (**Table 2**). After treatment with either Ejiao or placebo, the frequency of "moderate" to "mild" patients gradually increased (**Table 2**); however, the change in the Ejiao group was much more obvious. After treatment for 56 days, the frequency of "moderate" in the Ejiao group decreased to 6.5%, which is significantly better than the control group (14.5%). The frequency of "severe" decreased from 4.6% to 0 in the Ejiao group, while no change was observed in the placebo group. These results indicate that Ejiao treatment resulted in superior improvements of dizziness classification compared with the placebo ($p = 0.0043$).

The scores of TCM syndrome were also determined in our study. No differences were observed in the TCM syndrome scores between the two groups before treatment (3.02 ± 0.11 and 2.91 ± 0.14, respectively, $p > 0.05$). After treatment for 28 or 56 days, the scores decreased in both the Ejiao and placebo groups (**Figure 2**). All syndrome scores decreased significantly ($p < 0.01$) as compared with the baseline and more obviously after 56 days. After 4 weeks treatment with Ejiao, the scores decreased from 3.02 ± 0.11 to 2.31 ± 0.07, which further decreased to 2.13 ± 0.048 at the end of the trial. The scores in the Ejiao group showed statistical significance as compared to the control group. Other major syndromes of BDS, including pale complexion, palpitations, pale tongue, and thready pulse, were also evaluated in our study. However, Ejiao showed no significant improvement on these symptoms (**Supplementary Figure 1**). No significant difference was found in the secondary symptoms between the Ejiao and placebo groups after treatment (data not shown). The most possible reasons maybe that, first, in our study, we used only Ejiao instead of combinations, while in previous studies, Ejiao was usually combined with other TCM or Western drugs; second, the sample size is relatively small, and the treatment time is limited in our study, and more significant effects of Ejiao may be shown in studies with a bigger sample size and longer treatment time.

Effects of Ejiao on Peripheral Blood Cells

The quantities of different blood cells from the peripheral blood were investigated in our study. The results indicated that the decrease of

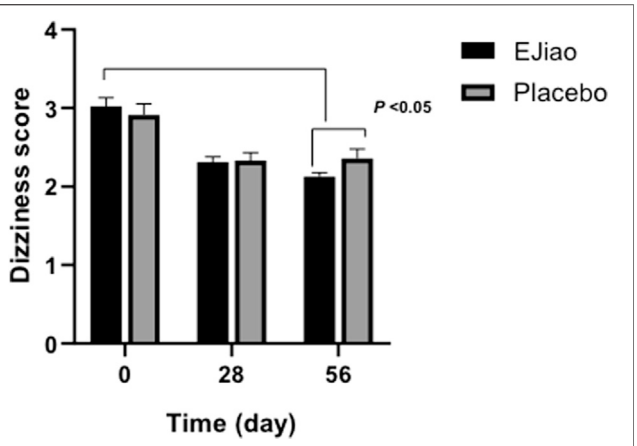

FIGURE 2 | The dizziness syndrome scores of the Placebo and Ejiao groups were evaluated after treatment for 28 and 56 days. After 56 days of administration of Ejiao (3 g, BID), there was a significant improvement in the symptoms of dizziness in the Ejiao group compared to the Placebo group and pretreatment state. Data are expressed as mean ± standard deviation. Intragroup and intergroup comparisons are calculated by means of paired samples t-test and independent samples t-test, respectively.

RBC and hematocrit (HCT) were alleviated, while the levels of white blood cells (WBCs) and absolute neutrophil count (ANC) were increased (**Table 3**). During the clinical trial, although the red blood cell numbers decreased in both groups, the decrease was greater in the control group (**Table 3**). The difference of the changes between the two groups was also analyzed. Our results indicated that the RBCs decreased significantly in the placebo group (−0.13 ± 0.029) as compared with the Ejiao group (−0.043 ± 0.02). For the HCT, the number decreased greatly (−1.35 ± 0.27) in the control group, which was significantly alleviated in the Ejiao group (−0.57 ± 0.21, $p = 0.036$). The quantities of the WBCs and ANC showed no obvious change in the control group, while the treatment of Ejiao increased the WBCs and ANC, but still within the normal values during the observed time period (**Figure 3**).

Ejiao Improved the Quality of Life in BDS Patients

To evaluate the health status of participants, the SF-36, a self-assessment health status questionnaire, was constructed, which contains 36 items (questions) about sociodemographic data, health, and personal behavior, grouped into eight multi-item domains (Chen et al., 2020). The mean (±SD) differences between the groups in the outcome visits at the baseline, 4 weeks, and 8 weeks are shown in **Table 4**. The mean baseline differences showed no statistically significant difference between the two groups ($p > 0.05$). After treatment, there were statistically significant differences in role physical, vitality, social function, role emotional, and the health transition of SF-36 between the two groups at 4 weeks ($p < 0.05$).

Changes on Fireness

The fire–heat symptoms scale, including the excess fire–heat score (EFS) and deficient fire–heat score (DFS), was used in

TABLE 3 | The changes of hematocrit and red blood cell numbers from the baseline over 56 days of treatment with Ejiao.

Item	Time point	Placebo		Ejiao	
		Outcome	Change from baseline	Outcome	Change from baseline
Hematocrit	Baseline	39.86±0.44	−1.35±0.27	39.11±0.28	−0.57 ± 0.21 (p = 0.036)
	56 days	38.41±0.41		38.42±0.32	
Red blood cell number	Baseline	4.41±0.042	−0.13±0.029	4.36±0.03	−0.043 ± 0.02 (p = 0.048)
	56 days	4.29±0.035		4.31±0.032	

Data are presented as mean ± SD. p *values were calculated by independent samples t-test.*

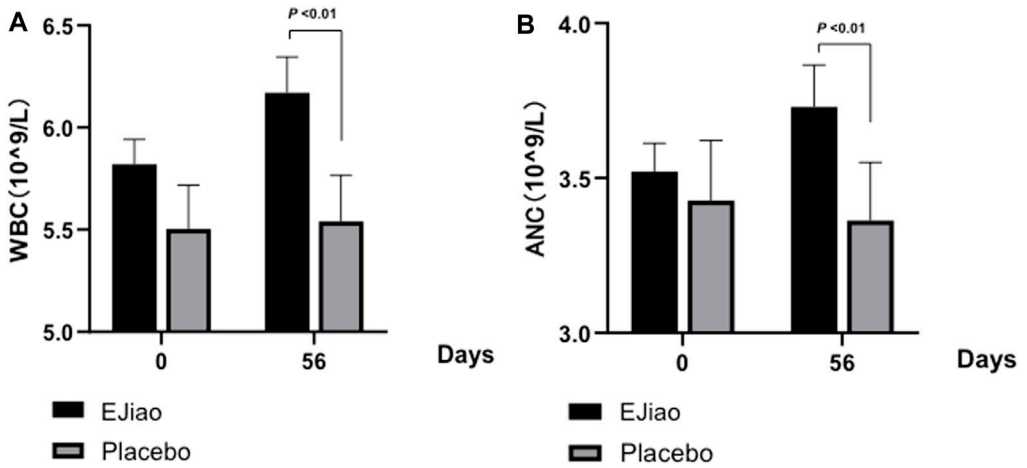

FIGURE 3 | Changes in WBCs and ANC between the Ejiao and placebo groups in 56 days. After 56 days of administration of Ejiao (3 g, BID), the WBCs **(A)** and ANC **(B)** were significantly increased in the Ejiao group compared to the pretreatment stage and placebo group, while there were no significant changes in the placebo group after the treatment of Ejiao. The data are expressed as mean ± standard deviation. p-value is analyzed by independent samples t-test.

TABLE 4 | The changes of SF-36 subscales during the 24 weeks.

Item	Groups	Baseline	28 days	56 days	Change 28 days	Change 56 days
Role physical	Placebo (n = 55)	70.454.68	97.272.01	86.363.6	26.824.71	15.914.55
	Ejiao (n = 108)	63.893.62	97.450.80	82.872.87	33.563.79*	18.984.09
Vitality	Placebo (n = 55)	66.362.11	73.181.33	73.091.77	6.822.42	6.732.08
	Ejiao (n = 108)	60.321.66	72.081.02	71.301.39	11.761.94**	10.971.88#
Social function	Placebo (n = 55)	105.232.58	97.051.65	111.142.01	-8.193.16	5.912.48
	Ejiao (n = 108)	98.501.91	97.341.19	106.831.78	-1.162.27**	8.332.21
Role emotional	Placebo (n = 55)	58.795.39	94.181.03	86.064.12	35.395.26	27.275.67
	Ejiao (n = 108)	50.614.00	92.901.58	78.703.29	42.284.26*	28.094.66
Health transition	Placebo (n = 55)	36.572.4	41.672.80	52.782.76	5.093.90	16.203.50
	Ejiao (n = 108)	35.421.97	47.692.01	53.702.22	12.272.93**	18.292.60

Data are presented as mean ± SD. p *values were calculated by independent samples t-test.*
For the difference between the changes of Ejiao versus placebo during 28 days, **p *< 0.01 compared with the control,* *p *< 0.05 compared with the control.*
For the difference between the changes of Ejiao versus placebo during 56 days, #p *< 0.05 compared with the control.*

our study. The baseline in the placebo and Ejiao groups were 17.96 ± 1.50 and 16.19 ± 1.19 for the total fire–heat score; 12.80 ± 0.98 and 11.94 ± 0.82 for the EFS; and 4.80 ± 0.58 and 4.42 ± 0.40 for the DFS, respectively. As shown in **Figure 4**, the results showed that all the scores were significantly decreased at day 28, which then increased at the end of the study, which might contribute to the reason that the baseline was determined mainly during June to September in 2019, and the time point day 28 was mainly detected during October to February of the next year, while the time point day 56 was mainly detected during March to June. According to the TCM theory, fireness is closely related to the weather, and it is easier for people to get fireness syndromes in summer than in winter. As shown in **Figure 4**, the trend of the change were the same for both the Ejiao and control groups, and there was no significant difference between the two groups.

TABLE 5 | Summary of AEs.

Item	Ejiao	Placebo	*p* value
Diarrhea	1 (0.9%)	1 (1.8%)	0.623
Sudden pneumothorax hospitalization	1 (0.9%)	0 (0.0%)	0.474
Poor appetite	2 (1.9%)	0 (0.0%)	0.309
Sudden herpes	0 (0.0%)	1 (1.8%)	0.159
Surgery for uterine fibroids	0 (0.0%)	1 (0.9%)	0.159
Facial acne	3 (2.78%)	1 (1.8%)	0.707
Anabrosis	5 (4.6%)	1 (1.8%)	0.364

Ejiao was administered (3 g twice a day [BID]) for 8 weeks. During the study period, all the AEs were documented. Data are expressed as the number of cases (%). p *values were calculated by Chi-square test.*

Safety Evaluation

The types and occurrences of AEs during both the Ejiao and placebo treatments were evaluated. 11 AEs occurred during Ejiao treatment, while 4 AEs occurred during the placebo treatment (**Table 5**). However, this difference was not statistically significant. The most frequent AEs occurring during Ejiao treatment were anabrosis (n = 5) and facial acne (n = 3). No moderate or severe AEs were reported during the whole clinical trial. Compared with the placebo, there was no significant difference on the index of the kidney and liver functions (Cr, ALT, and AST levels) after treatment with Ejiao for 56 days between the two groups (data not included). The results of electrocardiograms were also within the normal range. These results indicate that Ejiao treatment for 56 days has no obvious adverse reactions on patients with BDS.

DISCUSSION

BDS is a traditional Chinese clinical term, which is related to anemia in modern medicine. BDS is more prevalent in women as compared to men. There may be several reasons for this: first, women suffer from chronic blood loss due to monthly menstrual cycles. This blood loss, if not corrected by an appropriate diet, always leads to chronic BDS; second, women's hormones change at different stages of life, such as adolescence, pregnancy, lactation, and menopause. In all these stages, the demand for iron and calcium increases. If this increased demand is not met, they tend to suffer from BDS. Ejiao is thought to be the first choice for the treatment of BDS in women. In our clinical trial, although patients were recruited regardless of their sex, we found that among the 220 patients, 187 of them were females, which also indicated that Ejiao is more widely used in women. Therefore, the clinical study on safety and efficacy of Ejiao in females is important.

The hematopoietic effect of Ejiao has been well demonstrated from long-term clinical experience (Wang et al., 2014; Li et al., 2019). Studies have also proved that Ejiao promoted the recovery of hematopoietic function in 5-fluorouracil–induced blood deficiency in mice (Wu H. et al., 2007; Zhang Y. et al., 2019). Others have reported that the fractions from the enzyme-digested Ejiao might be the main active components, which are composed of different amino acids. In addition, the effect of Ejiao has also been compared with other traditional Chinese medicines. The leukocytopoiesis-promoting action of effective components from *Spatholobus suberectus Dunn* (Leguminosae; *Caulis spatholobi*) and Ejiao in rats with leukopenia induced by cyclophosphamide was compared, the results showed that the effective components from *C. spatholobi* could significantly promote leukocytopoiesis in rats, and the effect was equivalent with that of Ejiao (Ying et al., 2011). The effect of formulae containing Ejiao was also compared, the study showed that the formula containing Ejiao and *Ganoderma sinense* Zhao, Xu et Zhang (Polyporaceae; Ganoderma) had a better effect on the complex blood-deficient model, while the formulae containing *Angelica sinensis* (Oliv.) Diels (Umbelliferae; Angelicae Sinensis Radix), and *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (Leguminosae; Astragali Radix) showed better effects on the blood losing model (Chen et al., 2015).

Although these researches are important to prove the functions of Ejiao and explore the underlying mechanisms, the

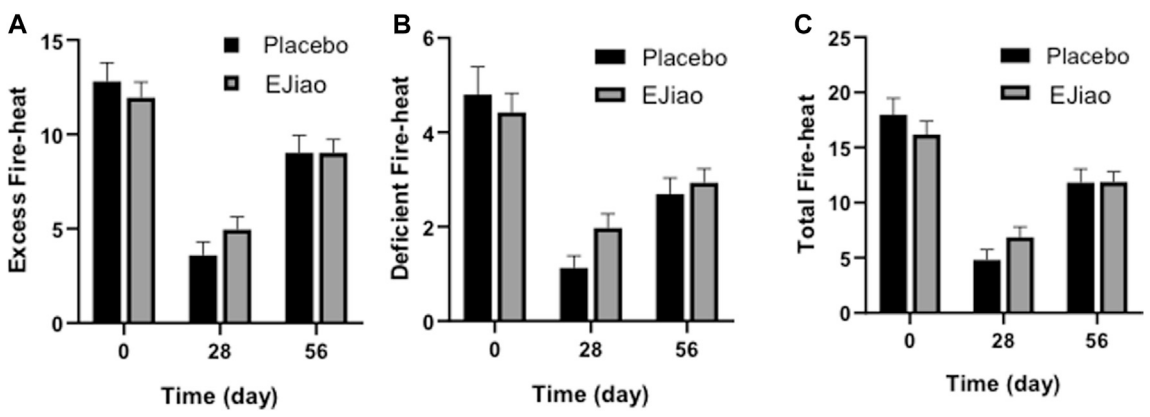

FIGURE 4 | fire–heat syndrome scores of the placebo and Ejiao groups were evaluated before and 4 and 8 weeks after treatment. Syndrome score for total symptom item of excess fire–heat **(A)**, deficient fire–heat **(B)**, and total fire–heat **(C)** are expressed as mean ± standard deviation.

evidence from the clinical trials is more direct and convincing. In clinical practice, Ejiao is usually used with a combination of other traditional Chinese medicine according to different constitutions and symptoms of the patients. Therefore, the randomized, double-blind, placebo-controlled trials were focused to explore the safety, effectiveness, and cost-effectiveness of the formulae which contained Ejiao (Zhang Y. et al., 2019). Our study is the first clinical study to provide systematic evidence for the blood nourishing effect of Ejiao in female patients with BDS. And here, we showed that the major symptoms of BDS were greatly improved after the treatment with Ejiao. The reduction of RBCs was controlled, while the numbers of WBCs were increased, which are consistent with previous *in vitro* studies. Our research provided scientific foundation for the application of Ejiao in BDS patients.

Fireness is a special term in TCM without objective diagnostic parameters (Lin et al., 2012; Xu and Dou, 2016). It is commonly thought that some of the traditional Chinese medicines can cause fireness, such as ginseng, Ejiao, and so on. Our previous study has already investigated whether the use of red ginseng, another famous traditional medicine used for thousands of years, would cause fireness. Our results showed that the proper use of ginseng in patients with deficiency syndrome is safe and will not cause fireness. Similar to ginseng, Ejiao is also a tonic medicine. Many people believe that the use of it can cause fireness, especially in summer. Although some studies reported that Ejiao could cause fireness, no systematic clinical trial has been conducted to study the direct relationship between Ejiao and fireness. Our study here is the first randomized, double-blind, placebo-controlled clinical trial to study the safety of Ejiao in female patients with BDS. Our results demonstrated that the fireness symptom scores showed no significant changes in the Ejiao group. Facial acne and anabrosis were thought to be symptoms related to fireness, although these cases were reported more in the Ejiao groups than in the placebo control groups, and statistical analyses showed that there were no significant differences between the two groups. Our data indicate that there is no safety concern with the consumption of Ejiao in female BDS patients.

There are some limitations in our study. First, our study only recruited participants with BDS. However, many people take Ejiao without considering about their constitution. Therefore, the results here cannot be generalized to all Chinese people. However, according to the TCM theory (Yi et al., 2017; Li et al., 2018), Ejiao is only suitable for patients with BDS. Therefore, it is more reasonable to choose patients with BDS as the research object. Second, 11 cases were lost and fell off due to the COVID-19 pandemic. However, we managed to control the cases lost within 20%. We took the questionnaire on the Internet or by means of telephone calls to reduce the risk of loss to follow-up. We delivered the research materials to participants by mail to maintain the trial. Third, we only studied the efficacy of Ejiao in our study, but the mechanisms of it have not been studied. However, our main objective was to provide direct evidence for the use of Ejiao in BDS patients, and our results have indicated that the major symptom of BDS was improved. Further studies will be conducted to elucidate the mechanism of the effect in the future.

In summary, the safety and efficacy of Ejiao in participants with BDS were first evaluated. Our results showed that Ejiao greatly alleviated the syndrome of dizziness, which is one of the major syndromes of BDS. The impact of Ejiao treatment on the peripheral blood indicated that Ejiao alleviated the decrease of RBC and HCT, while it increased the levels of WBCs and ANC. Safety evaluation indicated that Ejiao treatment was not associated with an increase in the fire–heat symptom score for people with BDS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Guanghua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (No. AF13v2-2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HX, LZ, JT, and JX conceived and designed the experiments; JZ, HF, and ZX performed the clinical trial; JT, PH, ZL, MY, and GC were responsible for data input and analyses; HL, DG, LX, and ZL wrote 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.2021.718154/full#supplementary-material

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Conflict of Interest: HL and DG were employed by the company Dong-E-E-Jiao Co. Ltd.

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

frontiers in Pharmacology

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An Effective Treatment of Perimenopausal Syndrome by Combining Two Traditional Prescriptions of Chinese Botanical Drugs

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Ethnopharmacological relevance: Two types of traditional Chinese formulas of botanical drugs are prescribed for treating perimenopausal syndrome (PMS), a disorder in middle-aged women during their transition to menopause. One is for treating PMS as kidney deficiency (KD) due to senescence and declining reproductive functions, and the other is for treating it as liver qi stagnation (LQS) in association with stress and anxiety. Despite the time-tested prescriptions, an objective attestation to the effectiveness of the traditional Chinese treatment of PMS is still to be established and the associated molecular mechanism is still to be investigated.

Materials and methods: A model for PMS was generated from perimenopausal rats with chronic restraint stress (CRS). The effectiveness of traditional Chinese formulas of botanical drugs and a combination of two of the formulas was evaluated based on ^{1}H NMR plasma metabolomic, as well as behavioral and physiological, indicators. To investigate whether the formulas contained ligands that could compensate for the declining level of estrogen, the primary cause of PMS, the ligand-based NMR technique of saturation transfer difference (STD) was employed to detect possible interacting molecules to estrogen receptors in the decoction.

Results: Each prescription of the classical Chinese formula moderately attenuated the metabolomic state of the disease model. The best treatment strategy however was to

Abbreviations: CSS, Chaihu-Shugan-San; ELISA, enzyme-linked immunosorbent assay; 1H NMR, proton nuclear magnetic resonance; HRT, hormone replacement therapy; KD, kidney deficiency; LQS, liver qi stagnation; OPLS-DA, orthogonal partial least-squares-discriminant analysis; PMS, perimenopausal syndrome; SPF, specific pathogen free; STD, saturation transfer difference; TCM, traditional Chinese medicine; VIP, variable influence on projection.

combine two traditional Chinese formulas, each for a different etiology, to adjust the metabolomic state of the disease model to that of rats at a much younger age. In addition, this attenuation of the metabolomics of the disease model was by neither upregulating the estrogen level nor supplementing an estrogenic compound.

Conclusion: Treatment of PMS with a traditional Chinese formula of botanical drugs targeting one of the two causes separately could ameliorate the disorder moderately. However, the best outcome was to treat the two causes simultaneously with a decoction that combined ingredients from two traditional prescriptions. The data also implicated a new paradigm for phytotherapy of PMS as the prescribed decoctions contained no interacting compound to modulate the activity of estrogen receptors, in contrast to the treatment strategy of hormone replacement therapy.

Keywords: perimenopausal syndrome, Chinese traditional medicine, phytotherapy, metabolomics, saturation transfer difference, ethnopharmacology

INTRODUCTION

Perimenopause is a transition state in middle-aged women with gradually declining ovarian function and irregular menstrual cycles (Zhang et al., 2013; Gemmell et al., 2017; Marques-Lopes et al., 2017; Zeng et al., 2018). With erratically fluctuating and descending estrogen level in the body, approximately 40–60% of perimenopausal women suffer from perimenopausal syndrome (PMS) characterized by disorders in the endocrine system and autonomic nervous system (Li et al., 2013; Xu et al., 2019). Clinical symptoms of PMS are diverse and include hot flashes, night sweats, insomnia, depression, irritability, fatigue, and cognitive impairment (Stearns et al., 2002; Elkins et al., 2008). The incidence of cardiovascular diseases, such as hypertension, atherosclerosis, myocardial infarction, and cerebral hemorrhage, increases in perimenopausal women with PMS (Nelson, 2008). The incidence of depression and osteoporosis is also higher in perimenopausal women with PMS than in healthy women (Whiteley et al., 2013).

The diagnosis of traditional Chinese medicine (TCM) is based on symptom-based pattern differentiation (also known as symptom differentiation, Zheng differentiation, pattern diagnosis, and pattern classification) (Liang et al., 2009; Liang et al., 2011; Li et al., 2015). Over the centuries of TCM practice, PMS is diagnosed as kidney deficiency (KD), a jargonistic term for the debility of bodily functions and loss of vitality, from senescence. Symptoms of insomnia, night sweat, lack of libido, back pain, and declining vitality, among others, were signs compatible with those of KD (Liang et al., 2009; Chen et al., 2020). However, KD does not seem to be the sole cause of PMS. As senescence is irreversible, aging could only deepen the degree of KD for middle-aged women. It is contradictory then that the symptoms of PMS will be naturally relieved in the later stage of perimenopause and after menopause as women get older. It is an indication that KD might not be the only cause for PMS. Obviously, other factors need to be taken into account in the diagnosis of PMS for better treatment.

The symptoms of hot flash, night sweat, insomnia, depression, irritability, fatigue, and cognitive impairment, which occur frequently in women suffering PMS, are compatible with those of liver qi stagnation (LQS), a diagnostic designation for illness correlated with anxiety and stress in pattern differentiation (Liang et al., 2010; Yu et al., 2018). There were demonstrations that prescriptions of decoctions for LQS were efficacious for treating PMS (Liang et al., 2010; Chen et al., 2014; Li et al., 2014; Li et al., 2015). For better prescription to treat PMS, it is important to investigate the physiological parameters for treating PMS as either KD, LQS, or a combination of both.

Herein, a rat model for PMS was generated with aging and stress. ^{1}H NMR metabolomics of plasma samples showed that a combined decoction for treating both KD and LQS could restore the metabolomic condition of the PMS rat model to that of rats at a younger age before entering the perimenopausal period. The data also indicated that the treatment adopted here modulated the metabolic state of the PMS rat without either altering the serum level of estrogen or supplementing a constituent capable of interacting with an estrogen receptor to attenuate its activity, implicating that TCM treatment of PMS might be under a different paradigm from the typical treatment strategy of hormone replacement therapy which relieves the symptoms of PMS by supplementing an estrogenic compound to modulate the activity of estrogen receptors.

METHODS

Preparing the Decoctions

The botanical drugs used to prepare the decoctions were purchased from the Third People's Hospital of Fujian University of Traditional Chinese Medicine (FJUTCM). The botanical drugs were authenticated by the staff in the Herb Identification, Teaching and Research Division of the College of Pharmacy of FJUTCM. The voucher specimens were kept in the College of Pharmacy of FJUTCM, Fujian, China. The decoctions, including those of Chaihu-Shugan-San (CSS), Yougui, and Zuogui, were prepared according to the Pharmacopoeia of the People's

TABLE 1 | The herbal composition of Chaihu-Shugan-San (CSS).

Chinese name	Botanical name (family name)	Pharmaceutical name	Amount (g)	Voucher specimens
Chai Hu	*Bupleurum chinense* DC. (Apiaceae)	Bupleuri radix	6	1000000618
Bai Shao	*Paeonia lactiflora* Pall. (Paeoniaceae)	Paeoniae radix alba	4.5	1000000608
Zhi Ke	*Citrus aurantium* L. (Rutaceae)	Aurantii fructus	4.5	1000000742
Chuan Xiong	*Ligusticum striatum* DC. (Apiaceae)	Chuanxiong rhizoma	4.5	1000000872
Chen Pi	*Citrus reticulata* Blanco (Rutaceae)	Citri reticulatae pericarpium	6	1000000674
Xiang Fu	*Cyperus rotundus* L. (Cyperaceae)	Cyperi rhizoma	4.5	1000001080
Zhi Gan Cao	*Glycyrrhiza uralensis* Fisch. (Fabaceae)	Glycyrrhizae radix et rhizoma praeparata cum melle	1.5	1000000884

TABLE 2 | The herbal composition of Yougui.

Chinese name	Botanical name (family name)	Pharmaceutical name	Amount (g)	Voucher specimens
Shu Di Huang	*Rehmannia glutinosa* (Gaertn.) DC. (Plantaginaceae)	Rehmanniae radix praeparata	9	1000001065
Shan Yao	*Dioscorea oppositifolia* L. (Dioscoreaceae)	*Dioscorea* rhizome	9	1000000908
Shan Zhu Yu	*Cornus officinalis* Siebold & Zucc. (Cornaceae)	Corni fructus	6	1000001097
Gou Qi Zi	*Lycium barbarum* L. (Solanaceae)	Lycii fructus	9	1000001087
Zhi Gan Cao	*Glycyrrhiza uralensis* Fisch. (Fabaceae)	Glycyrrhizae radix et rhizome	3	1000000884
Du Zhong	*Eucommia ulmoides* Oliv. (Eucommiaceae)	Eucommiae cortex	9	1000000792
Rou Gui	*Cinnamomum cassia* (L.) J.Presl (Lauraceae)	Cinnamomi cortex	3	1000001007
Fu Zi	*Aconitum carmichaeli* Debeaux (Ranunculaceae)	Aconiti lateralis radix praeparata	6	1000000606

TABLE 3 | The herbal composition of Zuogui.

Chinese name	Botanical name (family name)	Pharmaceutical name	Amount (g)	Voucher specimens
Shu Di Huang	*Rehmannia glutinosa* (Gaertn.) DC. (Plantaginaceae)	Rehmanniae radix praeparata	9	1000001065
Shan Yao	*Dioscorea oppositifolia* L. (Dioscoreaceae)	*Dioscorea* rhizome	6	1000000908
Shan Zhu Yu	*Cornus officinalis* Siebold & Zucc. (*Cornaceae*)	Corni fructus	6	1000001097
Gou Qi Zi	*Lycium barbarum L.* (Solanaceae)	Lycii fructus	6	1000001087
Zhi Gan Cao	*Glycyrrhiza uralensis* Fisch. (Fabaceae)	Glycyrrhizae radix et rhizome praeparata cum melle	3	1000000884
Fu Ling	*Poria cocos*[a] (Polyporaceae)	Poria	4.5	1000001020

[a]Poria cocos *is the dried sclerotia of* Wolfiporia cocos *(F. A. Wolf) Ryvarden & Gilb.*

TABLE 4 | The herbal composition of Zuogui-CSS.

Chinese name	Botanical name (family name)	Pharmaceutical name	Amount (g)	Voucher specimens
Chai Hu	*Bupleurum chinense* DC. (Apiaceae)	Bupleuri radix	6	1000000618
Bai Shao	*Paeonia lactiflora* Pall. (Paeoniaceae)	Paeoniae radix alba	4.5	1000000608
Zhi Ke	*Citrus* × *aurantium* L. (Rutaceae)	Aurantii fructus	4.5	1000000742
Chuan Xiong	*Ligusticum striatum* DC (Apiaceae)	Chuanxiong rhizoma	4.5	1000000872
Chen Pi	*Citrus reticulata* Blanco (Rutaceae)	Citri reticulatae pericarpium	6	1000000674
Xiang Fu	*Cyperus rotundus* L. (Cyperaceae)	Cyperi rhizoma	4.5	1000001080
Zhi Gan Cao	*Glycyrrhiza uralensis* Fisch. (Fabaceae)	Glycyrrhizae radix et Rhizoma praeparata cum melle	4.5	1000000884
Shu Di Huang	*Rehmannia glutinosa* (Gaertn.) DC. (Plantaginaceae)	Rehmanniae radix praeparata	9	1000001065
Shan Yao	*Dioscorea oppositifolia* L. (Dioscoreaceae)	*Dioscorea* rhizoma	6	1000000908
Shan Zhu Yu	*Cornus officinalis* Siebold & Zucc. (Cornaceae)	Corni fructus	6	1000001097
Gou Qi Zi	*Lycium barbarum* L. (Solanaceae)	Lycii fructus	6	1000001087
Fu Ling	*Poria cocos*[a] (Polyporaceae)	Poria	4.5	1000001020

[a]Poria cocos *is the dried sclerotia of* Wolfiporia cocos *(F. A. Wolf) Ryvarden & Gilb.*

Republic of China (2015 Edition). The combined decoction of Zuogui and CSS (Zuogui-CSS) was also prepared. The ingredients are listed in **Tables 1–4**.

For preparing the decoctions, botanical drugs for each formula were mixed and macerated in distilled water at room temperature for 1 h. The mixture was then decocted twice with distilled water

for half of an hour, at a ratio of 1:10 w/v and at a ratio of 1:5 w/v, respectively. The two resultant decoctions were combined, centrifuged, and filtrated. The decoctions of CSS, Yougui, Zuogui, and Zuogui-CSS were concentrated to 0.2777, 0.476, 0.304, and 0.5817 g/ml of the crude drugs, respectively. All decoctions were stored at −80°C.

Generation of the Rat Model for PMS With KD and LQS

SPF-grade (specific pathogen-free grade) female Sprague-Dawley (SD) rats (5 months old) were purchased from Shanghai Xipuer-Bikai Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed in individually ventilated cages and kept in an environmentally controlled room with a 12 h light/dark cycle at constant temperature (23°C) and humidity (55%). The rats had free access to the standard laboratory water and diet.

Perimenopausal rats were generated based on previous studies (Cai et al., 2016; Chen et al., 2020). These rats were with irregular menstrual cycles at the age of about 11–13 months. Forty of these perimenopausal rats were selected and randomly divided into five groups with eight rats each. The rat model with KD and LQS was generated by the method of chronic restraint stress (CRS) (Liang et al., 2015). The food intake, water intake, body weight, appearance, and activity of the rats were monitored weekly throughout the experiment. Plasma concentrations of estrogen (E2), adrenocorticotropic hormone (ACTH), cortisol (CORT), corticotropin-releasing hormone (CRH), 5-hydroxytryptamine (5-HT), dopamine (DA), and beta-endorphin (β-EP) were determined by ELISA (with kits from CUSABIO, China).

The human equivalent dose (HED) for rat has a multiplication factor of 6:37 (Reagan-Shaw et al., 2008). So the final concentration of crude drugs for Yougui, Zuogui, CSS, and Zuogui-CSS was adjusted to 0.476, 0.304, 0.2777, and 0.5817 g/ml, respectively. The decoctions were administered by gavage with a feeding tube once daily for 4 weeks at a dose of 1 ml for every 100 g of body weight. After overnight fasting, blood samples were collected and centrifuged. All plasma samples were stored at −80°C.

Sample Preparations and ^{1}H NMR Data Acquisition

The NMR spectra were acquired at 298 K on a 600 MHz Bruker AVANCE II NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600.13 MHz for ^{1}H signals. The ^{1}H NMR spectrum was acquired using a Carr-Purcell-Meiboom-Gill (CPMG, RD-90-(τcp-180-τcp)-acquisition) spin-echo pulse sequence to suppress the water with a total spin-spin relaxation delay (RD) of 320 ms to attenuate broad signals from proteins and lipoproteins due to their short transverse relaxation time. Each ^{1}H NMR spectrum was obtained with 80 scans with a spectral width of 12335.5 Hz, spectral size of 65,536 points, pulse width (PW) of 30° (12.7 μs), and RD of 2.0 s. The FIDs were Fourier transformed with LB = 0.3 Hz.

The ^{1}H NMR spectra were manually corrected for phase and baseline with the Topspin 3.2 software. Integrations of water resonance (4.70–5.15 ppm) in the spectra of aqueous samples were excluded. The metabolites were normalized to the total integrated spectral area (−0.55–8.55 ppm) for aqueous samples. The data sets were log-transformed and Pareto-scaled (mean-centered and divided by the square root of the standard deviation of each variable) prior to statistical analysis.

Multivariate Analysis

Multivariate analyses of the NMR spectra were carried out with the algorithm of PLS-DA (Partial Least Square Discriminant Analysis) implemented in MetaboAnalyst 4.0 (Chong et al., 2018). The model quality was validated based on two parameters, R^2 (goodness-of-fit parameter) and Q^2 (predictive ability). A model with a large R^2 (close to 1) and Q^2 ($Q^2 \geq 0.5$) was considered an excellent model. The PLS-DA model was also validated by the permutation test in which the class membership was randomly shuffled by 100 times for calculating the response values. The new R^2 and Q^2 values were lower than the original ones indicating that the model was not overfitting (Chang et al., 2007).

The important metabolites were identified based on their respective variable influence on projection (VIP) score in the PLS-DA analysis. Significant differences of the selected signals of the main metabolites, which were responsible for class discrimination, were analyzed using a *t*-test in GraphPad Prism 5 software. The data are presented as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Protein Production and Saturation Transfer Difference (STD) NMR Experiment

The human ligand-binding domain of estrogen receptor α (ERα LBD) containing residues 297–554 in the ERα sequence was expressed in *E.coli* with BL21 (DE) cells and a pET-22b vector. It was purified following an established protocol (Bruning et al., 2010). STD NMR (Mayer and Meyer, 2001; Meyer and Peters, 2003) was employed to detect the ERα LBD interaction with small molecules in the TCM decoctions at 298 K on an 850 MHz Bruker ADVANCE III spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a TCI CryoProbe. The RD was set to 2 s. Selective on-resonance irradiation frequency was set to 0.47 ppm with a saturation time of 2 s. The selective saturation was achieved by a train of 50 ms Gauss-shaped pulses separated by a 2 ms delay. The duration of the presaturation of 2 s was adjusted using n = 128 cycles. Off-resonance irradiation frequency for the reference spectrum was applied at 50 ppm. The decoction of Zuogui-CSS was mixed with the recombinant ERα LBD (20 μM) in phosphate buffer (50 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 5% glycerol) in the presence of 10% D_2O (Cambridge Isotope Laboratories, United States). The NMR data were analyzed with the Topspin 3.2 software.

In Vitro Gastrointestinal Digestion

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following the protocols in Pharmacopoeia of the People's Republic of China (2015 Edition). SGF was prepared by

mixing 16.40 ml of diluted hydrochloric acid, 800 ml of Milli-Q water, and 10 g of pepsin (Sigma-Aldrich, P7012) to a final volume of 1,000 ml. SGF thus prepared had a pH value of about 1.4. Solution A for SIF was prepared by dissolving 6.80 g of potassium dihydrogen phosphate in Milli-Q water to a final volume of 500 ml. The pH value of solution A was adjusted to 6.8 with a NaOH solution (0.1 mol/L). Solution B for SIF was prepared by dissolving 10 g of pancreatin (Sigma-Aldrich, P7545) in Milli-Q water. Solutions A and B were mixed and the final volume was adjusted to 1,000 ml with Milli-Q water to make SIF.

The SGF-treated Zuogui-CSS was prepared by incubating Zuogui-CSS with SGF at a ratio of 1:10 at 37°C with shaking at 100 rpm for 2 h. The sample was then dried using a rotary evaporator at 70°C. The SGF-treated samples were rehydrated with Milli-Q water. Similarly, Zuogui-CSS was treated with SIF by mixing Zuogui-CSS and SIF in a ratio of 1:4 for a 4 h incubation. It was similarly dried and then rehydrated as for the SGF-treated samples. Zuogui-CSS was also sequentially treated with SGF for 2 h and SIF for 4 h. The SGF- and SIF-treated samples were analyzed by ^{1}H NMR spectroscopy.

RESULTS

Perimenopause Was Accompanied by Metabolomic Changes

The perimenopausal rats (PM rats) were with irregular menstrual cycles starting at the age of about 12 months old and these rats were deemed to be with KD (Miao et al., 2018; Chen et al., 2020). The weight, sugar preference rate, and behavior indices of rats were of no significant difference between 6-month-old rats and perimenopausal rats, based on sugar preference test (SPT) and open-field test (OFT) (Strekalova et al., 2004; Kraeuter et al., 2019) (**Supplementary Figure S1**). While the levels of CRH, CORT, β-EP, and 5-HT showed no significant difference, the levels of E2 and DA were lower in perimenopausal rats, whereas the level of ACTH was upregulated (**Supplementary Figure S2**). These data were compatible with the physiological state of the perimenopausal rats (Cai et al., 2007; Fu et al., 2017).

In the preceding study, the metabolic profiles of rats entering the perimenopausal state and that of younger age were clearly different with metabolites for energy metabolism, such as lipid, glucose, trimethylamine-n-oxide, glutamine, pyruvate, acetoacetate, citrate, betaine, and acetone, influencing the shift of metabolomes (Chen et al., 2020).

Influence of LQS on Perimenopausal Rats

To generate a rat model for PMS, the perimenopausal rats were treated with CRS. These rats were considered to be with both KD and LQS (Li et al., 2015; Miao et al., 2018). The PMS rats were with a weight loss (**Figure 1A**), decreased sugar preference rate (**Figure 1B**), and reduced level of activity compared to either the 6-month-old or the PM rats without the stress (**Supplementary Figure S3**). The analyses also showed that the concentrations of E2, DA, β-EP, and 5-HT in the peripheral blood of the PMS rats decreased rapidly (**Figures 1C–E**), while the levels of ACTH and CORT in direct correlation with the HPA axis reactivity were elevated. These data indicated that PMS rats were under stress and depression.

The metabolomic states between PM rats and PMS rats were distinctly different (**Figure 2A**) and the metabolites that dictated the metabolomic change in the PMS rats were glucose and lipid (**Supplementary Figure S4** and **Figure 2B**). The levels of creatine, glycerol, trimethylamine-n-oxide, betaine, glutamine, pyruvate, acetoacetate, methionine, and glycoproteins increased in the plasma of PMS rats. In addition, the concentrations for unsaturated lipid, glycine, acetone, acetate, and isoleucine decreased (**Supplementary Figure S5** and **Supplementary Table S1**).

The metabolomic profile of PMS rats was also distinct from that of the 6-month-old rats (**Figure 2C**) with elevated levels of glucose, creatine, trimethylamine N-oxide, betaine, choline, creatinine, citrate, glutamine, succinate, pyruvate, acetoacetate, methionine, glycoprotein, acetate, and valine, but a downregulated level of glycine (**Figure 2D**, **Supplementary Figures S6, S7**, and **Supplementary Table S2**).

The above data indicated that the glucose metabolism and lipid metabolism of perimenopausal rats were upregulated from those of the 6-month-old rat. Entering the LQS state, the glucose metabolism was upregulated but the lipid metabolism was suppressed in PMS rats comparing to the perimenopausal rats. This aberrant state of glucose metabolism was in line with the fact that the rats were under stress and LQS was associated with downregulated lipid metabolism.

Influence of the Decoctions for KD on PMS Rats

After entering the PMS state, the metabolomic state changed rapidly without any treatment (**Figure 3A**). Since PMS would be with both KD and LQS by TCM diagnosis, it would be reasonable to treat the model with the corresponding prescriptions in TCM. KD was subdivided into kidney yin deficiency, kidney yang deficiency, and the combination of the two. Traditionally, Yougui, a decoction made from a mix of Chinese botanical drugs (**Table 2**), is prescribed for kidney yang deficiency and Zuogui (**Table 3**) is prescribed for kidney yin deficiency (Zhao et al., 2011; He et al., 2014; Fu et al., 2017; Chen et al., 2019). Previously, we discovered that both decoctions of Zuogui and Yougui could adjust the metabolomic states of PM rats (Chen et al., 2020). Metabolomic regulations of PMS rats by these two decoctions were also investigated.

PMS rats were treated with the decoction of Yougui (Yougui) for comparison with rats of 6-months old and of perimenopause. The treatment of Yougui showed little influence on the weight (**Figure 3B**) and the level of E2 of PMS rats (**Figure 3C**). However, the levels of ACTH and CORT were reduced, while the level of DA increased (**Figures 3D,E**). Overall, following the treatment with Yougui, the weight and the abovementioned endocrine indexes of PMS rats were not significantly changed. The E2 level was not significantly different either (**Figure 3**).

Yougui treatment did alter the metabolomic state of PMS rats by bringing the metabolomic state of PMS rats closer to that of perimenopausal rats (**Figures 4A,B**). The metabolomic difference

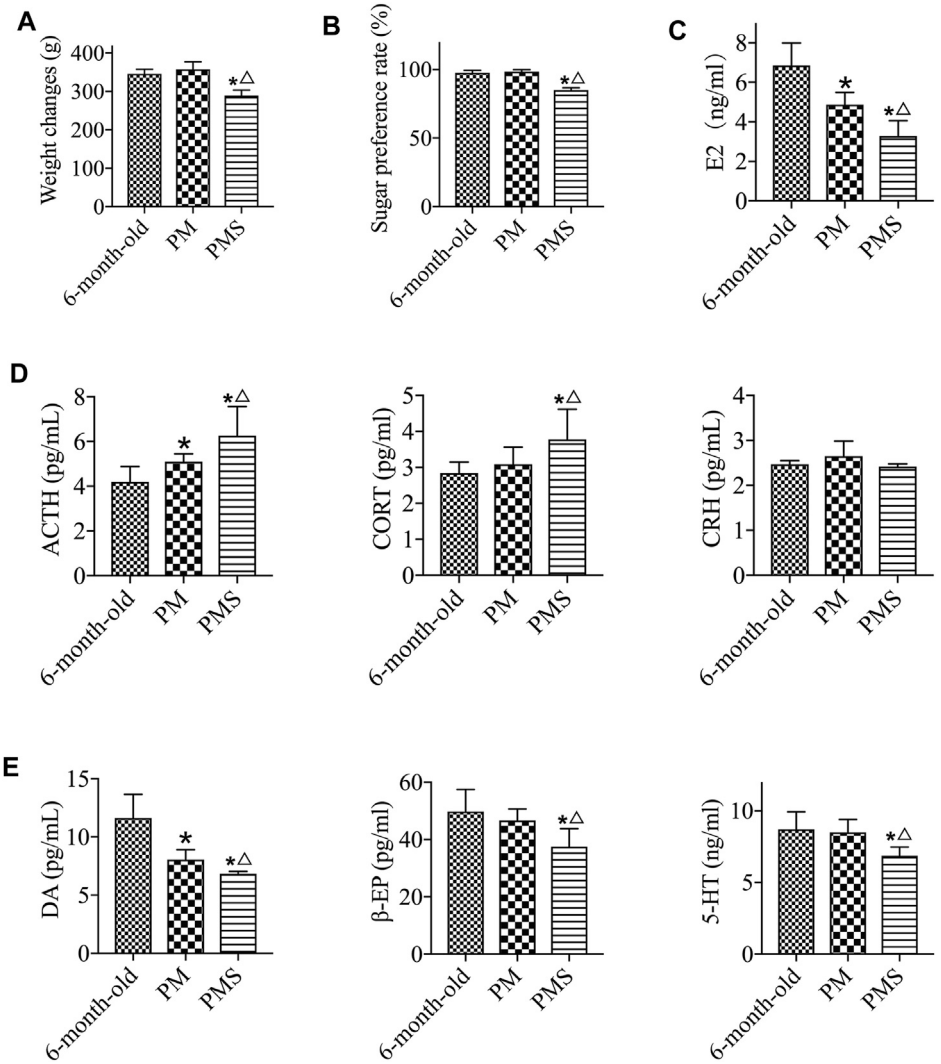

FIGURE 1 | PMS rats. **(A)** The body weights of 6-month-old, PM, and PMS rats. The PMS rats were with significant weight loss. **(B)** The PMS rats were of decreased sugar preference rate. **(C)** The level of estrogen in PMS rats was further decreased from that of perimenopausal rats. **(D)** The levels of ACTH and CORT in PMS rats were enhanced, while the level of CRH was not significantly altered. **(E)** The levels of β-EP, 5-HT, and DA were all lowered in PMS rats. "*" indicates the comparison with the 6-month-old rats [$p < 0.05$ (*)]; "△" indicates the comparison with the perimenopausal rats [$p < 0.05$ (△)].

between PMS rats treated with Yougui and rats of 6 months old though was still significant (**Figure 4C**). Overall, the data indicated that Yougui treatment improved the metabolomic state of PMS rats and brought its metabolomic profile closer to those of perimenopausal rats without LQS (**Figure 4D**). The dominant metabolites altering the metabolomic profile in the Yougui treatment were lipid, glucose, amino acids, and proteins (**Supplementary Figures S8–S10**).

After Yougui treatment, the levels of acetate and isoleucine increased significantly in the PMS rats, but the level of glucose decreased. In comparison to the 6-month-old rats, the levels of creatine, trimethylamine-n-oxide, choline, creatinine, glutamine, acetoacetate, glycoprotein, acetate, valine, isoleucine, and leucine were significantly higher (**Supplementary Figure S11** and **Supplementary Table S3**).

While Yougui is generally prescribed for kidney yang deficiency, the decoction of Zuogui (Zuogui) (**Table 3**) is for kidney yin deficiency in centuries of TCM practice (He et al., 2014). Treatment of PMS rats with Zuogui showed minimal influence on the body weight (**Supplementary Figure S12**). Similar to the treatment with Yougui, treatment with Zuogui did not lead to a significant change in the level of E2 in PMS rats (**Figure 5A**), but the levels of CORT and ACTH were reduced, and the level of DA was increased. Unlike Yougui, Zuogui treatment increased the level of β-EP (**Figures 5B,C**).

Zuogui modulated the metabolomic state of the PMS rats (**Figure 5D**). Lipid, glucose, proteins, and trimethylamine-n-oxide were important metabolites influencing the metabolomic profile (**Supplementary Figure S13**). Although the metabolomic profile moved in the direction of the perimenopausal rats, the

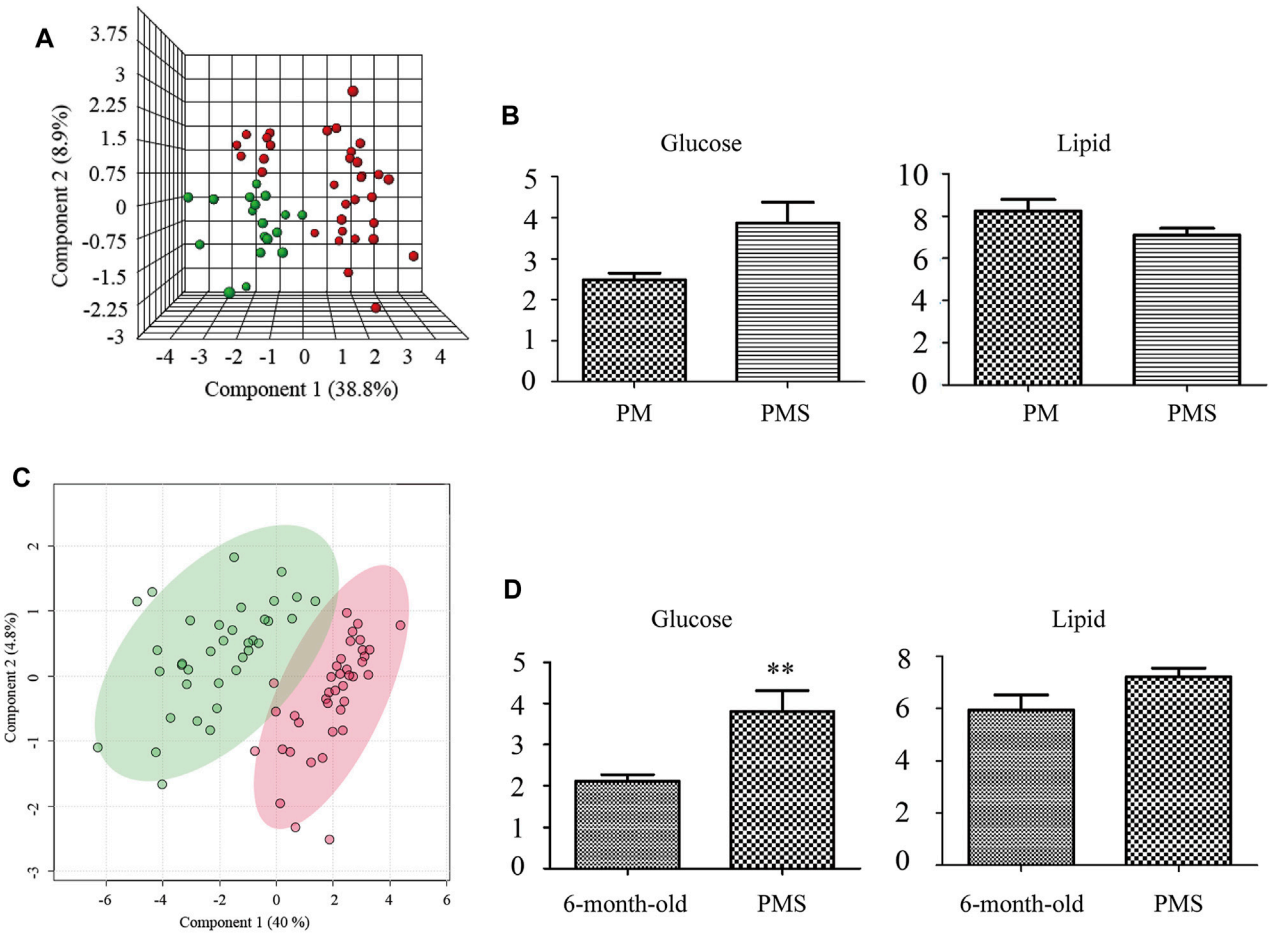

FIGURE 2 | Metabolomic profiles of 6-month-old, PM and PMS rats. **(A)** 3D representation of PLS-DA analysis of the PM (green) and PMS rats (red). The metabolomic profiles of the PMS rats changed significantly. **(B)** Transition from perimenopause to PMS, the levels of glucose were upregulated, while the levels of lipid were downregulated. **(C)** PLS-DA analysis of metabolomic profile from the plasma of 6-month-old rats (green) and PMS rats (red). **(D)** Compared with 6-month-old rats, the levels of glucose and lipid were upregulated. $p < 0.01$ (**).

adjustment was not sufficient to bring the metabolomic state of PMS rats to that of 6-months old (**Figure 5E**).

Zuogui upregulated the levels of unsaturated lipid, glutamine, acetate, valine, and lipid while downregulated the levels of creatine, trimethylamine-n-oxide, creatinine, glutamine, pyruvate, acetoacetate, and glycoproteins in the PMS rats. Notably, after the Zuogui treatment, the levels of creatine, creatinine, glutamine, pyruvate, glycoproteins, and citrate were brought to the metabolic levels of the 6-month-old rats (**Supplementary Figure S14** and **Supplementary Table S4**). It seemed that Zuogui attenuated the metabolism of PMS rats effectively, but it might also indicate that the PMS rats used here were more of kidney yin deficiency than kidney yang deficiency.

Influence of CSS on the PMS Rats

CSS (**Table 1**) is a classical prescription of TCM to treat LQS-associated illness. CSS was documented as early as 1624 in a book of TCM, Jing Yue Quan Shu. The decoction is an effective prescription for ameliorating depression and anxiety (Su et al., 2011; Su et al., 2014).

As shown in **Supplementary Figure S15**, not much change was observed in the weight of PMS rats treated with CSS as compared to that of the 6-month-old or perimenopausal rats. The changes in the levels of E2 and CORT were not significant either (**Figures 6A,B**). There was a significant reduction in the level of ACTH however and a considerable increase in the levels of β-EP, 5-HT, and DA in PMS rats treated with CSS, suggesting that CSS altered the endocrine parameters of PMS rats in the favorable direction (**Figure 6C**).

CSS treatment effectively modulated the metabolomic profile of PMS rats (**Figure 6D**). It brought the metabolomic state of the PMS rats closer to that of the PM rats but still not to that of the 6 months old (**Figure 6E**). It upregulated the levels of the unsaturated lipids, isoleucine, and lipid while downregulating the levels of glucose, creatine, betaine, creatinine, glutamine, pyruvate, and acetoacetate. The dominant metabolites for changing the metabolomic state by

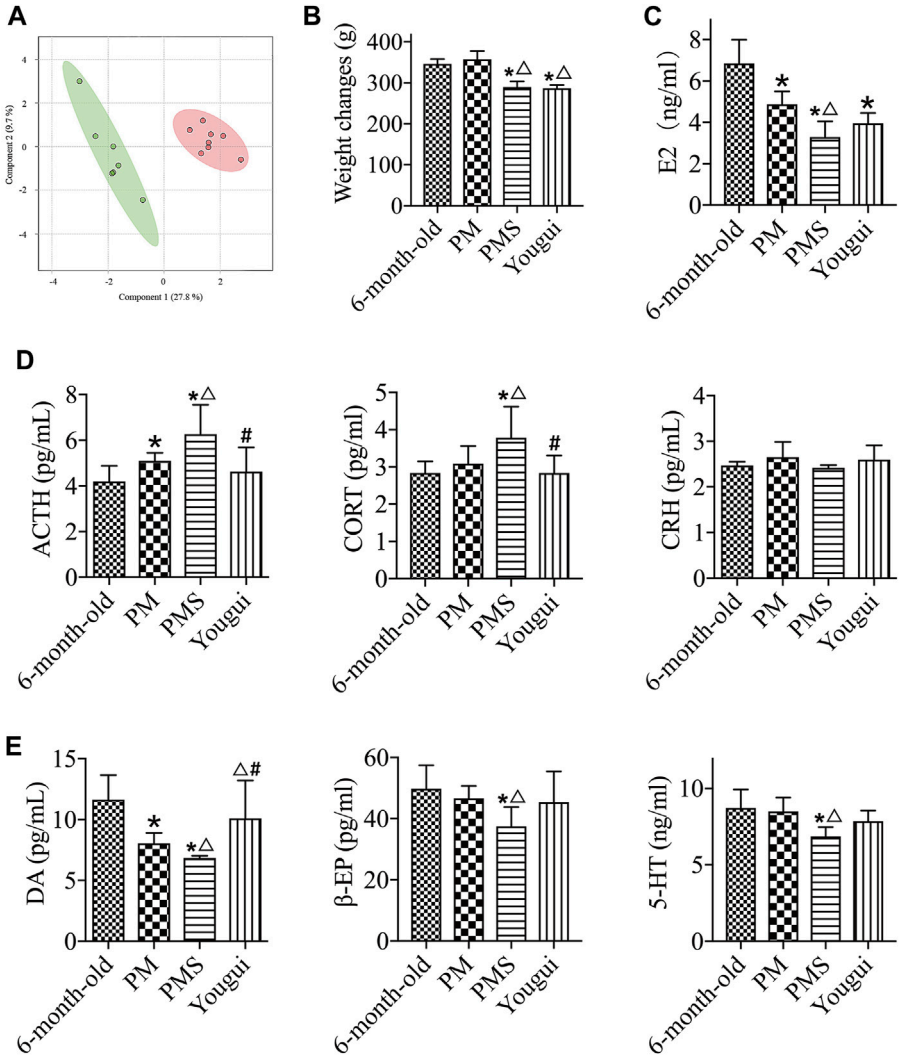

FIGURE 3 | Treatment with Yougui. **(A)** The metabolomics state of PMS (red) further progressed away from the original state after 4 weeks of gavage with a saline solution (green). The metabolic state changed rapidly. **(B)** Weight changes of PMS rats treated with Yougui. There was no statistically significant difference in the weight of PMS rats before and after treatment with Yougui. **(C)** Treatment of Yougui did not result in a statistically different E2 level in PMS rats. **(D)** ELISA results of ACTH, CORT, and CRH. Compared with PMS rats, the levels of ACTH and CORT were reduced after treatment with Yougui. **(E)** The levels of DA, β-EP, and 5-HT. Compared with PMS rats, the levels of DA were enhanced after treatment with Yougui. "*" indicates the comparison with 6-month-old rats [$p < 0.05$ (*)]; "△" indicates the comparison with the perimenopausal rats [$p < 0.05$ (△)]; "#" indicates the comparison with the PMS rats [$p < 0.05$ (#)].

CSS were glucose and lipid (**Supplementary Figure S16**). In comparison with the 6-month-old rats, the levels of most of the metabolites were higher, including trimethylamine-n-oxide, betaine, choline, citrate, glutamine, acetoacetate, acetone, methionine, glycoprotein, acetate, valine, isoleucine, leucine, and lipid. Moreover, the levels of glucose, creatine, pyruvate, glycerol, creatinine, and succinate were adjusted to that of the 6 months old **Supplementary Figure S17**, **Supplementary Table S5**). Overall, CSS upregulated the metabolism of lipid and downregulated the metabolism of glucose. Considering that LQS rats were with upregulated glucose metabolism and downregulated lipid metabolism, the data is consistent with the fact that CSS is with a tranquilizing potency.

A Combination Treatment for PMS

The aforementioned data indicated that both glucose and lipid metabolisms were upregulated in perimenopausal rats. In LQS rats, however, the glucose metabolism was further upregulated, while the lipid metabolism was downregulated. It was previously shown that the glucose and lipid metabolisms were both upregulated for the perimenopausal rats by the treatment with either Yougui or Zuogui (Chen et al., 2020). For the PMS rats though the glucose metabolism was downregulated, the lipid metabolism was upregulated for PMS rats treated with either Yougui, Zuogui, or CSS. It seemed that each of these decoctions was of various degrees of benefits in treating PMS rats based on the metabolomic indicators. However, none of these decoctions was effective enough to bring the metabolomic state of PMS rats to that of rats before entering the perimenopause.

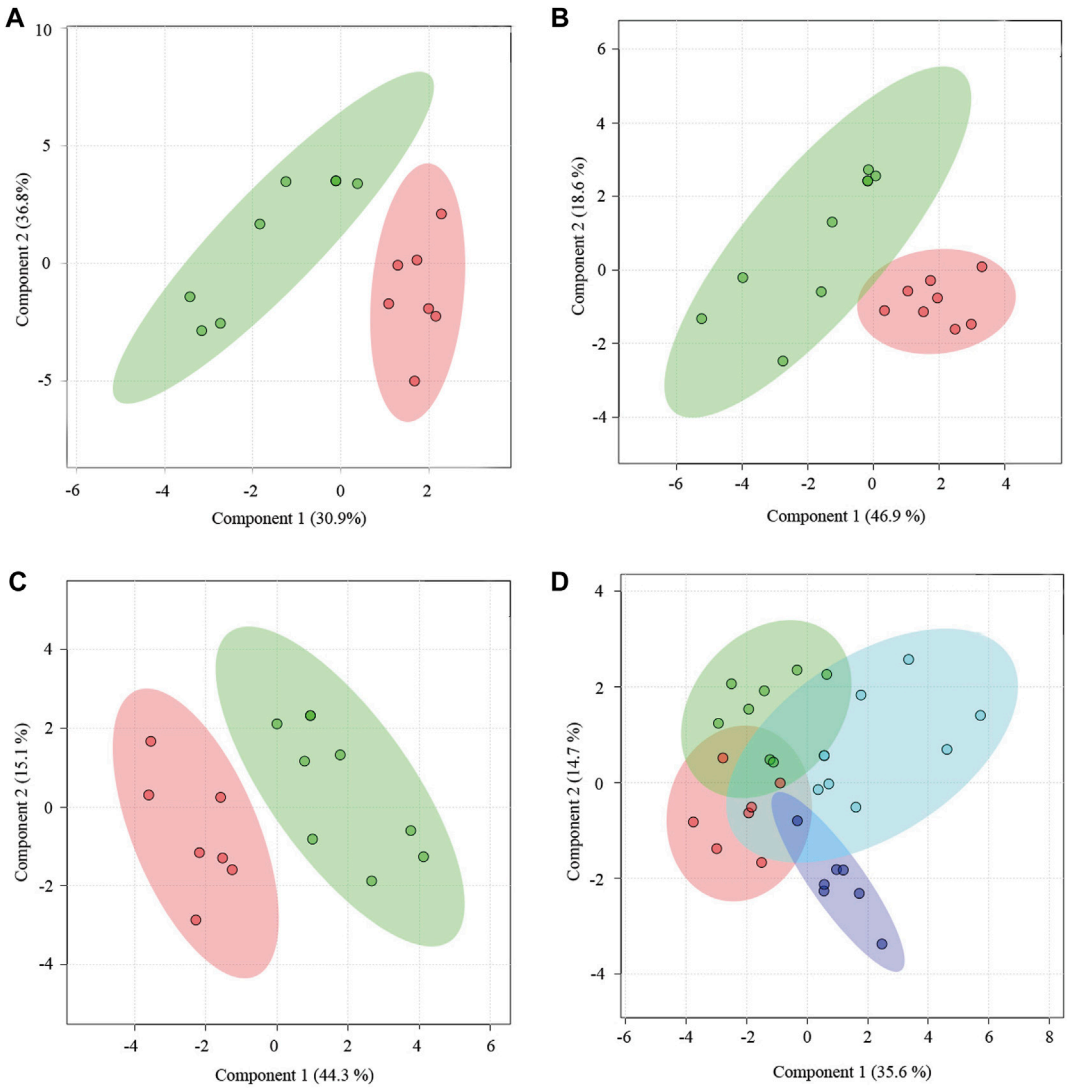

FIGURE 4 | Yougui regulated the metabolomic state of PMS rats. **(A)** PLS-DA analysis of metabolomic profile from the plasma of PMS rats before (red) and after (green) treatment with Yougui. **(B)** Metabolomic profile of the plasma of Yougui-treated PMS rats (green) and perimenopausal rats (red). **(C)** Metabolomic profile from the plasma of Yougui-treated PMS rats (green) and that of 6-month-old rats (red). **(D)** PLS-DA analysis of metabolomics profiles from the plasmas of PMS rats treated with (blue) and without Yougui (purple), perimenopausal (green) rats, and 6-month-old rats (red). Yougui treatment modulated the metabolomic state of PMS rats towards that of rats at younger ages.

As different decoctions for KD and LQS seem to adjust the metabolomics in different directions and attenuate different sets of metabolites, one would naturally wonder what would be the effect if both KD and LQS were ameliorated simultaneously. To this end, a decoction containing ingredients from Zuogui-CSS was prepared for the gavage of PMS rats.

The weight of PMS rats was not dramatically changed by the treatment with this decoction containing ingredients from Zuogui-CSS (**Supplementary Figure S18A**). The level of E2 was not significantly altered with the treatment either (**Supplementary Figure S18B**). The ACTH and CORT levels of peripheral blood dropped after the treatment, but the levels of DA, β-EP, and 5-HT increased (**Supplementary Figures S18C,D**).

The metabolomic analysis indicated that the combined treatment of Zuogui-CSS was effective for restoring a metabolic state of PMS (**Figure 7**). Treatment with Zuogui-CSS not only adjusted the metabolomic state of the PMS rats to that of the perimenopausal rats but also brought the metabolomic profile of PMS rats to the proximity of the metabolomic profile of the 6-month-old rats (**Figure 7B**). Further analysis indicated that Zuogui-CSS modulation of the metabolome of PMS rats was with lipid and glucose as the dominant factors (**Supplementary Figure S19**). The Zuogui-CSS treatment significantly lowered the levels of glucose, glycerol, trimethylamine-n-oxide, betaine, glutamine, acetoacetate, methionine, glycoprotein, valine, and leucine, but the levels were still higher than those of the 6-month-old rats. In

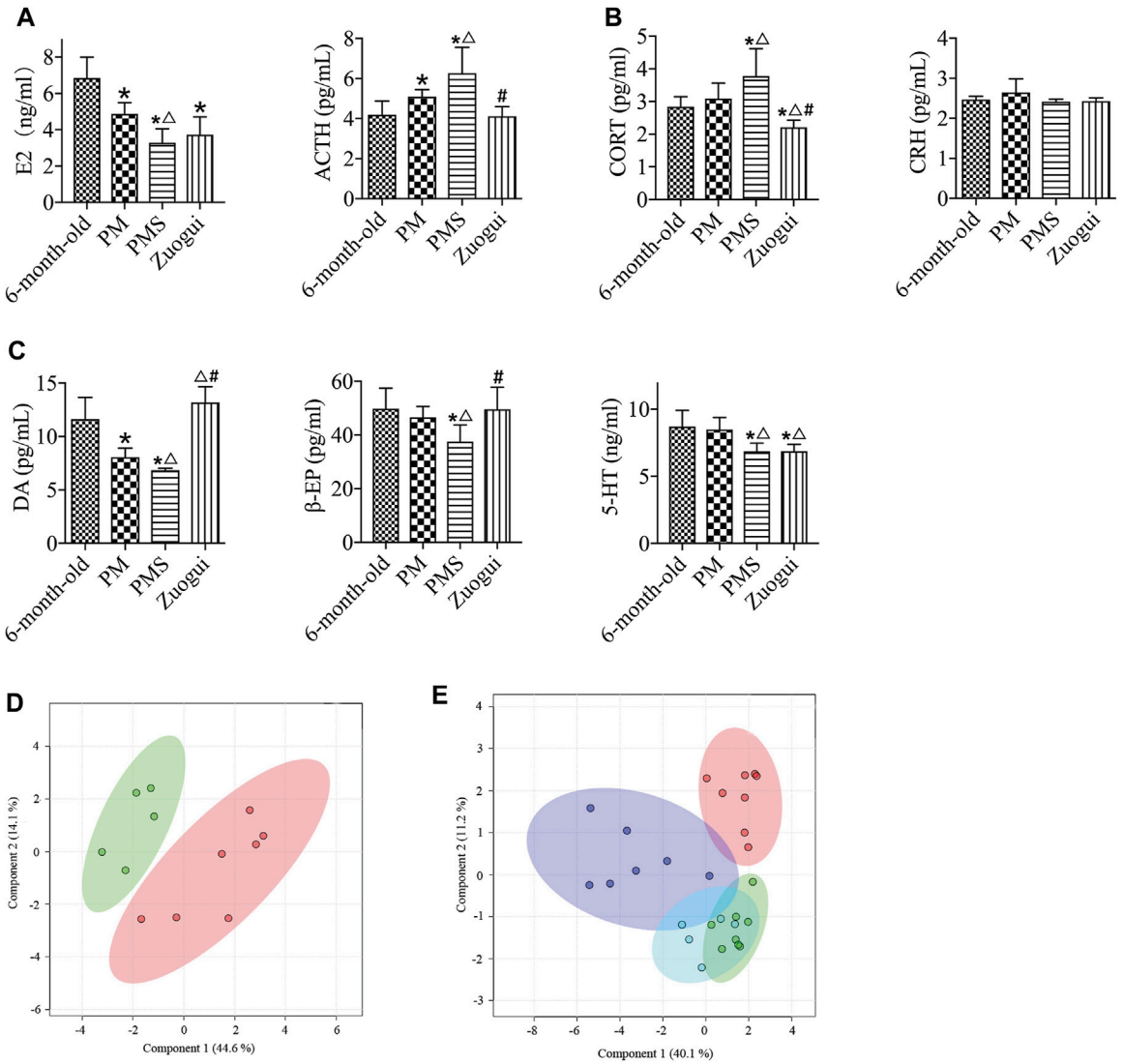

FIGURE 5 | Zuogui regulated the levels of metabolites and endocrine indexes in PMS rats. **(A)** The E2 levels of PMS rats treated Zuogui were not significantly different. **(B)** The levels of ACTH and CORT of PMS rats were reduced after the treatment with Zuogui. **(C)** The levels of DA and β-EP in PMS rats were enhanced after being treated with Zuogui. **(D)** PLS-DA analysis of metabolomics profiles for PMS rats treated with (green) and without Zuogui (red). **(E)** Metabolomic profiles of PMS rats (purple), PMS rats treated with Zuogui (blue), perimenopausal rats (green), and 6-month-old rats (red). "*" indicates the comparison with 6-month-old rats [$p < 0.05$ (*)]; "△" indicates the comparison with perimenopausal rats [$p < 0.05$ (△)]; "#" indicates the comparison with PMS rats [$p < 0.05$ (#)].

contrast, the pyruvate concentration in PMS rats was even lower than that in the 6-month-old rats after the treatment. Other metabolites, such as creatine, citrate, succinate, acetoacetate, acetone, glycine, and isoleucine, were of similar levels between PMS rats treated with Zuogui-CSS and the 6-month-old rats (**Supplementary Figure S20**; **Supplementary Table S6**).

No Detectable Estrogenic Compounds in the Decoction for Treating PMS

It was possible that the effective attenuation of the metabolomic state of PMS rats without significant change in the level of estrogen was through modulating the activity of estrogen receptors with interacting compounds in Zuogui-CSS. To address this possibility, the ligand-binding domain of estrogen receptor α (ERα LBD) was expressed and purified (**Supplementary Figure S21**). STD, a ligand-based NMR technique (Mayer and Meyer, 2001), was employed to detect the possible interacting molecules in the decoction. The STD experiment involves subtracting a spectrum, in which the protein was selectively saturated with magnetic radiation, from another spectrum without the protein saturation. In the difference spectrum, only the signals of those compounds that received the magnetic radiation transferred from the protein will remain. Other compounds that do not bind to the protein will not receive the saturation transfer so their characteristic signal will not

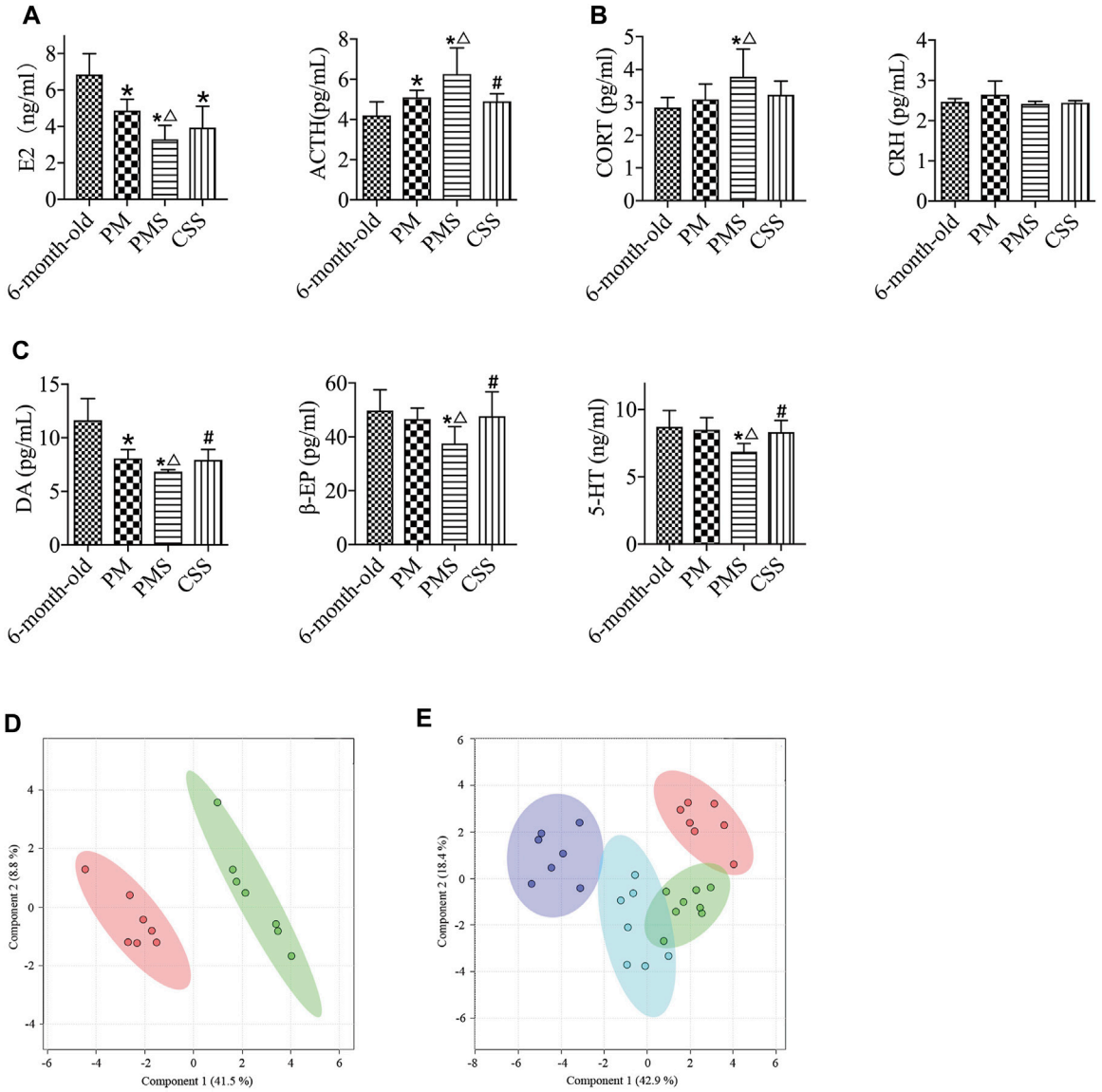

FIGURE 6 | PMS rats treated with CSS. **(A)** The E2 level of the PMS rats with treatment was not significantly different. **(B)** Compared with the PMS rats, the levels of ACTH were reduced after treatment with CSS. **(C)**. The levels of β-EP, 5-HT, and DA in PMS rats were enhanced after the treatment with CSS. **(D)** Metabolomic profiles of PMS rats with (red) and without (green) the CSS treatment. **(E)** PLS-DA analysis of the metabolomics of plasma from that CSS-treated PMS (blue), PMS (purple), perimenopausal (green), and 6-month-old (red) rats. "*" indicates the comparison with 6-month-old rats [$p < 0.05$ (*)]; "△" indicates the comparison with perimenopausal rats [$p < 0.05$ (△)]; "#" indicates the comparison with untreated PMS rats [$p < 0.05$ (#)].

appear in the difference spectrum (Meyer and Peters, 2003). The techniques can detect K_d values ranging between 10^{-3} and 10^{-8} M for the interacting compounds and is especially useful for detecting weak binders (Mayer and Meyer, 2001). Many phytoestrogens are with affinities in this range to estrogen receptors. For example, the K_d value for daidzein binding ERα is 5.9×10^{-6} M (van Lipzig et al., 2004) and that for coumestrol is 1×10^{-4} M (Qiu et al., 2020). So the technique is applicable to detecting the like compounds in the decoctions if they are of reasonable concentration. The spectrum without the saturation transfer from the protein was obtained for Zuogui-CSS (**Figure 8A**). In the STD difference spectrum (the upper spectrum in **Figure 8B**), the only observed peaks were those from glycerol (3.54, 3.63, and 3.77 ppm) and H_2O (4.70 ppm) in the solution (Lu et al., 2018). These were nonspecific peaks due to their high concentrations as they also were present in the spectrum without the protein (lower spectrum in **Figure 8B**). So, there was no detectable compound by STD in the decoction to interact with ERα.

As these decoctions were through the stomach and intestinal tracts after the gavage, the gastrointestinal digestion could yield interacting compounds. To test this possibility, Zuogui-CSS was treated with SGF and SIF for the STD NMR experiments. The treatment with either SGF or SIF indeed produced new

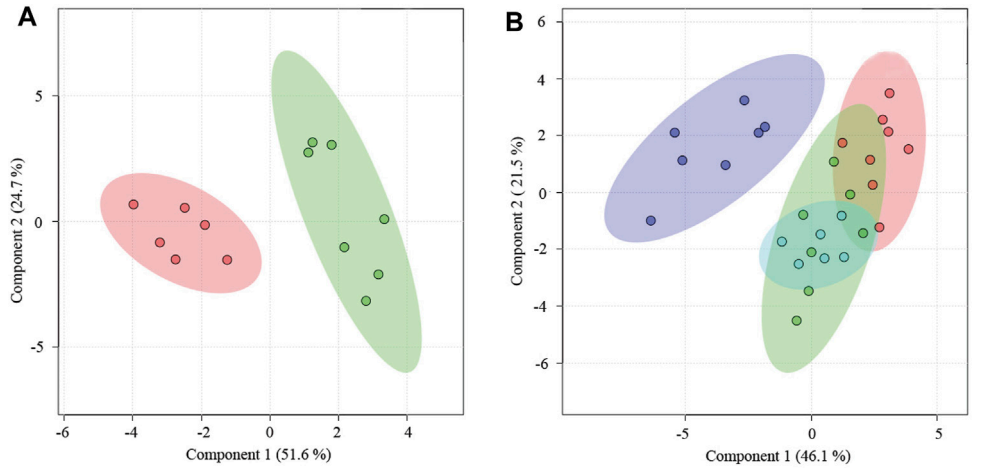

FIGURE 7 | A combination treatment for PMS. **(A)** PLS-DA analysis of metabolomics for the plasmas of PMS rats treated with (red) and without Zuogui-CSS (green). **(B)** Metabolomic profiles of the plasmas of PMS rats treated with Zuogui-CSS (blue), PMS rats (purple), perimenopausal rats (green), and rats of 6 months old (red).

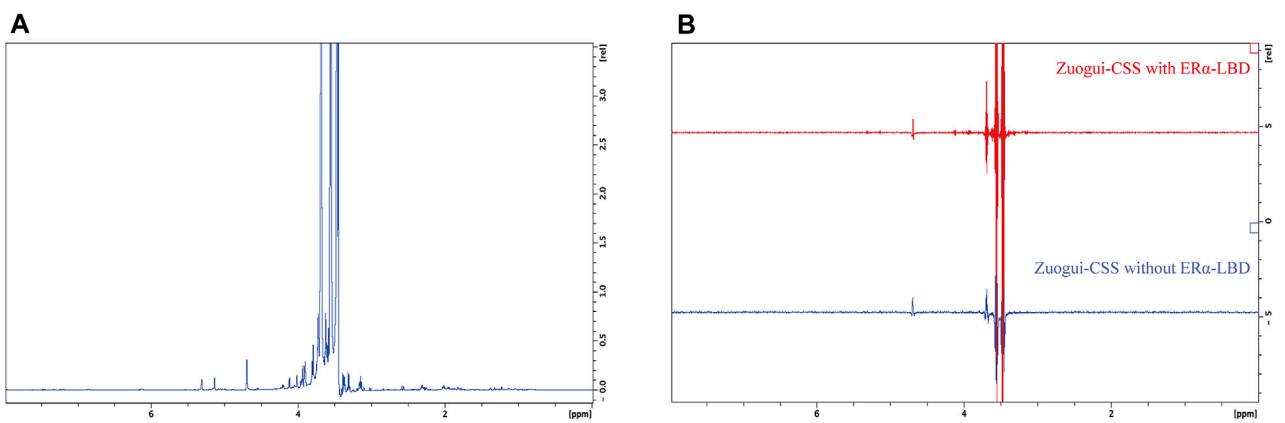

FIGURE 8 | STD NMR detection for estrogenic compounds in Zuogui-CSS. **(A)** The 1D ^{1}H NMR spectrum of Zuogui-CSS. **(B)** The STD spectra of Zuogui-CSS in the presence of ERα-LBD (upper red spectrum) and in the absence of ERα-LBD (lower blue spectrum). Only nonspecific solvent signals from glycerol (3.54, 3.63, and 3.77 ppm) and H_2O (4.70 ppm) were observed in the STD spectra.

compounds (**Figure 9A**). The decoction was also first treated with SGF and then with SIF simulating its passage through the gastrointestinal system. The resulting spectrum for the sequential treatment of Zuogui-CSS though was the superposition of spectra from Zuogui-CSS treated with SGF and SIF separately (**Figure 9A**). These newly generated compounds by the gastrointestinal digestion were with no detectable interaction with ERα LBD either based on the STD NMR analyses (**Figures 9B–D**).

DISCUSSION

Perimenopause is a period of transition to menopause characterized by irregularity in the menstrual cycle with fluctuating and overall declining levels of estrogen in women. As the body conditions going through a significant transformation with the gradual cessation of reproductive functions, many perimenopausal women suffer from PMS. Hormone replacement therapy (HRT) is an established clinical practice for treating PMS (Whiteley et al., 2013). However, serious side effects have also been reported for HRT (Hickey et al., 2005; Sare et al., 2008). For a transition to menopause, the declining level of estrogen and reproductive senescence is a natural process of aging. Supplementation of estrogenic compounds, such as that in HRT, could be countering a natural trend in the body. By directly compensating for the loss of estrogen, the PMS symptoms might be soothed but it could also be counterproductive and result in undesirable side effects. A better treatment strategy might be to palliate the

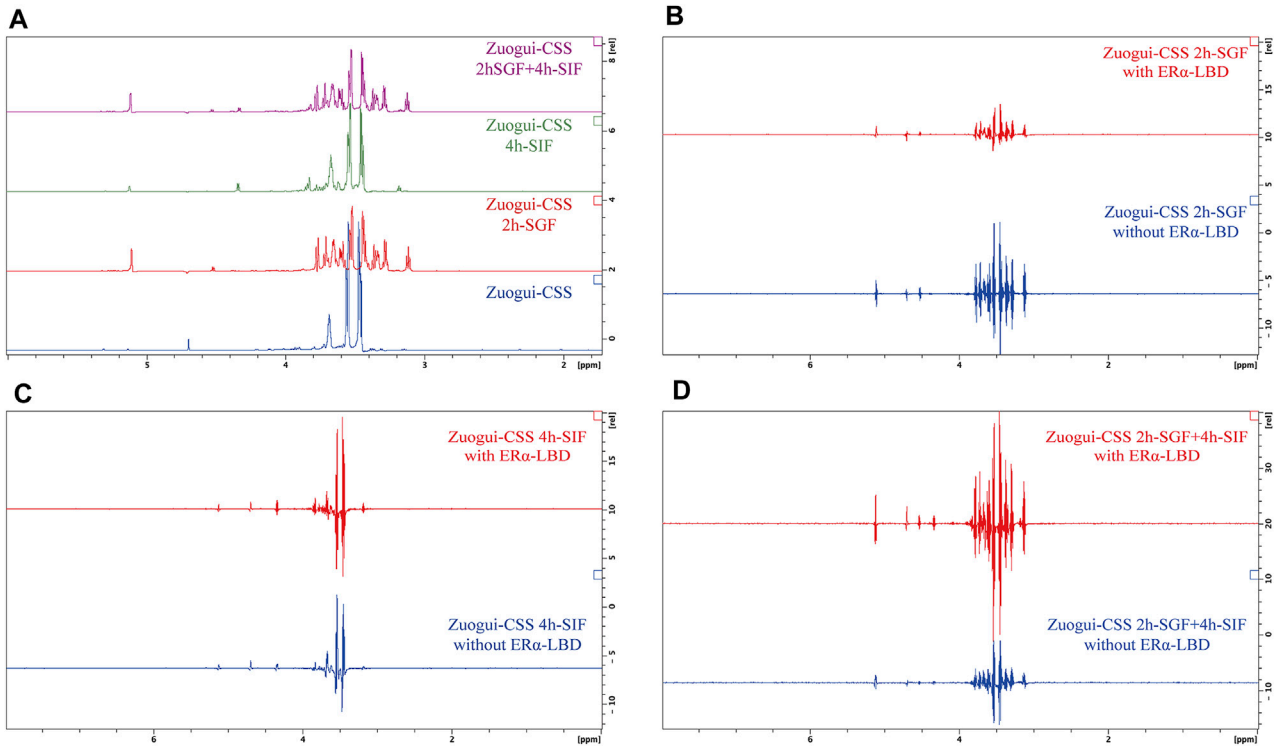

FIGURE 9 | Gastrointestinal digestion of Zuogui-CSS produced no estrogenic compound. **(A)** ^{1}H NMR spectra of Zuogui-CSS treated with simulated gastrointestinal fluids. The blue-colored spectrum was for Zuogui-CSS without the treatment. The green-colored spectrum was for Zuogui-CSS treated with SIF for 4 h. The red-colored spectrum was for Zuogui-CSS treated with SGF for 2 h. The purple-colored spectrum was for Zuogui-CSS sequentially treated with SGF for 2 h and SIF for 4 h. Peaks for new compounds appeared after Zuogui-CSS was treated with either SGF, SIF, or both. **(B)** The STD spectra of Zuogui-CSS treated with SGF for 2 h. The upper red-colored spectrum was from the sample with ERα-LBD and the lower blue-colored spectrum was from the sample without ERα-LBD. **(C)** The STD spectra of Zuogui-CSS treated with SIF for 4 h. The upper red-colored spectrum was from the sample with ERα-LBD and the lower blue-colored spectrum was from the sample without ERα-LBD. **(D)** The spectra of Zuogui-CSS were treated sequentially with SGF for 2 h and SIF for 4 h. The upper red-colored spectrum was from the sample with ERα-LBD and the lower blue-colored spectrum was from the sample without ERα-LBD. The STD spectra were with only solvent signal peaks (e.g., glycerol, H_2O, and self-degradation products of pepsin or pancreatin). There was no indication of any compound that could interact with ERα-LBD.

symptoms of PMS without interfering with the body's natural transition to menopause such as supplementing estrogenic compounds.

The association of PMS with KD and LQS in TCM seems enigmatic and jargonistic to the general practitioners of medicine. However, the fact that the subsequent prescriptions based on the theory of TCM are effective is an indication that it is with sound rationales. Our data here indicated that Zuogui, Yougui, and CSS were efficacious to various degrees for treating PMS, with Zuogui-CSS adjusting body's conditions to a more seemingly desirable direction in a PMS model that was deemed to be with both KD and LQS. The best strategy, however, seemed to be treating both KD and LQS simultaneously with a combined decoction of Zuogui and CSS for the PMS rats. This combination treatment could bring the metabolomic state of PMS close to that of rats at a younger age before entering perimenopause. It was even more remarkable that this amelioration was done seemingly neither by directly upregulating the estrogen level nor by supplementing an estrogenic compound.

The dose of phytoestrogens for treating PMS is in the range of hundreds of milligrams to grams for humans (Crisafulli et al., 2005; Xiao, 2008), which would be about milligrams to hundreds of milligrams for rats considering the difference in size and HED. Taking into accounts the volume of decoction feeding to the animal (4–5 ml) and the averaged molecular weight of phytoestrogens (~250), the useful concentration for phytoestrogens should be around a few millimolars to hundreds of millimolars. Since the STD technique employed here is capable of detecting interacting compounds in the range of low millimolars to nanomolars, or even lower, the detection of no interacting compound in Zuogui-CSS by STD indicates that either there was just no such compound or the amount of the compound was too small to effectively modulate the activity of estrogen receptors like those estrogenic compounds did in HRT. In light of the fact that the ingredients from two decoctions were to be mixed to yield the desirable results, it is possible that multiple factors are in play to attenuate the metabolomic state of the PMS model.

The doses used for this study seem to be an important factor to obtain the results in this study as a previous study showed that the increased dosage of Zuogui at daily applications of

either 13.78 g/kg, 20.67 g/kg, or 31 g/kg for each rat, as compared to the daily dose of 3.04 g/kg for each rat in this study, could lead to the increased serum level of estrogen in a dose-dependent manner (Zhao et al., 2011). It implies that a well-calibrated and moderate dose of botanical drugs, Zuogui in this particular case, is important for ameliorating the disorder while avoiding the side effects as it is desirable to treat PMS without elevating the level of estrogen to counter the natural trend of the body's transition to menopause.

The consistency between the metabolomic data in this study and the time-honored clinical practice of the abovementioned traditional Chinese prescriptions is an indication that the metabolomic indicators are applicable for assessing the efficacy of treating PMS with Chinese botanical drugs. It also implicates metabolomics as an invaluable tool to guide the use of traditional Chinese botanical drugs, in this case, by using a lower dose without elevating the level of serum estrogen for treating the disorder.

As effective as the treatment of the PMS model seems to be with the current prescription, the molecular mechanism of the treatment awaits further investigation. Does a ligand-independent ER pathway form the molecular basis of the TCM treatment of PMS? Or does the medicine applied here activate an ER-independent signaling pathway? In either case, the data presented here afford solid evidence for a better strategy to treat PMS with the traditional Chinese prescription of botanical drugs.

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 Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine (permission number: SYXK (Min) 2014-0005).

AUTHOR CONTRIBUTIONS

JL, XC, and CW performed the experiments and analyzed the data. ZZ, LH, LR, PZ, XY, CL, and SL were involved in maintaining the animals, generating the models, and collecting the samples. CY, SW, YD, and HR participated in the production of the recombinant proteins and the NMR experiments. W'nL, JS, HH, BG, and HY provided professional advice and helped in guiding the experiments. TL, XC, and CL designed and supervised the study and wrote the manuscript. All authors reviewed and approved the final version of 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.2021.744409/full#supplementary-material

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Clinical Effects of Korean Red Ginseng in Postmenopausal Women With Hand Osteoarthritis: A Double-Blind, Randomized Controlled Trial

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Background: Although many menopausal Asian women use herbal remedies for joint pain, there are no studies evaluating the efficacy of Korean red ginseng on osteoarthritis symptoms in postmenopausal women. The purpose of this study is to analyze antioxidant enzyme activity, oxidative stress markers, and pain scores before and after red ginseng consumption, to assess its effect in postmenopausal women. Methods. This prospective, double-blind, randomized controlled trial enrolled 52 postmenopausal women who presented with hand edema and/or pain and were diagnosed as degenerative arthritis of the hand. Patients were randomly assigned to the red ginseng (RG) group (supplemented with 3 g/d of RG for 12 weeks) or the placebo group. Changes in pain and Disability of the Arm, Shoulder, and Hand (DASH) scores, antioxidant enzyme, oxidative stress markers, serum estradiol levels, and endometrial thickness were analyzed. Results. The pain score and DASH score were significantly improved in the RG group (both $p < 0.05$). The improvement of pain score at rest, during work or sport, and DASH score was significant compared to that of the placebo group. The superoxide dismutase level increased ($p < 0.05$) and the malondialdehyde level decreased ($p < 0.05$) significantly in the RG group, while none of the antioxidative factors showed a significant change in the placebo group. Serum estradiol levels and endometrial thickness were not affected by RG supplementation. Conclusion. RG may be an effective dietary supplement for postmenopausal women with degenerative osteoarthritis of the hand. It may relieve pain and improve antioxidative activity without the risk of endometrial thickening.

Keywords: cartilage, osteoarthritis, pain, panax ginseng, postmenopausal state, randomized controlled trial

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INTRODUCTION

Many women experience vasomotor symptoms such as hot flushes and night sweats during the perimenopausal period. However, the prevalence of such symptoms and the perception of their discomfort are influenced by several different factors, including ethnicity, biological environment, lifestyle, overall health, and socioeconomic status (Obermeyer, 2000). There is evidence that the prevalence of menopausal symptoms is different in Asian women compared to that in Western

women. Asian women suffer less from vasomotor symptoms but more commonly report joint pain (Mccarthy, 1994; Hilditch et al., 1999; Haines et al., 2005).

Some observational studies have reported favorable effects of exogenous estrogen on joint pain (Nevitt et al., 1996; Wluka et al., 2001; Szoeke et al., 2006; Szoeke et al., 2008). In addition, post hoc analyses of the Women's Health Initiative (WHI) randomized controlled trial (RCT) have also shown that the use of estrogen alone significantly reduced joint pain in postmenopausal women (Chlebowski et al., 2018). Based on such results, hormone therapy may help alleviate joint pain. However, many women use herbal remedies instead of hormone therapy because of concerns about possible adverse effects of long-term hormone therapy.

Ginseng root (Panax ginseng C.A. Meyer) has been widely used in East Asian traditional medicine to improve general health and treat various conditions. Ginsenosides are the major constituent of ginseng root and exhibit a large variety of biological and pharmacological activities. Red ginseng (RG) is manufactured by steaming and drying fresh white ginseng (WG) and contains newly identified ginsenosides, which are believed to have more potent pharmacological activities than those of WG (Kim et al., 2000). Experimental studies have demonstrated that certain ginsenosides have the potential to be used as therapeutic agents in patients with osteoarthritis (OA) (Cheng et al., 2013; So et al., 2013; Lee et al., 2014). However, no clinical studies have investigated the effects of RG as an alternative therapy for OA symptoms in postmenopausal women.

A previous study has shown that RG did not relieve the vasomotor symptoms but did reduce the Kupperman index and Menopause Rating Scale scores (Kim et al., 2012). In addition, RG was demonstrated to have antioxidant effects, which may be of some benefit in preventing the destruction of articular cartilage as a result of oxidative stress (Seo et al., 2014). However, this study did not focus on whether RG can help relieve OA symptoms. This study was conducted to evaluate the effect of RG on joint pain related to OA in postmenopausal women in various circumstances. In addition, we assessed the cartilage markers such as cartilage oligomeric matrix protein (COMP) and C-terminal crosslinked telopeptide type II collagen (CTXII), oxidative stress, and hormone levels before and after RG or placebo consumption.

MATERIALS AND METHODS

Participants

This study recruited participants from among patients who visited the outpatient clinic of orthopedic surgery with the chief complaints of pain and edema of the hand and were diagnosed with degenerative OA by x-ray. All the patients were asked if they were menopaused and only the patients who were confirmed to be menopaused were asked to enroll to the study. Body measurements (height, weight, body mass index (BMI), blood pressure) and lipid profiles were obtained for all the study participants.

Patients with a diagnosis of rheumatoid arthritis, traumatic arthritis, or other orthopedic diseases such as rotator cuff tear, frozen shoulder, trauma, tenosynovitis, or peripheral neuritis were excluded because these diseases could affect the pain and function of the hand. Patients with chronic anti-inflammatory analgesic use for more than 1 month for degenerative arthritis were also excluded.

Study Design

This study was a single-center, double-blind RCT. After the initial screening visit and examination, all participants were allocated to either the RG or placebo group in a 1:1 ratio using a computer-generated random number sequence by Biostatistics Collaboration Unit (BCU) of Yonsei college of medicine. The random number sequence was kept secret to both investigators and participants until the end of trial to both investigators and participants. The permutation of random numbers generated by the SAS® system's Randomization program. The randomization table was devised and generated before clinical trials through SAS®.

RG and placebo capsules were provided by the Korea Ginseng Corporation (Daejeon, Korea) for the trial. The company packaged the tablets with the label according to the randomization number and supplied them to the testing institution before the trial. For double-blindness, the investigator provided the capsule with unique code consistent with the allocation number of the participants.

The RG group received 1 g of RG three times daily while the placebo group received identically shaped capsules composed of 95.25% cornstarch, 4% ginseng aromatic powder, 0.15% natural dye, and 0.6% caramel dye, to be taken three times a day for 12 weeks. Each RG capsule contained 500 mg of RG.

The ginsenoside composition in the RG was analyzed by high-performance liquid chromatography. 1 g of ginseng dried sample pulverized by 80–100 mesh was weighed in a centrifugation tube. 10 ml 50% MeOH was added and mixed uniformly. It was cooled for 1 h at 80°C and centrifugation (3,000 rpm, 10 min) was performed. The supernatant was taken. This process was repeated. The supernatant was vacuum-concentrated in a 50°C water tank using a rotary evaporator. The concentrate was dissolved in 2 ml of distilled water filtered with a 0.45 μm membrane filter. It was analyzed by a validated method of high-performance liquid chromatography. According to the standard method required by Health Functional Food Acts of South Korea, HPLC analysis was performed with Halo® RP-Amide column (4.6 × 150 mm, 2.7 μm, Advanced Materials Technology, Inc., DE, United States) at 50°C (Fuzzati, 2004). Mobile phase was 0–6 min: 27–28% acetonitrile; 6–10 min: 28%; 10–30 min: 28–34%; 30–33 min: 34–80%; 33–35 min: 27% acetonitrile and gradient eluted. The flow rate was 0.5–0.8 ml/min and the absorbance was measured at the 203 nm wavelength of the UV detector . It was found to contain Rg1 (2.61 mg/g), Rb1 (4.26 mg/g), Rb2 (1.65 mg/g), Rg2s (0.20 mg/g), Rg3s (0.13 mg/g), Rc (1.80 mg/g), Rd (0.29 mg/g), Re (1.71 mg/g), Rf (0.67 mg/g), and Rh1 (0.11 mg/g).

For the HPLC analysis, reference standard method was used. Regarding the precision of the HPLC, the results and statistical values of the three repeated tests by adjusting the amount of ginsenoside Rg1, Rb1 and Rg3s to 0.25, 0.5, and 0.75 g was presented in **Table 1**. Regarding the accuracy and percentage recovery, we added the saponin fraction corresponding to 100%, 150%, and 200% of the amount of ginsenoside Rg1, Rb1, and Rg3s present in 0.5 g of the sample and the test was repeated 3 times. The

TABLE 1 | Precision of HPLC analysis.

	Rg1		Rb1		Rg3s	
	Mean ± SD	**Precision (%RSD)**	**Mean ± SD**	**Precision (%RSD)**	**Mean ± SD**	**Precision (%RSD)**
Level 1 (0.25 g)	2.808 ± 0.009	0.33	4.994 ± 0.020	0.39	0.228 ± 0.004	1.60
Level 2 (0.5 g)	2.697 ± 0.073	2.70	4.758 ± 0.123	4.65	0.211 ± 0.005	2.13
Level 3 (0.75 g)	2.654 ± 0.007	0.25	4.641 ± 0.012	0.26	0.211 ± 0.003	1.47
Between-level	2.720 ± 0.038	1.38	4.798 ± 0.062	1.30	0.217 ± 0.001	0.32

SD, standard deviation; SD, standard deviation; RSD, releative standard deviation.

TABLE 2 | Accuracy and percentage recoveray of HPLC analysis.

	100% (Mean ± SD)	**150% (Mean ± SD)**	**200% (Mean ± SD)**
Rg1	100.0 ± 0.40	101.9 ± 0.87	100.8 ± 1.03
Rb1	99.9 ± 0.55	101.7 ± 0.51	101.0 ± 1.40
Rg3s	91.8 ± 1.36	95.4 ± 0.82	97.5 ± 1.96

SD, standard deviation; SD, standard deviation.

TABLE 3 | LLOD and LLOQ of HPLC analysis.

Components	**LLOD(μg/mL)**	**LLOQ(μg/mL)**
Rg1	1.74	5.8
Rb1	0.89	2.87
Rg3s	0.34	1.12

LLOD, lower limits of detection; LLOQ, lower limits of quantification.

statistical values and recovery rates was presented in **Table 2**. The lower limits of detection (LLOD) and lower limits of quantification (LLOQ) of Rg1, Rb1 and Rg3s was presented in **Table 3**. All the other information about the HPLC analysis was presented in **Supplementary Material**.

Measurements

The pain score was obtained *via* self-report questionnaire. The participants were asked to score the pain in certain circumstances according to Visual Analog Scale (VAS) at the baseline (week 0) and final (week 12) visits. The Disabilities of the Arm, Shoulder and Hand (DASH) score was also assessed by a self-reported questionnaire (Hudak et al., 1996; Williams, 2013). The questionnaire consists of 30 items among which 21 questions evaluate the difficulty of a specific task, 5 questions evaluate symptoms, and 4 questions evaluate social function, work function, sleep, and confidence. The DASH score range is from 0 to 100, and the higher the score, the higher the upper limb disability. Anthropometric measurements were obtained, and blood was drawn for laboratory testing at the baseline (week 0) and final (week 12) visits. Body weight and height were measured with the participants in light indoor clothing with InBody analyzer (Inbody Co., Ltd., Seoul, South Korea) that also calculated BMI as the weight divided by the height squared (kg/m2). Blood pressure was measured with the participant in the sitting position, after 5 min of rest, using an automated device (TM2665P; A&D Co., Ltd., Tokyo, Japan). Blood samples were collected in sterile tubes from an antecubital vein and were centrifuged at 300 g for 10 min. The serum samples were stored at -80.1°C until analysis.

Enzyme-linked immunosorbent assays were performed using various commercial kits. Serum superoxide dismutase (SOD) (Cayman Chemical Company, Ann Arbor, MI, United States) was measured to assess antioxidative enzyme activity. Malondialdehyde (MDA) was measured as an oxidative stress marker (Cell Biolabs Inc., San Diego, CA, United States). COMP (Kamiya biomedical company, Tukwila, WA, United States) and urinary CTXII (UCSN Life Sciences, Inc., Wuhan, China) were also measured.

Statistical Analysis

Data were analyzed by intention-to-treat analysis and expressed as mean ± SD. Primary validation variables included those related to pain and function: the pain score in VAS and the DASH score. The secondary validation variables were cartilage damage index, antioxidant enzyme activity, and oxidative stress markers. A paired *t*-test was used to compare the mean changes from baseline to 12 weeks within each group, and a Two sample *t*-test was used to compare the RG and placebo groups. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 15.0 software (SPSS Inc., Chicago, IL, United States). *p*-values ≤0.05 were considered statistically significant.

Ethical Approval

This study was approved by the institutional review board of Severance Hospital (IRB No. 4-2013-0713) and performed in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

This trial has been registered with the Clinical Research Information Service [CRIS, (http://cris.nih.go.kr)], Republic of Korea (KCT0006326).

RESULTS

A total of 52 participants were enrolled, with 26 participants randomly assigned to each study group (**Figure 1**). There were three participants in the RG group and six in the placebo group who dropped out of the study and failed to attend the follow-up session. **Table 4** shows the baseline characteristics of the participants. There was no significant difference between the two groups in their age, height, weight, BMI, systolic and diastolic blood pressure, pain scores at rest, daily activities, physical activity at work or sports, baseline DASH scores, and the laboratory variables such as SOD and MDA.

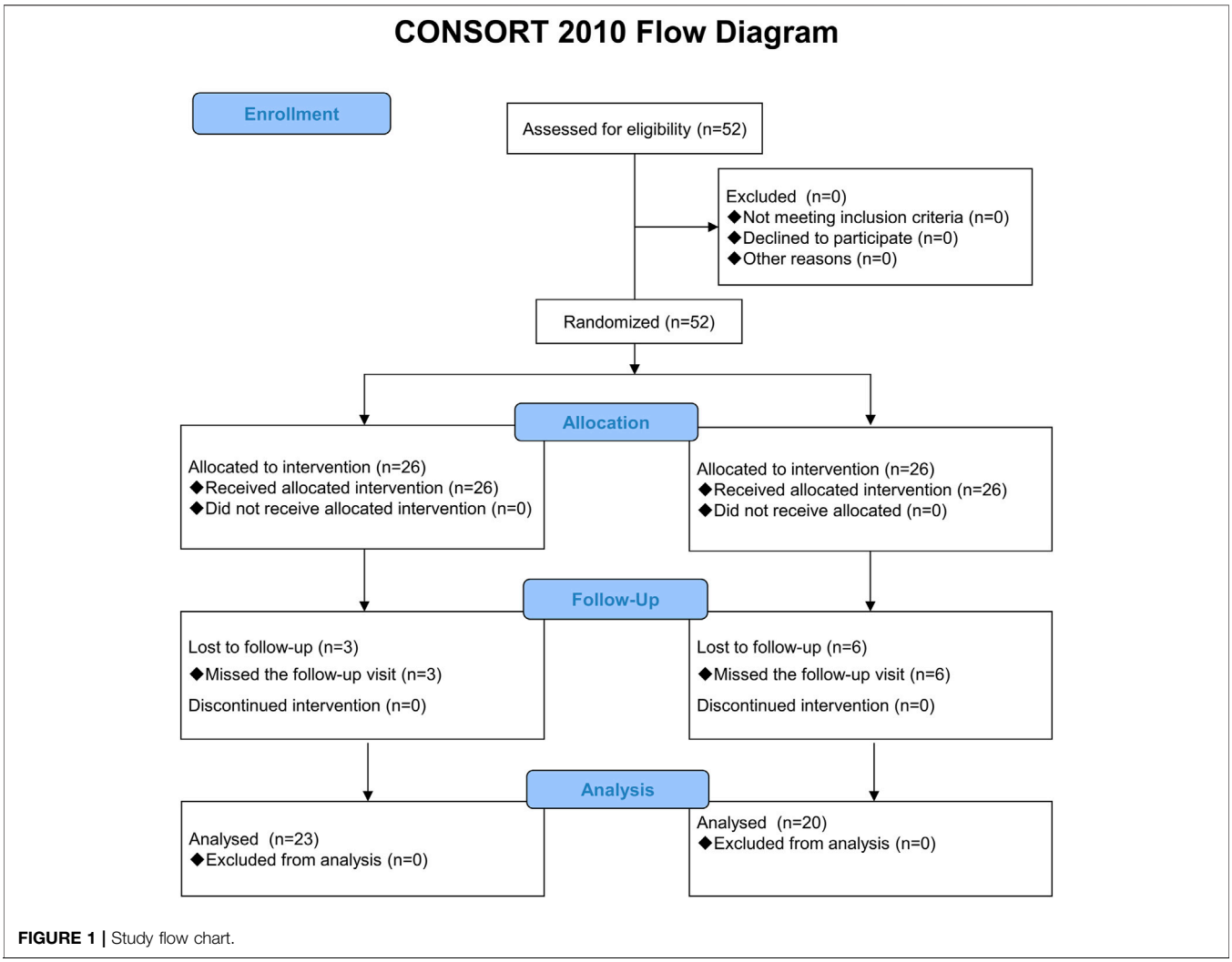

FIGURE 1 | Study flow chart.

TABLE 4 | Baseline demographic and clinical characteristics of participating postmenopausal women.

Variables	RG group (N = 23)	Placebo group (N = 20)	*p*-value
Age (y)	60.17 ± 9.49	60.55 ± 7.86	0.889
Height (cm)	154.90 ± 4.30	156.13 ± 5.14	0.466
Weight (kg)	56.64 ± 7.90	58.47 ± 6.17	0.477
BMI (kg/m^2)	23.58 ± 2.98	23.99 ± 2.47	0.675
Systolic blood pressure (mmHg)	127.29 ± 11.63	125.53 ± 7.72	0.623
Diastolic blood pressure (mmHg)	77.17 ± 8.15	74.93 ± 8.37	0.449
Pain score at rest	2.52 ± 1.24	3.30 ± 1.59	0.208
Pain score at daily activities	4.70 ± 1.64	4.85 ± 1.57	0.754
Pain score at work or sports	6.00 ± 1.62	6.20 ± 2.07	0.794
DASH score	38.34 ± 15.58	36.47 ± 17.74	0.715
SOD (U/mL)	266.11 ± 35.60	280.11 ± 42.01	0.243
MDA (nmol/mg)	235.89 ± 46.25	204.89 ± 41.81	0.421
Estradiol (pg/ml)	11.17 ± 5.63	11.16 ± 5.50	0.991
Endometrial thickness (mm)	0.30 ± 0.09	0.28 ± 0.09	0.618

RG, red ginseng; BMI, body mass index; DASH, disabilities of arm, Shoulder and Hand; SOD, superoxide dismutase; MDA, malondialdehyde.

Table 5 shows the pain scores in various circumstances and DASH scores for both groups at baseline and after 12 weeks of treatment. The pain score at rest for the RG group decreased significantly after treatment ($p < 0.05$), and the improvement was statistically significant compared to that of the placebo group ($p < 0.05$). The pain score at various circumstance were all

TABLE 5 | Pain score and disability of the arm, shoulder, and hand (DASH) scores at baseline and week 12.

	RG group (N = 23)	Placebo group (N = 20)	*p*-value[a]
Pain score at rest	Baseline: 2.52 ± 1.24 Week 12: 1.04 ± 0.88 *p*-value[b]: <0.001	Baseline: 3.30 ± 1.59 Week 12: 2.50 ± 1.57 *p*-value[b]: 0.006	0.040
Pain score at daily activities	Baseline: 4.70 ± 1.64 Week 12: 4.30 ± 1.10 *p*-value[b]: <0.001	Baseline: 4.85 ± 1.57 Week 12: 3.75 ± 2.02 *p*-value[b]: 0.012	0.486
Pain score at work or sports	Baseline: 6.00 ± 1.62 Week 12: 3.87 ± 1.89 *p*-value[b]: <0.001	Baseline: 6.20 ± 2.07 Week 12: 5.15 ± 2.13 *p*-value[b]: 0.006	0.023
DASH score	Baseline: 38.34 ± 15.58 Week 12: 20.87 ± 12.56 *p*-value[b]: <0.001	Baseline: 36.47 ± 17.74 Week 12: 30.33 ± 17.69 *p*-value[b]: 0.139	0.021

RG, red ginseng; DASH, disabilities of arm, shoulder and hand.
[b]*Paired t-test comparison between means at baseline and at week 12 within groups.*
[a]*Comparison of supplementation effects by comparing the change from baseline to week 12 between groups by two-sample t-test.*

TABLE 6 | Antioxidant enzyme activity and oxidative stress markers at baseline and week 12.

	RG group (N = 23)	Placebo group (N = 20)	*p*-value[a]
SOD (U/mL)	Baseline: 266.11 ± 35.60 Week 12: 312.10 ± 51.40 *p*-value[b]: 0.002	Baseline: 280.11 ± 42.01 Week 12: 301.95 ± 38.04 *p*-value[b]: 0.095	0.190
oxLDL (U/L)	Baseline: 31.54 ± 14.58 Week 12: 33.07 ± 0.10 *p*-value[b]: 0.458	Baseline: 33.03 ± 17.54 Week 12: 36.31 ± 18.99 *p*-value[b]: 0.285	0.431
MDA (nmol/mg)	Baseline: 215.89 ± 46.25 Week 12: 182.08 ± 50.51 *p*-value[b]: 0.018	Baseline: 204.89 ± 41.82 Week 12: 190.65 ± 50.20 *p*-value[b]: 0.333	0.322

RG, red ginseng; SOD, superoxide dismutase; oxLDL, oxidized low-density lipoprotein; MDA, malondialdehyde.
[b]*Paired t-test comparison between means at baseline and at week 12 by group.*
[a]*Comparison of supplementation effects between two groups by two-sample t-test.*

significantly improved in RG group after 12 weeks of red ginseng consumption ($p < 0.05$). Even though the pain score at rest, during daily activity and during work or sport was also improved in placebo group ($p < 0.05$), the change from baseline to week 12 were significantly different between RG group and placebo group in all variables except for the pain score during daily activity ($p < 0.05$ and $p = 0.49$, respectively). After RG supplementation, the DASH score decreased by approximately 26% in the RG group ($p < 0.05$), but this change was not statistically significant compared with that of the placebo group ($p = 0.37$).

Table 6 shows the change in antioxidant enzyme activity and oxidative stress markers before and after the 12 weeks of treatment. After 12 weeks of treatment, the SOD level increased, and the MDA level decreased significantly in the RG group (both $p < 0.05$), while these levels did not change significantly in the placebo group ($p = 0.09$, $p = 0.33$, respectively). However, the changes in SOD and MDA levels were not significantly different between the two groups ($p = 0.19$, $p = 0.32$, respectively). The oxidized low-density lipoprotein (oxLDL) level did not change in either group after 12 weeks of treatment ($p = 0.46$, RG group; $p = 0.29$, placebo group).

Table 7 shows the changes in estradiol level and endometrial thickness after the treatment. In both groups, the estradiol level did not change after treatment ($p = 0.63$, RG group; $p = 0.28$, placebo group). Moreover, the endometrial thickness, which has been well documented to be affected by hormone levels, did not increase in either group ($p = 0.71$, RG group; $p = 0.43$, placebo group).

Figure 2 shows the change in COMP serum level in each group after 12 weeks of treatment. COMP is well accepted as a diagnostic as well as prognostic indicator of OA (Tseng et al., 2009). After 12 weeks of treatment, the COMP level of the RG group decreased, but the change was not statistically significant ($p = 0.06$). However, the COMP level in the placebo group increased without statistical significance ($p = 0.11$), and the change after 12 weeks was significantly different between the two groups ($p = 0.01$). **Figure 3** presents the CTXII levels of the two groups after 12 weeks of treatment. CTXII is another marker of cartilage degradation. The change in both groups was not statistically significant after treatment ($p = 0.82$, RG group; $p = 0.86$, placebo group).

DISCUSSION

This study evaluated the effect of RG supplementation on OA pain, cartilage markers, oxidative stress, and hormone levels in

TABLE 7 | Serum estradiol levels and endometrial thickness at baseline and week 12.

	RG group (N = 23)	Placebo group (N = 20)	*p*-value[a]
Estradiol (pg/ml)	Baseline: 11.17 ± 5.62 Week 12: 10.72 ± 5.71 *p*-value[b]: 0.634	Baseline: 11.16 ± 5.50 Week 12: 9.86 ± 6.27 *p*-value[b]: 0.278	0.569
Endometrial thickness (mm)	Baseline: 0.30 ± 0.09 Week 12: 0.29 ± 0.10 *p*-value[b]: 0.709	Baseline: 0.28 ± 0.09 Week 12: 0.30 ± 0.08 *p*-value[b]: 0.430	0.447

RG, red ginseng.
[b]*Paired t-test comparison between means at baseline and at week 12 by group.*
[a]*Comparison of supplementation effects between two groups by two-sample t-test.*

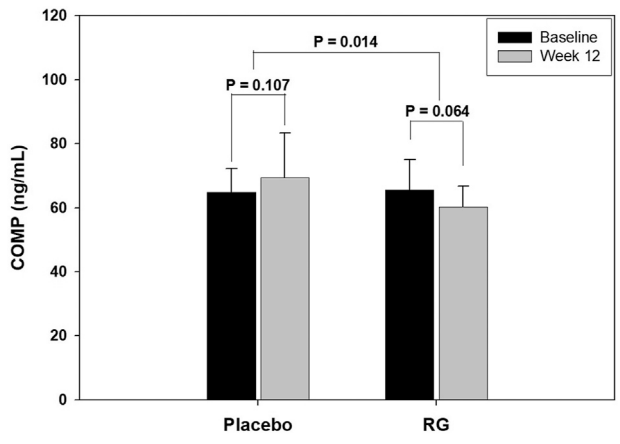

FIGURE 2 | Change in cartilage oligomeric matrix protein (COMP) level after 12 weeks of treatment.

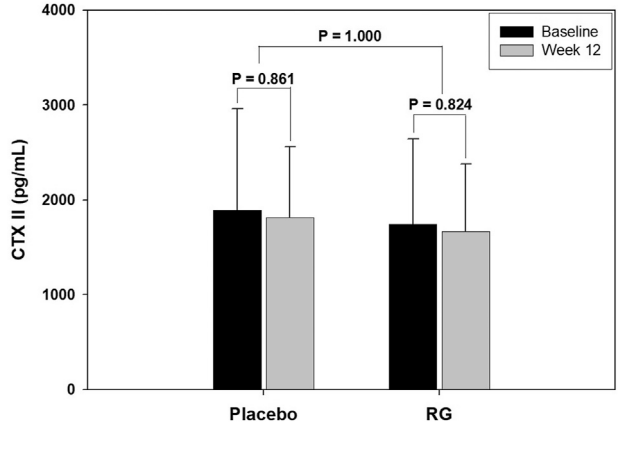

FIGURE 3 | Change in crosslinked telopeptide type II collagen (CTXII) level after 12 weeks of treatment.

postmenopausal women. The 12-weeks RG treatment improved the pain score at rest, during daily activity and work or sport, and the DASH score in the RG group. The improvement in pain score at rest, during work or sport and DASH score was statistically significant compared to that of the placebo group. Further, SOD levels increased, and MDA levels deceased significantly in the RG group after the treatment, indicating a reduction in oxidative stress. Lastly, the COMP level, one of the cartilage degradation markers, showed a decrease in the RG group after 12 weeks of treatment, although this was not statistically significant.

In 2002, the WHI reported the effects of combined conjugated equine estrogens (CEE) and medroxyprogesterone acetate in postmenopausal women in their first report about hormone replacement therapy (HRT), and their later report compared the effects of CEE monotherapy compared to placebo (Rossouw et al., 2002; Stefanick et al., 2006). After those reports showed an increased risk of breast cancer in women with HRT, the use of HRT decreased substantially worldwide (Maclennan et al., 2004).

As a result, the search for new therapies to overcome menopausal symptoms increased. As a result, complementary and alternative medicine, and nonhormone pharmaceuticals such as soy-derived products, St. John's wort, and evening primrose oil gained more attention (Acog, 2001). However, none of these alternative medical treatments have been proven as being effective in treating vasomotor symptoms.

In East Asian countries, ginseng root has been widely used as an alternative medicine to improve general health. Ginsenoside is the major constituent of ginseng root and that in RG has been proven to have a wide range of biological and pharmacological activities (Kim et al., 2000). In a previous study, Kim et al. evaluated the effects of RG on menopausal symptoms and cardiovascular risk factors in postmenopausal women. There was a significant improvement in the Kupperman index and Menopause Rating Scale in the RG group compared to those of the placebo group (Kim et al., 2012). However, RG did not improve vasomotor symptoms. In contrast to women in Western countries, postmenopausal women in Asian countries report more joint pain than vasomotor symptoms (Mccarthy, 1994; Hilditch et al., 1999; Haines et al., 2005). Therefore, we evaluated the effect of RG on symptoms of degenerative OA in postmenopausal Asian women.

Previous *in vitro* and animal studies have shown the protective activity of RG against cartilage degradation (Kim et al., 2010; Endale et al., 2014; Lee et al., 2014; Lee et al., 2015). Some ginsenoside-enriched fractions inhibit both matrix metalloproteinase-13 (MMP-13) expression in IL-1β-treated human chondrocytes and the release of glycosaminoglycans from rabbit cartilage culture. MMP-13 is known to play an

important role in the pathogenesis of OA by degrading type II collagens (Lee et al., 2015). In addition, red ginseng saponin extract has been shown to improve the severity of mouse collagen-induced arthritis (Kim et al., 2010). Ginsenosides including Rc, Rd, Rf, Rg1, Rg3, and F4 have inhibitory effects on MMP-13 expression in human chondrocytes. Ginsenosides Rg3 and F4 are contained only in RG (Endale et al., 2014; Lee et al., 2014). Based on these results, RG is believed to be effective in treating cartilage degradation-related disorders, but there has been no clinical study demonstrating this so far.

In this study, the pain score at various circumstances and DASH score improved with statistical significance in the RG group and the improvement in pain score at rest, during work or sport, and DASH score was significantly different compared to those of control group. Furthermore, the COMP level tended to decrease in the RG group after 12 weeks of treatment, and the change was statistically significant when compared to that in the placebo group, which showed an increase without significance. Serum COMP is one of the most consistent biomarkers associated with the diagnosis and prognosis of OA. Furthermore, serum COMP is influenced by various treatments and can be used as an indicator to evaluate the effectiveness of certain therapies in patients with OA (Tseng et al., 2009; Hosnijeh et al., 2015). Therefore, the COMP level results imply that RG can be a potential therapeutic option for OA in postmenopausal women.

A previous study reported that the activity of serum SOD was significantly increased after 12-weeks RG supplementation compared with the placebo group (Kim et al., 2012). In the present study, serum SOD levels were also significantly increased after 12-weeks RG supplementation, but these changes were not statistically significant compared with the placebo group. The discrepancy in results can be explained by the smaller sample size and greater age of participants, who were approximately 10 years older in the current study. Women experience OA more often after menopause than before. Women over 50 years of age experience estrogen deficiency after menopause and this may result in a higher prevalence and greater severity of cartilage degeneration of the joints. Therefore, a study with younger postmenopausal women is required to document the effects of age.

The limitations of this study include the small sample size and the single national background of the participants. Further studies on larger, more diverse ethnic populations are needed to apply the results to the general population. In addition, the pharmacokinetics of RG are not fully understood, so the mechanism for OA symptom improvement remains unknown. The side effects of RG are not well-studied yet. Even though RG is commonly accepted as a health supplement in Korea for people of all ages, its side effects include nervousness, insomnia, dizziness, and vaginal bleeding. Therefore, further investigations are needed to assess the safety and efficacy of RG in postmenopausal women.

To our knowledge, this is the first RCT to investigate the effect of RG on joint pain and function in postmenopausal women with degenerative OA. We also attempted to investigate whether biochemical markers of cartilage degradation were affected by RG. Given that previous studies have shown favorable effects of RG on antioxidative stress, RG may contribute to a decrease in pain and other degenerative OA symptoms as well as oxidative stress (Seo et al., 2014). In many OA patients, various anti-inflammatory analgesics are prescribed to relieve symptoms. However, gastrointestinal tract complications and bleeding are common side effects. Alternative medicines such as RG may present a brand-new, sustainable treatment option with fewer side effects for those suffering from degenerative OA.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of Severance Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HK curated the data, and wrote the original draft. SC investigated and collected the data. KS analysed the data, curated the data, reviewed and edited the draft. SS conceptualized this study, analysed the data, reviewed and edited the draft, and supervised this study. Y-RC conceptualized this study, reviewed and edited the draft, supervised this study, acquired the funding and administered this project. All authors have read and agreed to the published version of the article.

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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.2021.745568/full#supplementary-material

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Ethnobotanical Survey on Skin Whitening Prescriptions of Traditional Chinese Medicine in Taiwan

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The increasing interest and demand for skin whitening products globally, particularly in Asia, have necessitated rapid advances in research on skin whitening products used in traditional Chinese medicine (TCM). Herein, we investigated 74 skin whitening prescriptions sold in TCM pharmacies in Taiwan. Commonly used medicinal materials were defined as those with a relative frequency of citation (RFC) > 0.2 and their characteristics were evaluated. Correlation analysis of commonly used medicinal materials was carried out to identify the core component of the medicinal materials. Of the purchased 74 skin whitening prescriptions, 36 were oral prescriptions, 37 were external prescriptions, and one prescription could be used as an oral or external prescription. After analysis, 90 traditional Chinese medicinal materials were obtained. The Apiaceae (10%; 13%) and Leguminosae (9%; 11%) were the main sources of oral and external medicinal materials, respectively. Oral skin whitening prescriptions were found to be mostly warm (46%) and sweet (53%), while external skin whitening prescriptions included cold (43%) and bitter (29%) medicinal materials. Additionally, mainly tonifying and replenishing effects of the materials were noted. Pharmacological analysis indicated that these medicinal materials may promote wound healing, treat inflammatory skin diseases, or anti-hyperpigmentation. According to the Spearman correlation analysis on interactions among medicinal materials with an RFC > 0.2 in the oral skin whitening prescriptions, *Paeonia lactiflora* Pall. (white) and *Atractylodes macrocephala* Koidz. showed the highest correlation (confidence score = 0.93), followed by *Ziziphus jujuba* Mill. (red) and *Astragalus propinquus* Schischkin (confidence score = 0.91). Seven medicinal materials in external skin whitening prescriptions with an RFC > 0.2, were classified as Taiwan *qī bái sàn* (an herbal preparation), including *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., *Wolfiporia extensa* (Peck) Ginns, *Bletilla striata* (Thunb.) Rchb. f., *Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Paeonia lactiflora* Pall. (white), and *Bombyx mori* Linnaeus. Skin whitening prescriptions included multiple traditional Chinese medicinal materials. Despite the long history of use, there is a lack of studies concerning skin whitening products, possibly due to the complex composition of

traditional Chinese medicine. Further studies are required to assess the efficacy and safety of these traditional Chinese medicinal materials for inclusion in effective, safe, and functional pharmacological products.

Keywords: skin whitening, ethnobotanical, Taiwan, traditional Chinese medicine pharmacy, traditional Taiwanese medicine

1 INTRODUCTION

The global cosmetics market is undergoing an unprecedented boom due to economic development and growing aesthetic needs. Rapidly expanding Asian cosmetic markets, of which China, Japan, South Korea, and India are major consumer countries, are following in the footsteps of European and American countries with the ubiquitous use of cosmetic products, particularly skin whitening products. Moreover, a recent survey noted the increasing prevalence of males using skin whitening products in addition to females (Pillaiyar et al., 2017; Hu et al., 2020).

Skin whitening prescriptions can not only be used to lighten skin tone, but also clinically treat hyperpigmentary disorders by decreasing melanin synthesis (Gillbro and Olsson, 2011). Melanin, one of the important pigments, is synthesized in melanocytes in the basal layer of epidermis and can protect the skin from ultraviolet-induced damage (Brenner and Hearing, 2008). Epidermal melanin content is intimately associated with anthropological origins. As the level of ultraviolet radiation is higher in low-latitude regions, people in these regions have higher melanin content; conversely, melanin content is lower in people at high-latitude regions, hence they have whiter skin (Slominski et al., 2004). Melanogenesis involves the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by tyrosinase before further conversion to L-dopaquinone. Finally, L-dopaquinone undergoes a series of chemical reactions to form melanin (**Supplementary Figure S1**). Tyrosinase is a rate-limiting enzyme in melanogenesis and is considered an important target for the development of therapies in treating hyperpigmentation (Sonthalia et al., 2016; Ullah et al., 2019; Zaidi et al., 2019).

At present, there are a variety of skin whitening products on the market classified as either "inhibiting melanogenesis" or "inhibiting melanogenesis and promoting melanin removal." In Taiwan, only 13 skin whitening components (**Supplementary Table S1**) (Food and Drug Administration, 2014) have been approved for use in cosmetic preparations. While ascorbic acid (vitamin C) is a common component, it is unstable and easily oxidized which limits its direct use. In order to prevent premature degradation, derivatives such as magnesium ascorbyl phosphate, ascorbyl glucoside, and ascorbyl tetraisopalmitate (Balaguer et al., 2008) are often utilized. Caution must be exercised when using various skin whitening components as improper use may lead to dermatitis, erythema, burns, and other skin injuries (Nordin et al., 2021). Thus, manufacturers have begun to seek natural alternatives to develop gentle, hypoallergenic skin whitening products derived from traditional Chinese medicine (TCM).

In traditional Chinese medicine books, many words describe dark skin or spots on the face, such as miàn gǎn zèng. In addition, there are ancient descriptions regarding the use of TCM for skin whitening. The Shennong Materia Medica, an extant medicinal text published in 100 B.C., recorded that *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. promotes skin growth and has moisturizing effects. Of additional interest is qī bái sàn, a well-known skin whitening herbal prescription. However, its composition varies among different historical dynasties, geographical regions, and environments. For example, different compositions of qī bái sàn can be found in related prescriptions such as yǒng lèi qián fāng, pǔ jì fāng, and tài píng shèng huì fāng (**Table 1**). To date, there is still no comprehensive study on skin whitening prescriptions in traditional Chinese medicine pharmacies in Taiwan. Therefore, the aim of this study was to examine the composition of skin whitening prescriptions sold in traditional Chinese medicine pharmacies in Taiwan to understand the usage, methods of preparation, and principles of skin whitening prescriptions in Taiwan.

2 MATERIALS AND METHODS

2.1 Ethical Review

The research for this study was conducted from March 2020 to April 2021. The study was approved by the Central Regional Research Ethics Committee of China Medical University (CRREC-109-125) (**Supplementary Figure S2**).

2.2 Research Process

This study complied with the ethnobotanical research guidelines (Rivera et al., 2014; Mullane et al., 2015; Heinrich et al., 2018; Heinrich et al., 2020), and could mainly divided into field investigation, medicinal material identification, and analysis. The complete study methods are shown in the study flowchart (**Figure 1**).

2.2.1 Field Investigation

Taiwan is an island located at the intersection between Northeast Asia and Southeast Asia and a total area of 36,000 km^2 (Tourism Bureau, 2021). The study was conducted over 12 months and we randomly visited the TCM pharmacies that appropriately represented the use of TCM medicine in Taiwan. The number of TCM pharmacies selected was directly proportional to the number of TCM pharmacies in each county and city published by the government (Ministry of Health and Welfare, 2015). A total of 75 TCM pharmacies were visited, included 16 pharmacies were visited but no prescription was obtained.

TABLE 1 | Qī bái sàn-related prescriptions in ancient books.

Name of prescription	Composition	Source
qī bái sàn (七白散)	*Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Bombyx mori* Linnaeus, *Paeonia lactiflora* Pall., *Ipomoea nil* (L.) Roth, *Sauromatum giganteum* (Engl.) Cusimano & Hett	yǒng lèi qián fang (永類鈐方)
qī bái wán (七白丸)	*Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Bletilla striata* (Thunb.) Rchb.f., *Bombyx mori* Linnaeus, *Wolfiporia extensa* (Peck) Ginns, *Sauromatum giganteum* (Engl.) Cusimano & Hett	pǔ jì fang (普濟方)
qī bái sàn xǐ miàn yào (七白散洗面藥)	*Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Bombyx mori* Linnaeus, *Paeonia lactiflora* Pall., *Ipomoea nil* (L.) Roth, *Wolfiporia extensa* (Peck) Ginns	pǔ jì fang (普濟方)
qī bái tǐng zǐ gāo (七白挺子膏)	*Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Asarum heterotropoides* F.Schmidt f. *mandshuricum* (Maxim.) Kitag., *Bletilla striata* (Thunb.) Rchb.f., *Wolfiporia extensa* (Peck) Ginns, *Sauromatum giganteum* (Engl.) Cusimano & Hett (This prescription must be mixed with egg white for use)	tài píng shèng huì fang (太平聖惠方)

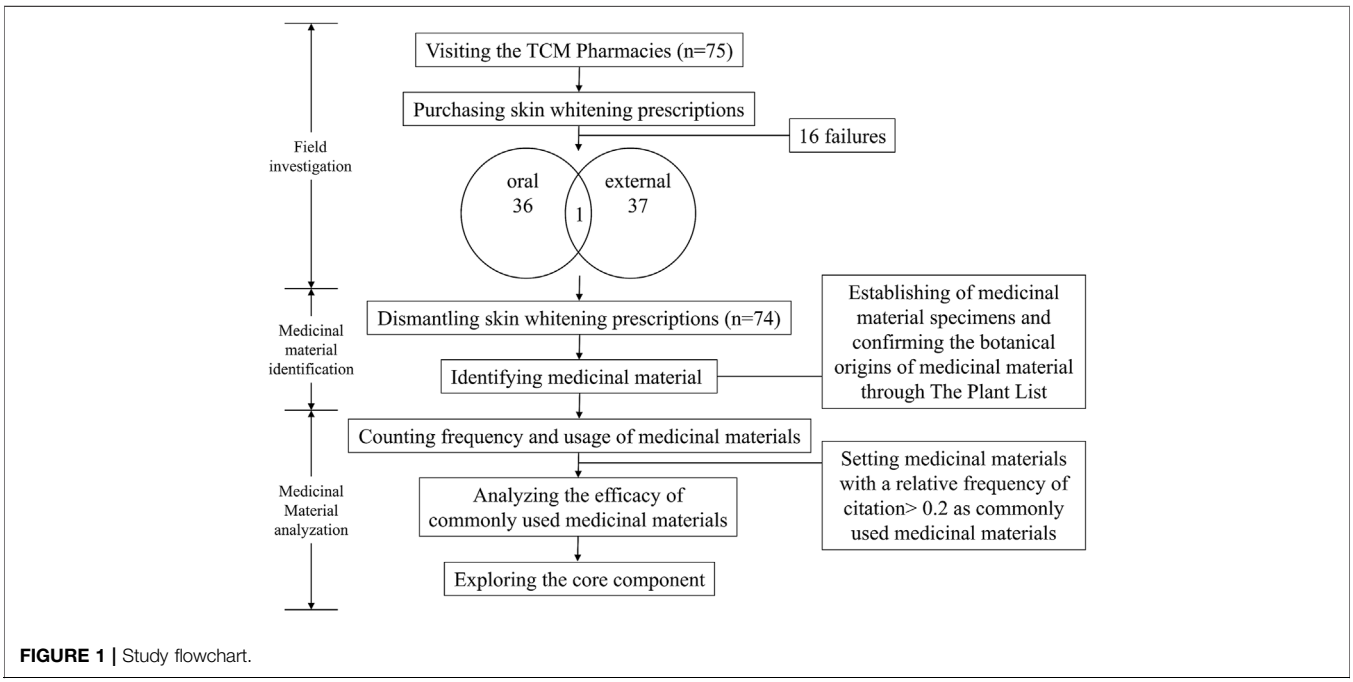


FIGURE 1 | Study flowchart.

Overall, 74 skin whitening prescriptions were obtained (with 13 TCM pharmacies providing more than one prescription) (**Figure 2**), including 36 oral prescriptions, 37 external prescriptions, and one prescription which could be used as an oral or external prescription.

2.2.2 Identification of Medicinal Materials

This study examined the purchased medicinal materials and performed the five-sense identification to identify the origins and plant parts of the materials, and compared them with the medicinal material standards to distinguish authentic or misused medicinal materials (**Figure 3**). We also photographed the materials and recorded the weight of each material. Finally, the materials were numbered and stored in the herbarium of the China Medical University, Taiwan.

2.2.3 Analysis of Medicinal Materials

The various medicinal materials were analyzed and collated based on biological taxonomy, relative frequency of citation (RFC), efficacy of traditional use, and skin-related pharmacological effects. Biological taxonomy included the Scientific name of the crude drug, family, and color. The Plant List (http://www.theplantlist.org/) was used as a source of botanical information. Medicinal materials with RFC > 0.2 were defined as commonly used medicinal materials. The RFC formula was defined as follows (Vitalini et al., 2013; Dixit and Tiwari, 2020; Abbas et al., 2021):

$$\mathrm{RFC}_i = FC_i/N \left(0 \leq \mathrm{RFC}_i \leq 1\right)$$

Where RFC_i is the relative frequency count of i species and it is commonly used in ethnopharmacology papers. FC_i defines the

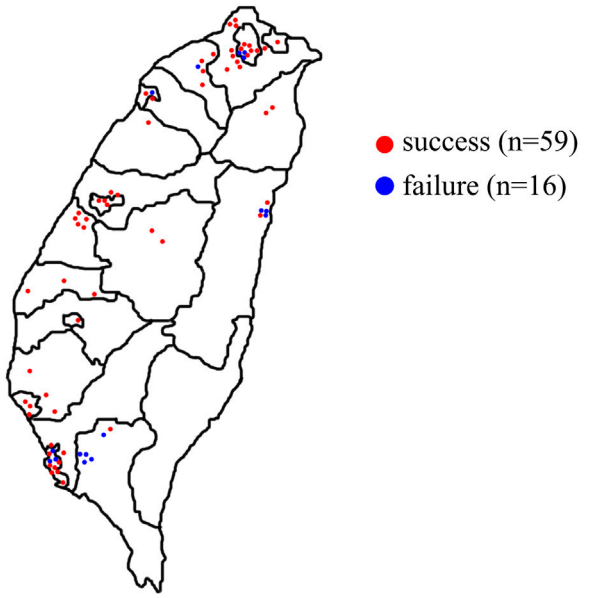


FIGURE 2 | Geographical distribution of pharmacies visited for skin whitening prescriptions in Taiwan. All dots were based on latitude and longitude coordinates. Red dots indicated successful pharmacies; blue dots indicated failed pharmacies.

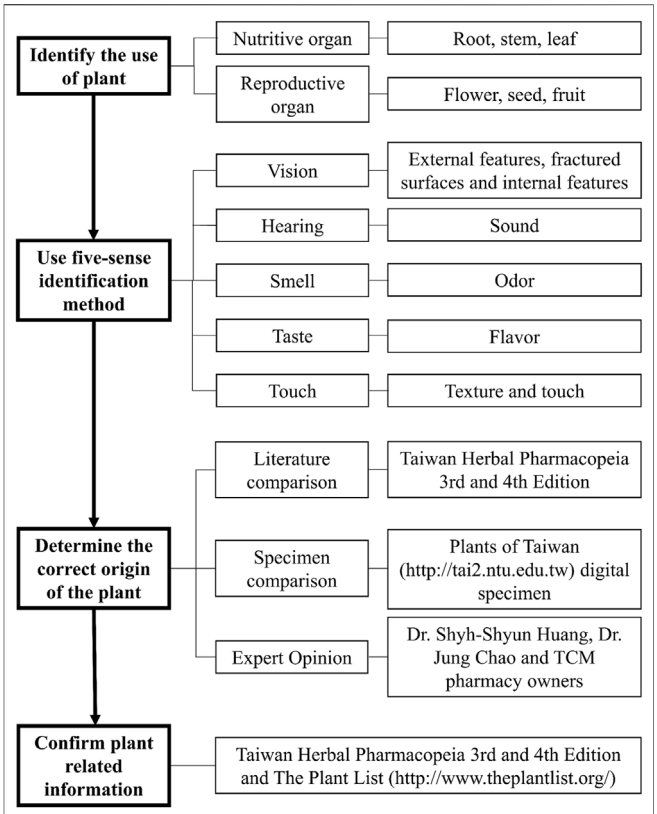


FIGURE 3 | Flowchart for medicinal materials identification.

count of prescriptions which used species *i*. N denotes the total number of prescriptions.

The medicinal materials were indexed against the Taiwan Herbal Pharmacopeia (3rd, 4th edition) (Taiwan Herbal Pharmacopeia 3rd Edition Committee, 2018; Taiwan Herbal Pharmacopeia 4th Edition Committee, 2021), Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia Commission, 2020), and Chinese Materia Medica (State Administration of Traditional Chinese Medicine, 1999). The effects, properties, and flavors of traditional Chinese medicine were cited from the Taiwan Herbal Pharmacopeia (3rd, 4th edition). The PubMed database was systematically searched from Jan 2010 to May 2021 for skin-related pharmacological effects, utilizing keywords such as "skin" and the scientific names of medicinal materials.

2.2.4 Analysis

GraphPad Prism 9.0 (GraphPad Prism version 9.0 for Windows, GraphPad Software, San Diego, California, USA) was used to plot a heat map for Spearman correlation analysis of commonly used medicinal materials used in oral skin whitening prescriptions. The colors of the squares in the heat map were based on the visualization of Spearman correlation matrix of the two medicinal materials. The more intense red hue, the higher the correlation between the two medicinal materials. Conversely, the lighter the color, the lower the correlation between the two medicinal materials (Vacanti, 2019).

3 RESULTS

3.1 Biological Taxonomic Characteristics of Medicinal Materials Used in Skin Whitening Prescriptions

During this study, 74 skin whitening prescriptions were purchased from 59 TCM pharmacies in Taiwan, of which 36 were oral prescriptions, 37 were external prescriptions, and one prescription could be used orally or externally. Oral prescription use method is to add appropriate amount of water to decoct; external prescription use method is to mash the medicinal materials, then add water, honey or milk, and apply to the face. Among the oral and external skin whitening prescriptions, 79 and 56 medicinal materials were found respectively. Overall, a total of 90 medicinal materials were obtained from the 74 prescriptions, and 6 misused medicinal materials were found (**Supplementary Table S2**). The majority of these medicinal materials were Plantae (93.33%), 3 medicinal materials (3.33%) were obtained from Animalia [*Bombyx mori* Linnaeus, *Crassostrea gigas* (Thunberg), and *Pteria martensii* (Dunker)] and 3 (3.33%) were from Fungi [*Tremella fuciformis*, *Wolfiporia extensa* (Peck) Ginns cum pini radix, and *Wolfiporia extensa* (Peck) Ginns] (**Figure 4A**).

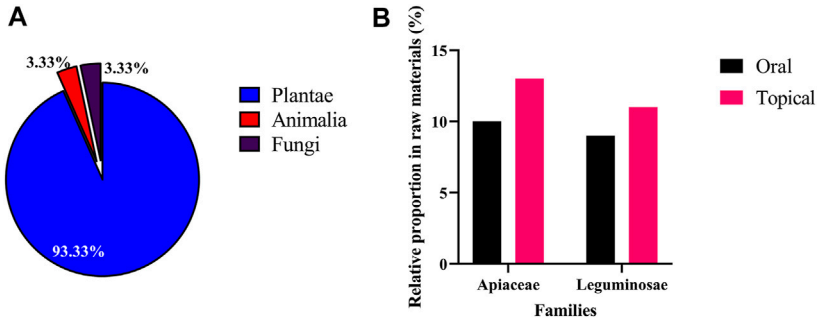


FIGURE 4 | Taxonomy of 90 medicinal materials in 74 skin whitening prescriptions. **(A)** Kingdoms and **(B)** families.

The most used of medicinal material in oral skin whitening prescriptions was *Wolfiporia extensa* (Peck) Ginns (RFC = 0.51), followed by *Glycyrrhiza uralensis* Fisch. and *Paeonia lactiflora* Pall. (white) (RFC = 0.41), while *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. (RFC = 0.89) was the most commonly used of medicinal material in external skin whitening prescriptions. When classified by family, the most common families in both oral and external skin whitening prescriptions were Apiaceae (10 and 13% respectively) and Leguminosae (9 and 11% respectively) (**Figure 4B**).

3.2 Analysis of Traditional Efficacy, Skin-Related Pharmacological Effects and Dosage of Commonly Used Medicinal Materials in Skin Whitening Prescriptions

Commonly used medicinal materials were defined as those with RFC > 0.2. Thirteen and seven commonly used medicinal materials were obtained from oral and external skin whitening prescriptions, respectively (**Table 2**).

With regards to the properties (**Figure 5A**), commonly used medicinal materials used in oral skin whitening prescriptions were mostly warm (46%) and plain (31%), while those used in external were mostly cold (43%), followed by warm (29%) and plain (29%). About the flavors (**Figure 5B**), commonly used medicinal materials used in oral skin whitening prescriptions were mostly sweet (53%), while those used in external were mostly bitter (29%).

About classification by traditional effect (**Figures 5C,D**), commonly used medicinal materials used in both oral and external skin whitening prescriptions were mostly tonifying and replenishing. Integrated the modern research related to skin, including pharmacological effects, clinical studies and intervention studies, it is found that most of these medicinal materials could promote wound healing, treat inflammatory skin diseases, or anti-hyperpigmentation (**Figure 5E**).

The analysis of the various medicinal materials dosage used in prescriptions is presented in **Supplementary Table S3**. In commonly used oral medicinal materials (**Figure 6A**), the average dose of *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf was the highest and that *Glycyrrhiza uralensis* Fisch. was the lowest. *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf showed the largest dose difference across the various TCM pharmacies, while dose differences of the *Angelica sinensis* (Oliv.) Diels were the smallest. In commonly used external medicinal materials (**Figure 6B**), the average dosage of *Wolfiporia extensa* (Peck) Ginns was the highest, while the *Bletilla striata* (Thunb.) Rchb. f. and *Paeonia lactiflora* Pall. (white) were the lowest. The dosages of *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. had the largest differences across various TCM pharmacies, while the dosages of the *Paeonia lactiflora* Pall. (white) exhibited the smallest difference.

3.3 Correlation Analysis of Commonly Used Medicinal Materials Used in Oral Skin Whitening Prescriptions

Spearman correlation analysis was performed for commonly used medicinal materials in oral skin whitening prescriptions and a heatmap was plotted (**Figure 7A**). The highest correlation was detected between *Paeonia lactiflora* Pall. (white) and *Atractylodes macrocephala* Koidz. (confidence score = 0.93), followed by the correlation between *Ziziphus jujuba* Mill. (red) and *Astragalus propinquus* Schischkin (confidence score = 0.91). In contrast, low correlation was observed between *Dioscorea polystachya* Turcz. and *Glycyrrhiza uralensis* Fisch (confidence score = −0.7). *Dioscorea polystachya* Turcz. also demonstrated a low correlation with *Paeonia lactiflora* Pall. (white) and *Atractylodes macrocephala* Koidz. Therefore, *Dioscorea polystachya* Turcz. is less likely to be present when *Glycyrrhiza uralensis* Fisch., *Paeonia lactiflora* Pall. (white), or *Atractylodes macrocephala* Koidz. are present. When network analysis was performed on medicinal materials with RFC > 0.2 in oral skin whitening prescriptions (**Figure 7B**) and two medicinal materials with confidence score >0.6 were connected by lines, it was found that *Paeonia lactiflora* Pall. (white) and *Atractylodes macrocephala* Koidz. frequently appeared together with *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. or *Wolfiporia extensa* (Peck) Ginns and *Glycyrrhiza uralensis* Fisch.;

TABLE 2 | Medicinal properties and skin-related modern research of commonly used medicinal materials used in skin whitening prescriptions (RFC>0.2).

Scientific name/Latin name of crude drug/Local name/sample number	RFC[a]	Family	Color	Flavor and property	Traditional usage	Literature on skin modern research (PubMed)[b]
Oral						
Wolfiporia extensa (Peck) Ginns/Poria/白茯苓/CMU2021SWP	0.51	Polyporaceae	white	Sweet and plain; plain	Dampness-draining diuretic	Hyperpigmentation (Ho et al., 2021); inhibition of melanogenesis (Lee and Cha, 2018; Kang et al., 2019); moisturizing and increased skin barrier function (Choi et al., 2019); oxidative stress associated skin aging effects and inflammatory skin diseases (Kang et al., 2019; Fang et al., 2021)
Glycyrrhiza uralensis Fisch./Glycyrrhizae radix et rhizoma/甘草/CMU2021SWGrr	0.41	Leguminosae	brown	Sweet; plain	Tonifying and replenishing	Accelerate wound healing and promote neovascularization (Hao et al., 2020) anti-photoaging effects (Kim et al., 2017; Xuan et al., 2017); human dermal fibroblasts (Kim et al., 2017); inflammatory skin diseases (Jeong et al., 2015; Cha et al., 2016; Lee et al., 2020); inhibition of melanogenesis (Lim et al., 2018); protection of skin barrier (Cha et al., 2017); viral skin diseases (Wang et al., 2013a)
Paeonia lactiflora Pall. (white)/Paeoniae radix alba/白芍/CMU2021SWPra	0.41	Paeoniaceae	white	Bitter and sour; cold	Tonifying and replenishing	Ameliorated vascular damage (Chen et al., 2013); anti-photoaging effects (Lu et al., 2020); hyperpigmentation (Qiu et al., 2016; Ho et al., 2021); inflammatory skin diseases (Chen et al., 2011; Jeong et al., 2015; Kim et al., 2021); inhibition of melanogenesis (You et al., 2017); psoriasis (Sun et al., 2015a; Choi et al., 2015; Li et al., 2019); skin itching Zhu et al., 2019)
Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav./Angelicae dahuricae radix/白芷/CMU2021SWAdr	0.35	Apiaceae	white	Pungent; warm	Exterior-releasing	Accelerate wound healing and promote neovascularization (Bai et al., 2012; Yang et al., 2017; Yang et al., 2020a); acne (Hwang et al., 2016); inflammatory skin diseases (Lee et al., 2012; Ku et al., 2017); inhibition of melanogenesis (Kim et al., 2016); melanoma (Hwangbo et al., 2020); skin itching (Zhu et al., 2019)
Astragalus propinquus Schischkin/ Astragali radix/黃耆/CMU2021SWApr	0.32	Leguminosae	brown	Sweet; warm	Tonifying and replenishing	Accelerate wound healing (Luo et al., 2016; Zhao et al., 2017); anti-photoaging effects (Hong et al., 2013; Berezutsky et al., 2019; Shan et al., 2019); hyperpigmentation (Tsao et al., 2017); inflammatory skin diseases (Kim et al., 2013; Deng et al., 2019)
Angelica sinensis (Oliv.) Diels/Angelicae sinensis radix/當歸/CMU2021SWAsr	0.32	Apiaceae	brown	Sweet and pungent; warm	Tonifying and replenishing	Accelerate wound healing (Hsiao et al., 2012; Zhao et al., 2012; Wang et al., 2013b); inflammatory skin diseases (Gong et al., 2015; Choi et al., 2016; Lee et al., 2016; Saba et al., 2016; Nam et al., 2021); skin itching (Lee at al., 2016; Zhu et al., 2019); melanoma (Gao et al., 2018)
Coix lacryma-jobi var. ma-yuen (Rom.Caill.) Stapf/Coicis semen/白薏仁/ CMU2021SWCs	0.32	Poaceae	white	Sweet and plain; cool	Dampness-draining diuretic	Accelerate wound healing (Kalekhan et al., 2021); anti-photoaging effects (Shan et al., 2012); chapped skin and warts (Chung et al., 2011; Byun et al., 2016; Son et al., 2019); inhibition of melanogenesis (Huang et al., 2014; Amen et al., 2017)
Ziziphus jujuba Mill. (red)/Jujubae fructus (red)/紅棗/CMU2021SWJf	0.3	Rhamnaceae	red	Sweet; warm	Tonifying and replenishing	Accelerate wound healing (Fazio et al., 2020); anti-wrinkle (Son and Lee, 2020); melanoma (Hung et al., 2012)
Atractylodes macrocephala Koidz./ Atractylodis macrocephalae rhizoma/白朮/ CMU2021SWAmr	0.3	Compositae	white	Bitter and sweet; warm	Tonifying and replenishing	Hyperpigmentation (Ho et al., 2021); skin itching (Zhu et al., 2019)

(Continued on following page)

TABLE 2 | (*Continued*) Medicinal properties and skin-related modern research of commonly used medicinal materials used in skin whitening prescriptions (RFC>0.2).

Scientific name/Latin name of crude drug/Local name/sample number	RFC[a]	Family	Color	Flavor and property	Traditional usage	Literature on skin modern research (PubMed)[b]
Dioscorea polystachya Turcz./Dioscoreae rhizoma/白山藥/CMU2021SWDr	0.24	Dioscoreaceae	white	Sweet; plain	Tonifying and replenishing	Inflammatory skin diseases (Jegal et al., 2017; Jegal et al., 2018); skin cancer (Tsukayama et al., 2018)
Lycium chinense Mill./Lycii fructus/枸杞子/CMU2021SWLf	0.24	Solanaceae	red	Sweet; plain	Tonifying and replenishing	Anti-photoaging effects (Yi et al., 2013; Im et al., 2016; Li at el., 2017; Liang et al., 2018; Neves et al., 2020; Neves et al., 2021); inflammatory skin diseases (Wu et al., 2020); melanoma (Cenariu et al., 2021); moisturizing (Meng et al., 2020)
Ophiopogon japonicus (Thunb.) Ker Gawl./Ophiopogonis radix/麥門冬/CMU2021SWOr	0.24	Asparagaceae	white	Sweet and bitter; cold	Tonifying and replenishing	Inflammatory skin diseases (Kitahiro et al., 2018; Mainzer et al., 2019; An et al., 2020)
Ligusticum striatum DC./Chuanxiong rhizoma/川芎/CMU2021SWCr	0.22	Apiaceae	brown	Pungent; warm	Tonifying and replenishing	Inflammatory skin diseases (Lee et al., 2016; Yan et al., 2020); skin itching (Zhu et al., 2019)
External						
Angelica dahurica (Hoffm.) Benth. & Hook.f. ex Franch. & Sav./Angelicae dahuricae radix/白芷/CMU2021SWAdr	0.89	Apiaceae	white	Pungent; warm	Exterior-releasing	Accelerate wound healing and promote neovascularization (Bai et al., 2012; Yang et al., 2017; Yang et al., 2020b): acne (Hwang et al., 2016); inflammatory skin diseases (Lee et al., 2012; Ku et al., 2017); inhibition of melanogenesis (Kim et al., 2016); melanoma (Hwangbo et al., 2020); skin itching (Zhu et al., 2019)
Wolfiporia extensa (Peck) Ginns/Poria/白茯苓/CMU2021SWP	0.82	Polyporaceae	white	Sweet and plain; plain	Dampness-draining diuretic	Hyperpigmentation (Ho et al., 2021); inhibition of melanogenesis (Lee and cha, 2018; Kang et al., 2019); moisturizing and increased skin barrier function (Choi et al., 2019); oxidative stress associated skin aging effects (Kang et al., 2019; Fang et al., 2021)
Bletilla striata (Thunb.) Rchb.f./Bletillae rhizoma/白及/CMU2021SWBr	0.58	Orchidaceae	white	Bitter, sweet, and astringent; cold	Hemostatic	Accelerate wound healing (Yu et al., 2011; He et al., 2017; Song et al., 2017; Zhang et al., 2019; Yang et al., 2020a); chapped skin (He et al., 2017); hyperpigmentation (Ho et al., 2021)
Atractylodes macrocephala Koidz./Atractylodis macrocephalae rhizoma/白朮/CMU2021SWAmr	0.53	Compositae	white	Bitter and sweet; warm	Tonifying and replenishing	Hyperpigmentation (Ho et al., 2021); skin itching (Zhu et al., 2019)
Ampelopsis japonica (Thunb.) Makino/Ampelopsis radix/白蘞/CMU2021SWAr	0.37	Vitaceae	white	Bitter and pungent; cold	Heat-clearing	Accelerate wound healing and promote neovascularization (Lee et al., 2015); hyperpigmentation (Fong and Tong, 2012; Ho et al., 2021)
Paeonia lactiflora Pall. (white)/Paeoniae radix alba/白芍/CMU2021SWPra	0.32	Paeoniaceae	white	Bitter and sour; cold	Tonifying and replenishing	Ameliorated vascular damage (Chen et al., 2013); anti-photoaging effect (Lu et al., 2020); hyperpigmentation (Qiu et al., 2016; Ho et al., 2021); inflammatory skin diseases (Chen et al., 2011; Jeong et al., 2015; Kim et al., 2020); inhibition of melanogenesis (You et al., 2017); psoriasis (Sun et al., 2015b; Choi et al., 2015; Li et al., 2019); skin itching (Zhu et al., 2019)
Bombyx mori Linnaeus/Bombyx batryticatus/白殭蠶/CMU2021SWBb	0.26	Bombycidae	white	Salty and pungent; plain	Liver-pacifying and wind-extinguishing	Accelerate wound healing (Tariq et al., 2021)

[a]*RFC, relative frequency of citation.*

[b]*Literature on skin modern research (PubMed) included pharmacological effects, clinical research and intervention research.*

Ziziphus jujuba Mill. (red) and *Astragalus propinquus* Schischkin were used in combination with *Lycium chinense* Mill. or *Ligusticum striatum* DC.; and *Angelica sinensis* (Oliv.) Diels, *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf, and *Dioscorea polystachya* Turcz. was a prescription. These combinations could be used as a reference for oral skin whitening prescriptions.

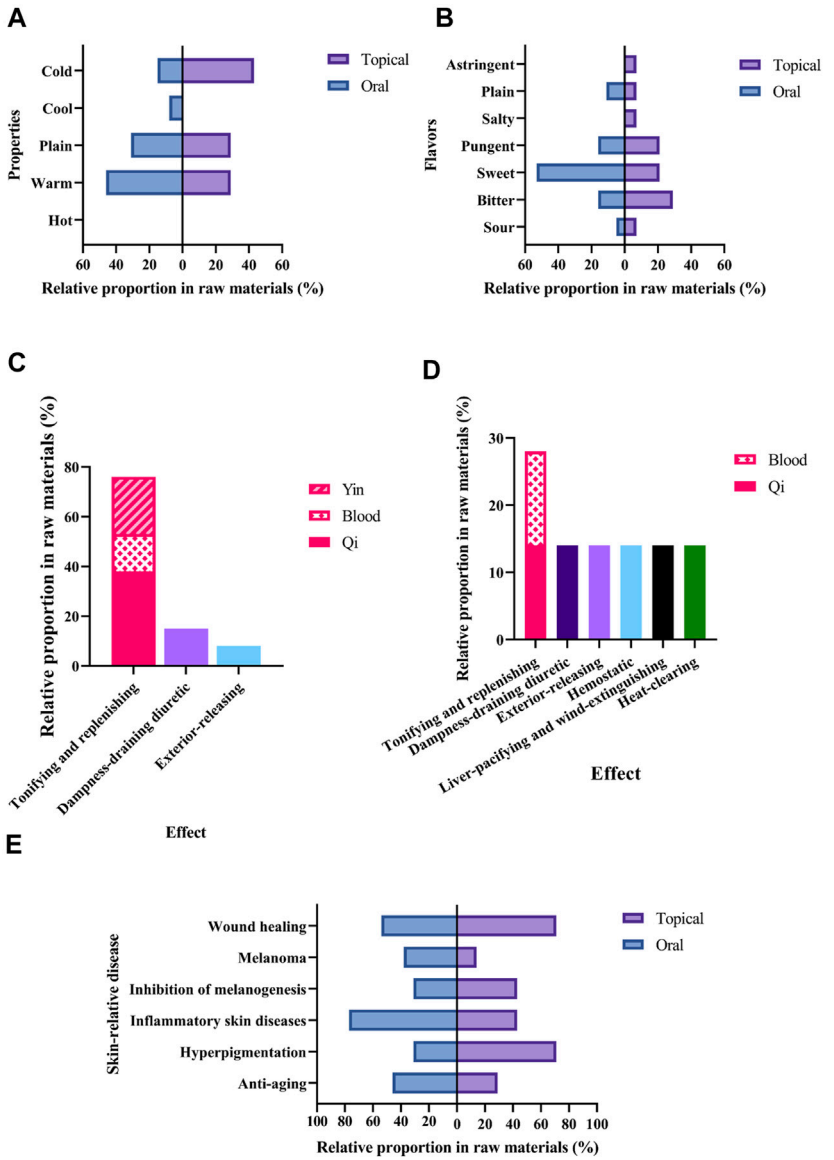


FIGURE 5 | Characteristics of medicinal materials with an RFC > 0.2 in skin whitening prescriptions. **(A)** Properties and **(B)** Flavors. Histogram of traditional efficacy classifications. **(C)** oral prescriptions and **(D)** external prescriptions. **(E)** Modern research related to skin.

3.4 Venn Diagram Analysis of Commonly Used Medicinal Materials Used in External Skin Whitening Prescriptions

In this study, medicinal materials with RFC >0.2 in external skin whitening prescriptions were defined as Taiwan *qī bái sàn* (**Figure 8A**). Taiwan qī bái sàn consists of *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., *Wolfiporia extensa* (Peck) Ginns, *Bletilla striata* (Thunb.) Rchb. f., *Atractylodes macrocephala* Koidz., *Ampelopsis japonica* (Thunb.) Makino, *Paeonia lactiflora* Pall. (white), and *Bombyx mori* Linnaeus. Venn diagram analysis of these medicinal materials with qī bái sàn-related prescriptions in yǒng lèi qián fāng, pǔ jì fāng, and tài píng shèng huì fāng found that Taiwan qī bái sàn is the addition and subtraction formula from the qī bái sàn mentioned in ancient books (**Figure 8B**).

3.5 Misuse of Medicinal Materials in Skin Whitening Prescriptions

Due to the wide variety of traditional Chinese medicinal materials, some medicinal materials may have the same vernacular name but are composed of different materials, whereas some medicinal materials may have different names but same origin. During integration and analysis of medicinal materials used in skin whitening prescriptions, it was found that *Ampelopsis japonica* (Thunb.) Makino, *Astragalus propinquus* Schischkin, *Reynoutria multiflora* (Thunb.) Moldenke, *Rosa*

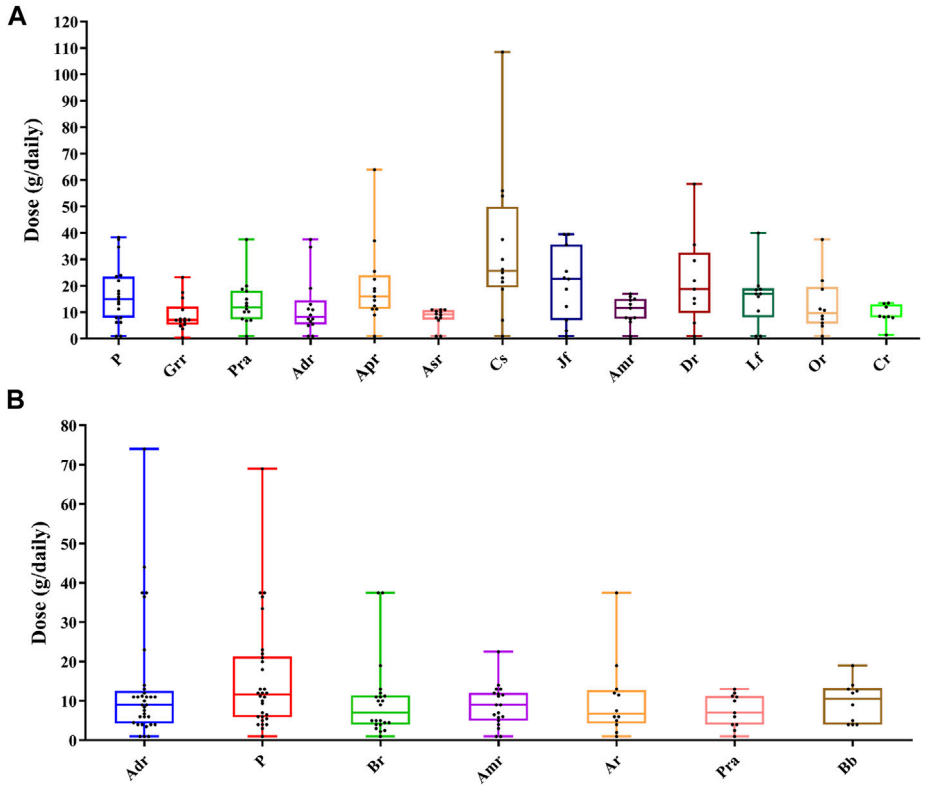


FIGURE 6 | Box plots of dose ranges of commonly used medicinal materials. The top line represents the maximum value, and the bottom line represents the minimum value; the bottom of each box represents the first quartile (Q1), the middle line represents the second quartile (Q2), and the top of each box represents the third quartile (Q3). The black dots represent the doses in the collected samples. **(A)** Oral prescriptions and **(B)** external prescriptions. Adr, *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.; Amr, *Atractylodes macrocephala* Koidz; Apr, *Astragalus propinquus* Schischkin; Ar, *Ampelopsis japonica* (Thunb.) Makino; Asr, *Angelica sinensis* (Oliv.) Diels; Cr, *Ligusticum striatum* DC.; Cs, *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf; Dr, *Dioscorea polystachya* Turcz.; Grr, *Glycyrrhiza uralensis* Fisch.; Jf, *Ziziphus jujuba* Mill. (red); Lf, *Lycium chinense* Mill.; Or, *Ophiopogon japonicus* (Thunb.) Ker Gawl.; P, *Wolfiporia extensa* (Peck) Ginns; Pra, *Paeonia lactiflora* Pall. (white).

rugosa Thunb., *Scutellaria baicalensis* Georgi, and *Tribulus terrestris* L. had misused sound alike or look alike medicinal materials (**Table 3**).

4 DISCUSSION

4.1 Field Investigation Sites

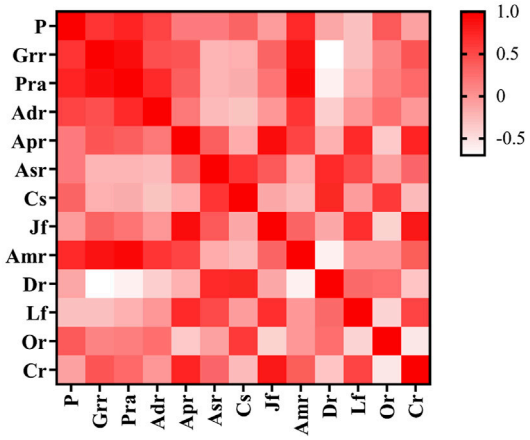
In this study, field investigation was employed to study skin whitening prescriptions sold in TCM pharmacies in Taiwan. Field investigations are mostly used in sociology, geography, or cultural studies and was previously employed to examine the drug treatment habits for certain diseases in some regions, such as traditional Chinese medicine composition used in galactagogues prescriptions (Chao et al., 2020), herbal composition of Qīng-Cǎo-Chá tea (Huang et al., 2020), and medicinal materials used for hypertension (Baharvand-Ahmadi et al., 2016). Traditional Chinese Medicine, the mainstay of Asian culture, is a form of experience-based therapies, and is a medical care system for diagnosing, preventing, and treating diseases (Xu et al., 2013). Therefore, traditional Chinese pharmacies in Taiwan are important sites for retaining TCM culture.

4.2 Types and Biological Taxonomic Characteristics of Medicinal Materials in Skin Whitening Prescriptions

This study on the composition of skin whitening prescriptions used in Taiwan found that most medicinal materials were from Apiaceae, including *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., *Angelica sinensis* (Oliv.) Diels and *Ligusticum striatum* DC., followed by Leguminosae, including *Glycyrrhiza uralensis* Fisch. and *Astragalus propinquus* Schischkin. Apiaceae and Leguminosae plants can inhibit tyrosinase activity, thereby reducing melanogenesis. These plants are rich in phenolic compounds and flavonoids that are proven to have significant antioxidant activity. The previous experiments also showed that they can inhibit matrix metalloproteinases (MMPs), delay skin photoaging, and stimulate keratinocyte and fibroblast migration, which has significant effects on skin regeneration (Tundis et al., 2015; Waqas et al., 2015; Zofia et al., 2020).

Melanin is an important pigment that determines skin, hair, and eye colors, and can be mainly divided into pheomelanin and eumelanin (Zanetti et al., 2001). Melanin synthesis is intimately associated with tyrosinase. Pigmentation is an important

A



	P	Grr	Pra	Adr	Apr	Asr	Cs	Jf	Amr	Dr	Lf	Or	Cr
P		0.64	0.75	0.55	0.17	0.15	0.31	-0.05	0.71	-0.12	-0.30	0.40	-0.10
Grr	0.64		0.89	0.46	0.40	-0.22	-0.18	0.32	0.87	-0.70	-0.30	0.11	0.41
Pra	0.75	0.89		0.71	0.35	-0.21	-0.16	0.20	0.93	-0.63	-0.19	0.14	0.28
Adr	0.55	0.46	0.71		0.19	-0.26	-0.31	-0.02	0.63	-0.39	-0.02	0.23	-0.02
Apr	0.17	0.40	0.35	0.19		0.35	-0.17	0.91	0.53	-0.20	0.71	-0.36	0.76
Asr	0.15	-0.22	-0.21	-0.26	0.35		0.63	0.40	-0.15	0.71	0.50	-0.09	0.30
Cs	0.31	-0.18	-0.16	-0.31	-0.17	0.63		-0.15	-0.26	0.72	-0.07	0.60	-0.26
Jf	-0.05	0.32	0.20	-0.02	0.91	0.40	-0.15		0.31	-0.13	0.67	-0.41	0.83
Amr	0.71	0.87	0.93	0.63	0.53	-0.15	-0.26	0.31		-0.62	-0.04	-0.02	0.37
Dr	-0.12	-0.70	-0.63	-0.39	-0.20	0.71	0.72	-0.13	-0.62		0.28	0.26	-0.33
Lf	-0.30	-0.30	-0.19	-0.02	0.71	0.50	-0.07	0.67	-0.04	0.28		-0.42	0.54
Or	0.40	0.11	0.14	0.23	-0.36	-0.09	0.60	-0.41	-0.02	0.26	-0.42		-0.55
Cr	-0.10	0.41	0.28	-0.02	0.76	0.30	-0.26	0.83	0.37	-0.33	0.54	-0.55	

B

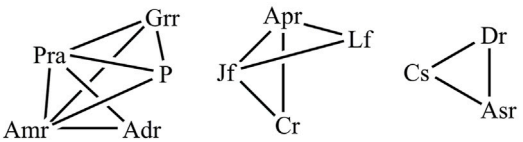


FIGURE 7 | Spearman correlation analysis of commonly used medicinal materials used in oral skin whitening prescriptions. **(A)** Heat map and **(B)** Network map. Adr, *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.; Amr, *Atractylodes macrocephala* Koidz.; Apr, *Astragalus propinquus* Schischkin; Asr, *Angelica sinensis* (Oliv.) Diels; Cr, *Ligusticum striatum* DC.; Cs, *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf; Dr, *Dioscorea polystachya* Turcz.; Grr, *Glycyrrhiza uralensis* Fisch.; Jf, *Ziziphus jujuba* Mill. (red); Lf, *Lycium chinense* Mill.; Or, *Ophiopogon japonicus* (Thunb.) Ker Gawl.; P, *Wolfiporia extensa* (Peck) Ginns; Pra, *Paeonia lactiflora* Pall. (white).

photoprotective factor, and its regulatory mechanism is extremely complex and still not completely understood. However, a large volume of data shows that ultraviolet-induced DNA damage and its repair will activate tyrosinase in melanocytes, resulting in melanogenesis (Gilchrest and Eller, 1999; Brenner and Hearing, 2008; Lai et al., 2018). Therefore, inhibition of tyrosinase can inhibit melanogenesis.

4.3 Analysis of Effects and Pharmacology of Commonly Used Medicinal Materials Used in Skin Whitening Prescriptions

TCM has a unique theory where medicinal materials are classified by properties (hot, warm, plain, cool, and cold) and flavors (sour, bitter, sweet, pungent, salty, plain, and astringent). Most TCM materials comprise a combination of flavors (Liao et al., 2008). The ^{1}H-NMR spectrum was used to identify the properties of the medicinal materials, and it was found that their ingredients were very different (Zhang et al., 2020). A previous report showed that warm and hot medicinal materials can regulate the immune system; cold and cool medicinal materials can inhibit cell growth and proliferation (Liang et al., 2013); sweet medicinal materials have supplementation, moderation, and harmonization effects (He et al., 2012), while bitter medicinal materials mostly contain alkaloids with anti-inflammatory effects (Chen et al., 2015). The results of this study found that commonly used medicinal materials used in oral skin whitening prescriptions are mostly warm and sweet while those used in external skin whitening prescriptions are mostly cold and bitter. In combination with previous studies, it can be deduced that oral skin whitening prescriptions mostly focus on immune regulation while external skin whitening prescriptions focus on inflammation alleviation.

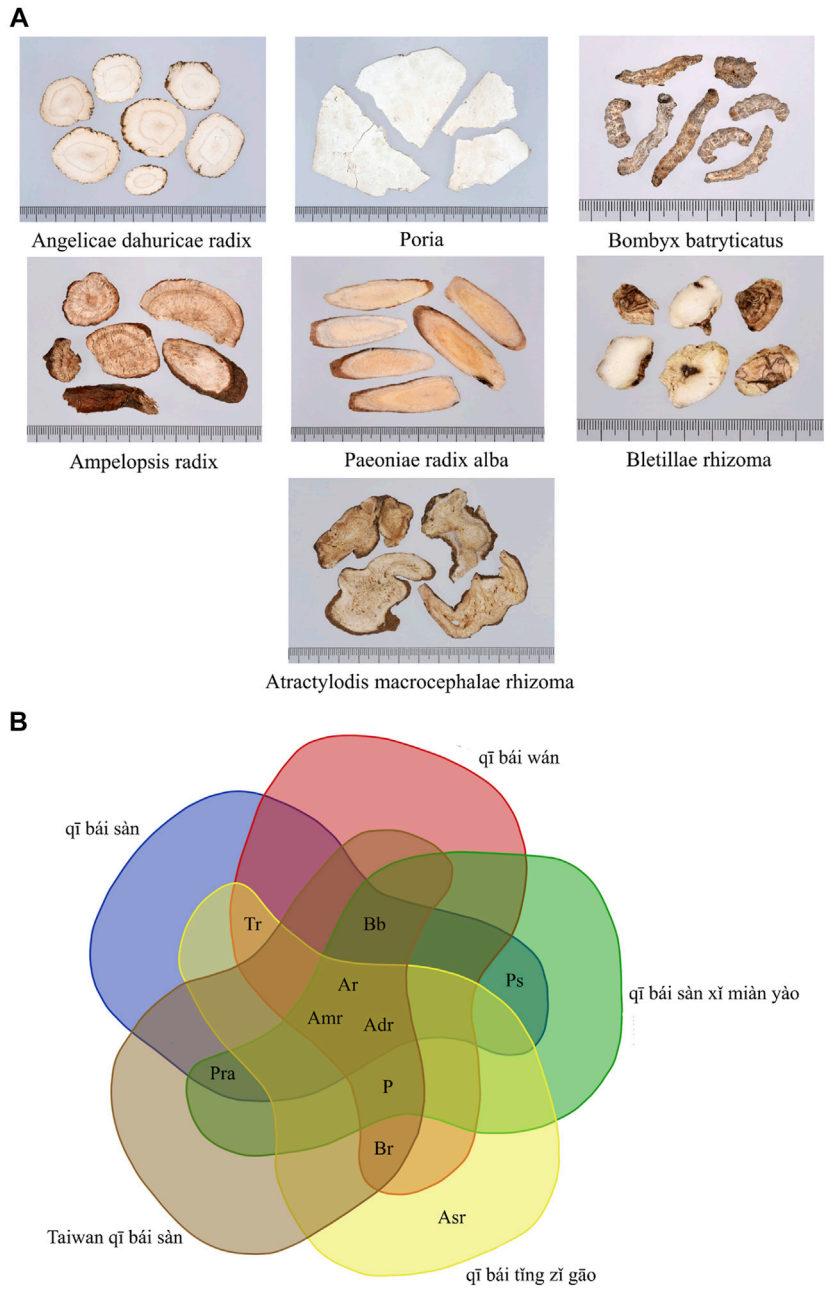


FIGURE 8 | (A) Pictures of medicinal materials of Taiwan *qī bái sàn*. **(B)** Venn diagram of *qī bái sàn*-related prescriptions in ancient books and Taiwan *qī bái sàn*. Adr, *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav.; Amr, *Atractylodes macrocephala* Koidz.; Ar, *Ampelopsis japonica* (Thunb.) Makino; Asr, *Asarum heterotropoides* F. Schmidt f. *mandshuricum* (Maxim.) Kitag.; Bb, *Bombyx mori* Linnaeus; Br, *Bletilla striata* (Thunb.) Rchb. f.; P, *Wolfiporia extensa* (Peck) Ginns; Pra, *Paeonia lactiflora* Pall. (white); Ps, *Ipomoea nil* (L.). Roth; Tr, *Sauromatum giganteum* (Engl.) Cusimano & Hett.

4.4 Analysis of Commonly Used Medicinal Materials Used in Oral and External Skin Whitening Prescriptions

Wolfiporia extensa (Peck) Ginns, *Paeonia lactiflora* Pall. (white), *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav., and *Atractylodes macrocephala* Koidz. are commonly used medicinal materials used in oral and external skin whitening prescriptions. *Wolfiporia extensa* (Peck) Ginns regulates tyrosinase activity to inhibit melanogenesis (Lee and Cha, 2018). In past studies had found that *Paeonia lactiflora* Pall. (white) can be used to treat allergic dermatitis and reduce facial wrinkles. Paeoniflorin in *Paeonia lactiflora* Pall. (white) can reduce the expression of microphthalmia-associated transcription factor (MITF) and melanogenic enzymes (including tyrosinase, TRP-1, and TRP-2) by regulating the p38 MAPK pathway, thereby inhibiting

TABLE 3 | Summary of medicinal materials that tend to be misused in skin whitening prescriptions.

Latin name of crude drug	Authentic or misused	Scientific name	Family	Look alike or sound alike[a]	Frequency/Use ratio (%)
Ampelopsis radix	authentic	*Ampelopsis japonica* (Thunb.) Makino	Vitaceae	look alike	11/73%
	misused	*Momordica cochinchinensis* (Lour.) Spreng	Cucurbitaceae		4/27%
Astragali radix	authentic	*Astragalus propinquus* Schischkin [*Astragalus membranaceus* (Fisch.) Bunge][b]	Leguminosae	look alike	1/7%
	misused	*Hedysarum polybotrys* Hand. -Mazz	Leguminosae		13/93%
Reynoutriae multiflorae radix	authentic	*Reynoutria multiflora* (Thunb.) Moldenke [*Polygonum multiflorum* Thunb.][c]	Polygonaceae	look alike	0/0%
	misused	*Pteroxygonum giraldii* Dammer and Diels	Polygonaceae		1/100%
Rosae rugosae flos	authentic	*Rosa rugosa* Thunb	Rosaceae	look alike	1/50%
	misused	*Rosa chinensis* Jacq	Rosaceae		1/50%
Scutellariae radix	authentic	*Scutellaria baicalensis* Georgi	Lamiaceae	look alike	3/60%
	misused	*Scutellaria amoena* C.H.Wright	Lamiaceae		2/40%
Tribuli fructus	authentic	*Tribulus terrestris* L.	Zygophyllaceae	sound	5/83%
	misused	*Astragalus complanatus* Bunge	Leguminosae	alike	1/17%

[a] *"Look alike" refers to the similar appearance of two confusing medicinal materials, thus causing misuse. "Sound alike" means that the local names of two confusing medicinal materials are similar in pronunciation, which causes misuse.*

[b] Astragalus membranaceus *(Fisch.) bunge is a commonly used synonym of* astragalus propinquus *schischkin.*

[c] Polygonum multiflorum *Thunb. is a commonly used synonym of* Reynoutria multiflora *(Thunb.) moldenke.*

melanogenesis (Qiu et al., 2016). *Angelica dahurica* (Hoffm.) Benth. & Hook. f. ex Franch. & Sav. is a good immunomodulatory agent, and it can significantly increase phagocytosis and the secretion of cytokines by macrophages (Wang et al., 2021). It can also inhibit melanogenesis. It is involved inhibition of tyrosinase synthesis, but it does not inhibit tyrosinase activity (Cho et al., 2006). A study highlighted that the extract of *Atractylodes macrocephala* Koidz. has immune-enhancing activities (Sun et al., 2015a). Its active ingredients are Atractylenolide I and 14-acetoxy-12-senecioyloxytetradeca-2E, 8E, 10E-trien-4,6-diyn-1-ol. They are both alkaloids that demonstrate anti-inflammatory effects and are tyrosinase inhibitors (Li et al., 2007; Ye et al., 2010).

4.5 Misuse of Medicinal Materials in Skin Whitening Prescriptions

The Taiwan Herbal Pharmacopeia is a codex of management regulations for TCM in Taiwan. The medicinal materials recorded in monographs should comply with the pharmacopeia standards before being manufactured, sold, and dispensed for medical treatment and health care. Medicinal materials recorded in the Taiwan Herbal Pharmacopeia are defined as authentic medicinal materials, while the origins of medicinal materials not included in the pharmacopeia are regarded as misused or fake medicinal materials (Taiwan Herbal Pharmacopeia 3rd Edition Committee, 2018; Taiwan Herbal Pharmacopeia 4th Edition Committee, 2021). Although the government releases information about authentic medicinal materials every year, medicinal materials' misuse is still commonly seen in markets in Taiwan. Such misused materials were also found in skin whitening prescriptions that were collected in this study, including medicinal materials that look alike, which refers to the similar appearance of two confusing medicinal materials, thus causing misuse, such as *Momordica cochinchinensis* (Lour.) Spreng. that was used instead of *Ampelopsis japonica* (Thunb.) Makino, *Hedysarum polybotrys* Hand-Mazz. that was used instead of *Astragalus propinquus* Schischkin, *Scutellaria amoena* C.H.Wright that was used instead of *Scutellaria baicalensis* Georgi, *Pteroxygonum giraldii* Dammer & Diels that was used instead of *Reynoutria multiflora* (Thunb.) Moldenke, and *Rosa chinensis* Jacq. that was used instead of *Rosa rugosa* Thunb.; and medicinal materials that sound alike, which means that the local names of two confusing medicinal materials are similar in pronunciation, thus causing misuse, such as *Astragalus complanatus* Bunge that was used instead of *Tribulus terrestris* L (**Figure 9**). Even though misused medicinal materials in skin whitening prescriptions do not seem to cause immediate harm to the human body, there is no way to prove whether they will have adverse interactions with other medicinal materials. Therefore, simple and clear pictures showing that appearance and properties of medicinal materials should be created, and rapid, simple, and convenient identification methods should be developed to disseminate knowledge for identification of misused medicinal materials and remind traditional Chinese medicine users to be cautious and prevent misuse of medicinal materials.

4.6 Limitations and Future Directions

This study had certain limitations that should be addressed in future research. The first limitation is that this study was a field survey study, which only investigated skin whitening prescriptions sold in Chinese medicine stores in Taiwan. The skin whitening prescriptions investigated in this study reflect local usage in Taiwan. Further research will be needed to examine the whitening prescriptions used in traditional Chinese medicine in other regions. The second limitation is that this study only analyzed the core medicinal materials and dosages of the prescriptions but not the efficacy of these botanical drugs. Therefore, animal or cell experiments can be used in future

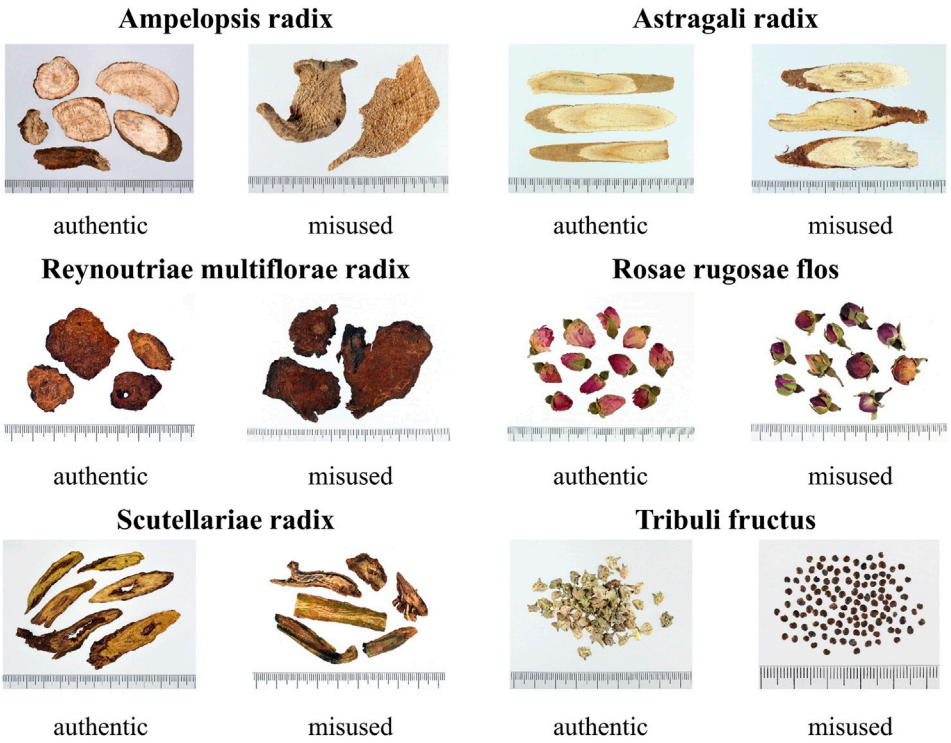


FIGURE 9 | Pictures of authentic or misused medicinal materials.

research to verify the effectiveness of the skin whitening medicinal materials or prescriptions. The last limitation is that the Spearman correlation analysis only shows the correlations between prescription drugs and not the drug interactions between them. When discussing prescriptions in future research, we may have to consider both the theoretical significance of traditional Chinese medicine and the practical significance. We should also conduct *in vivo* and *in vitro* experiments to further explore the efficacy and the interactions between the medicinal materials.

5 CONCLUSION

In Asian countries, whiter skin color is synonymous with beauty for women and many Asian women look for natural and without side effects skin whitening products in order to reduce cutaneous pigmentation. This study is the first ethnobotanical survey on skin whitening prescriptions collected from TCM pharmacies in Taiwan. The purpose is to preserve the use of TCM for skin whitening in Taiwan. Although the use of TCM in skin whitening has been widely recorded and many skin whitening medicinal materials were collected from TCM pharmacies in this study, the ingredients of TCM are extremely complex and tends to be affected by many factors, may also be used in misused medicinal materials. Moreover, the efficacy and safety of most medicinal materials have not been scientifically validated. Further studies are needed to support the conversion of traditional skin whitening prescriptions to effective functional products. Thus, the results of this study may provide significant foundational data for subsequent studies The Royal Botanic Gardens, 2013, Li et al., 2017, Institute of Ecology et al., 2021.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CRREC-109-125. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

C-YK, P-YC, JC, and S-SH performed the field investigation and organized and analyzed the database. C-YK, JC, S-SH, TM, and C-YL contributed conception and design of the study. S-SH and S-YS identified the botanical materials. C-YK, S-SH, JC, S-YS, TM, and C-YL drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

S-SH, JC, S-YS, and H-CC provided guidance for the project and supervised the experiment and manuscript review.

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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.2021.736370/full#supplementary-material

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Ethnopharmacological Survey of Traditional Chinese Medicine Pharmacy Prescriptions for Dysmenorrhea

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Chinese herbal medicines have long been used for the treatment of dysmenorrhea. The treatment experiences of traditional Chinese medicine (TCM) pharmacies passed down through generations have contributed to a wealth of prescriptions for dysmenorrhea that have achieved significant therapeutic effects in countless Taiwanese women. Therefore, surveying and analyzing these prescriptions may enable us to elucidate the core medication combinations used in TCM prescriptions for dysmenorrhea. In the present study, a field investigation was conducted on various TCM pharmacies in Taiwan. A total of 96 TCM pharmacies were sampled, and 99 prescriptions for dysmenorrhea containing 77 different medicinal materials were collected. Compositae (8%) was the most common botanical source of the medicinal materials, and the predominant TCM property and flavor of the materials were warm (45%) and sweet (73%), respectively. The blood-activating and stasis-dispelling effect (23%) and the qi-tonifying effect (23%) were the most prevalent traditional effects, and the modern pharmacological effects most commonly found in the materials were anti-inflammatory (73%), antitumor (59%), and analgesic (12%) effects. Network analysis of the 77 medicinal materials used in the prescriptions, which was performed using the Traditional Chinese Medicine Inheritance Support System, yielded seven core medicinal materials and the corresponding network diagram. The seven core medicinal materials ranked in order of relative frequency of citation (RFC) were *Angelica sinensis* (Oliv.) Diels (Dang Gui), *Ligusticum chuanxiong* Hort (Chuan Qiong), *Rehmannia glutinosa* Libosch (Di Huang), *Paeonia lactiflora* Pall (Bai Shao), *Hedysarum polybotrys* Hand.-Mazz (Hong Qi), *Lycium chinense* Mill (Gou Qi Zi), and *Cinnamomum cassia* (L.). J. Presl (Gui Zhi). A total of 58 combinations, each consisting of two to five of the seven medicinal materials and 107 association rules among the materials, were identified. This study provides a record of valuable knowledge on TCM pharmacy prescriptions for dysmenorrhea. The rich medicinal knowledge of TCM pharmacies in Taiwan is worthy

of further exploration, and the results of this study can serve as a basis for future pharmacological research and the development of naturally derived medications for dysmenorrhea.

Keywords: dysmenorrhea, ethnopharmacology, Taiwan, traditional Chinese medicine pharmacy, Chinese herbal medicines

1 INTRODUCTION

"Dysmenorrhea" is a Greek term meaning "difficult menstrual flow" (Burnett and Lemyre, 2017). Dysmenorrhea can be classified as primary and secondary dysmenorrhea, with the former defined as pain occurring with menses in the absence of pelvic pathology, and the latter as menstrual pain associated with underlying pelvic pathology, such as endometriosis (Burnett and Lemyre, 2017). It is a common condition among women of reproductive age, and the severe pain experienced by dysmenorrhea sufferers often causes interference with daily and educational activities (Durand et al., 2021). A review of relevant literature published between 1944 and 2015 revealed that primary dysmenorrhea affects 45–95% of menstruating women worldwide (Iacovides et al., 2015). Other studies have shown prevalence rates of 74–94% in European countries (Abreu-Sánchez et al., 2020; Barcikowska et al., 2020; Durand et al., 2021), 72.1% among young Asian women (Abubakar et al., 2020), and 65.3% in Taiwan (Yeh et al., 2019).

Treatment strategies for dysmenorrhea are broadly classified into three categories, namely pharmacological, surgical, as well as complementary and alternative therapies. Pharmacological therapy can be further divided into non-hormonal medical therapy, which includes various analgesics, and hormonal therapy, which includes combined hormonal contraceptives and progestin regimens (Burnett and Lemyre, 2017). This therapy is adopted by a large proportion of dysmenorrhea sufferers, with analgesics being used by up to 79.5% of affected women. However, pharmacological therapy produces side effects. For instance, non-steroidal anti-inflammatory drugs cause adverse effects in the gastrointestinal tract and central nervous system; hormonal treatment leads to side effects, such as nausea, breast tenderness, and headaches (Rosenberg et al., 1995), as well as an increased risk of venous thromboembolism (Vinogradova et al., 2014). Surgical management is indicated for secondary dysmenorrhea, and it involves open or laparoscopic surgery for eliminating pelvic pathology after a confirmed diagnosis has been made by pelvic ultrasound, magnetic resonance imaging (MRI), cystoscopy, or colonoscopy (Burnett and Lemyre, 2017). Complementary and alternative therapy is the most popular form of therapy among women with dysmenorrhea, with up to 95.1% of dysmenorrhea sufferers adopting non-pharmacological and non-surgical methods, such as heat application, hot shower/bath, exercise (Durand et al., 2021), transcutaneous electrical nerve stimulation, acupuncture and acupressure, behavioral interventions, and dietary supplements (Burnett and Lemyre, 2017).

In countries with widespread use of herbal medications, there have been reports of the adoption of herbs as a form of complementary and alternative therapy for dysmenorrhea. For instance, the use of *Marantodes pumilum* (family Primulaceae) to alleviate dysmenorrhea is popular in traditional Malay medicine (Aladdin et al., 2020). A study conducted in Turkey showed that the consumption of dry figs over three menstrual cycles decreased pain severity and increased quality of life (Amanak, 2020). Many ethnomedical studies have also shown that *Paeonia lactiflora* (Li et al., 2021), *Sparganium stoloniferum* (Jia et al., 2021), and *Foeniculum vulgare* (Lee et al., 2020) can alleviate pain in dysmenorrhea. Other studies have reported that *Tetradium ruticarpum* (Li and Wang, 2020) has been used to treat dysmenorrhea and pelvic inflammation in clinical practice for thousands of years, and that the fruit of *Akebia quinate* is widely used as a folk medicine to treat primary dysmenorrhea by the Tujia minority in China (Ma et al., 2021). Cinnamon, fennel, and ginger can effectively reduce pain intensity in primary dysmenorrhea, with cinnamon also being able to shorten the duration of pain (Xu Y. et al., 2020). A case-control study conducted in Ethiopia found that thyme tea drinking and consumption of vegetables and fruits have a primary dysmenorrhea-related pain-relieving tendency (Zeru and Muluneh, 2020).

In Taiwan, Chinese herbal medicines are the most popular form of complementary and alternative therapy used for treating dysmenorrhea, with approximately 75.2% of women aged 13–19 years and 63.3% of women aged 19–45 years in Taiwan seeking TCM treatment when suffering from dysmenorrhea (Huang, 2012). A previous survey showed that the majority of Taiwanese women purchased Chinese herbal medicines from community TCM pharmacies (Ho et al., 2011). However, as most of these TCM pharmacies are family-owned businesses, the Chinese herbal medicine knowledge of the pharmacists, including the processing methods, formulae, dosages, and administration methods, is only handed down by apprenticeship and has not been rigorously recorded or published (Huang et al., 2020). Hence, to gather and retain important medical knowledge regarding dysmenorrhea treatment that is currently only passed down from one generation to another in TCM pharmacies, we surveyed and gathered prescriptions for dysmenorrhea treatment from TCM pharmacies across Taiwan for the first time. Subsequently, the compositions of the prescriptions were analyzed to screen for the frequently used medicinal materials. The TCM property and flavor, traditional effects, and modern pharmacological effects

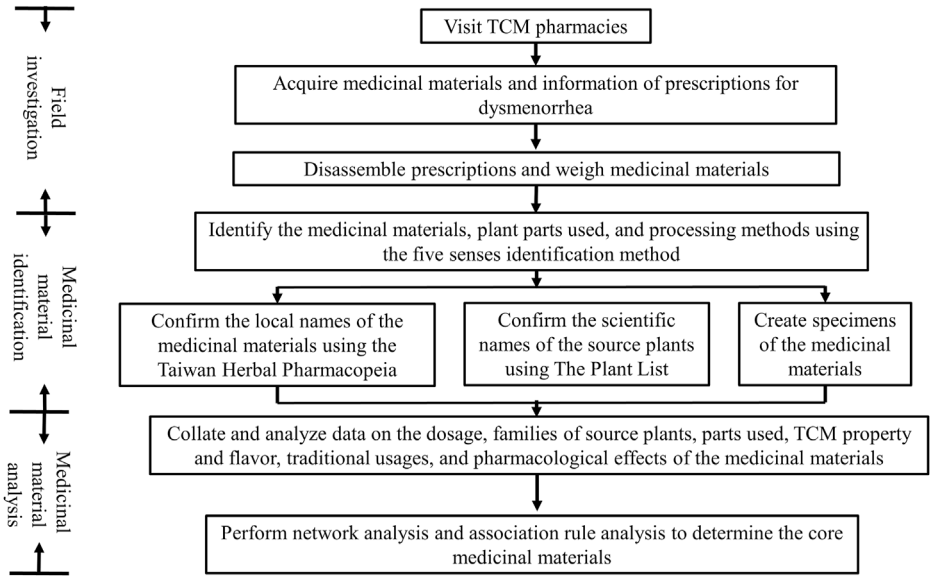

FIGURE 1 | Research process flow chart.

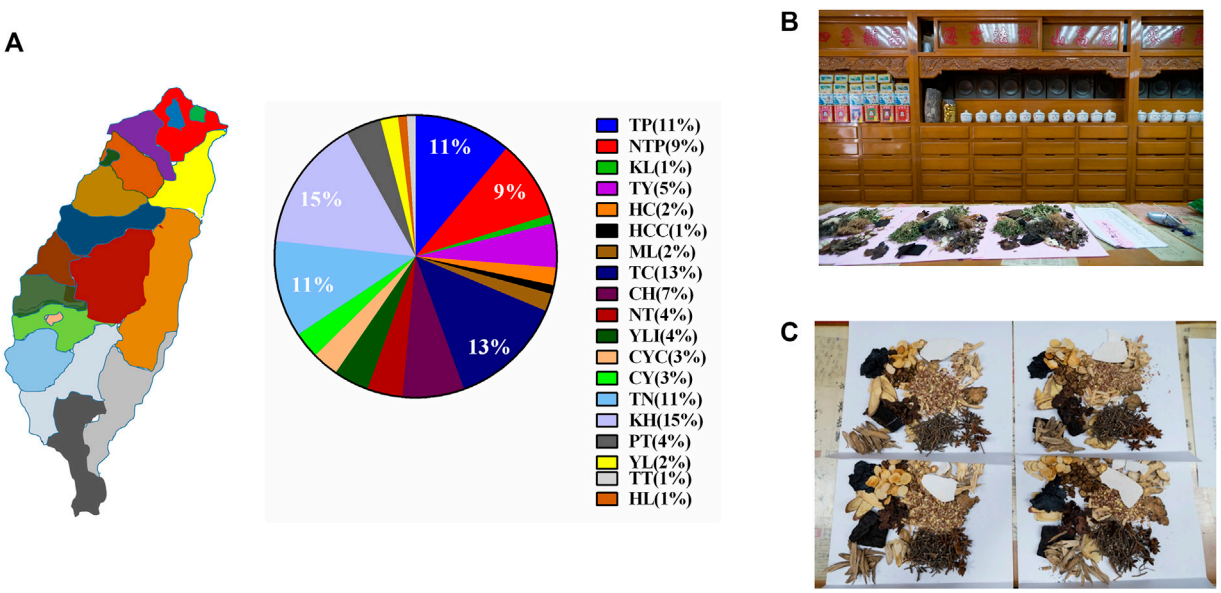

FIGURE 2 | Regions investigated for prescriptions for dysmenorrhea (**A**) Distribution of TCM pharmacies across different regions in the main island of Taiwan; (**B**) Photograph of a typical TCM pharmacy in Taiwan; (**C**) Examples of TCM prescriptions collected during field investigation. CH, Changhua County; CYC, Chiayi City; CY, Chiayi County; HCC, Hsinchu City; HC, Hsinchu County; HL, Hualien County; KH, Kaohsiung City; KL, Keelung City; ML, Miaoli County; NT, Nantou County; NTP, New Taipei City; PT, Pingtung County; TC, Taichung City; TN, Tainan City; TP, Taipei City; TT, Taitung County; TY, Taoyuan City; YL, Yilan County, and YLI, Yunlin County.

of the frequently used medicinal materials were then subjected to statistical analysis and network analysis to determine the frequently used medicinal combinations and the core medicinal material network of the prescriptions. Our results may provide a scientific basis for future pharmacological research and the development of naturally derived medications for dysmenorrhea.

2 MATERIALS AND METHODS

2.1 Ethical Review

The present study was conducted from October 2020 to April 2021 and was approved by the Central Regional Research Ethics Center of China Medical University prior to commencement (Approval No.: N/A/CRREC-109-125) (**Supplementary Figure S1**).

2.2 Research Process

The research process consisted of three main steps, namely field investigation, medicinal material identification, and medicinal material analysis (**Figure 1**).

2.3 Field Investigation

The field investigation of this study was performed on TCM pharmacies across the main island of Taiwan.

The main island of Taiwan is located at 22–25°N, 120–122°E, and has a land area of approximately 36,000 km^2. It measures approximately 395 km from north to south and has a maximum width of 144 km from east to west. The island country, located in the western side of the Pacific Ocean, has a combination of tropical and subtropical climates. Currently, the main island comprises six special municipalities, 10 counties, and three cities. TCM pharmacies across Taiwan were sampled using a ratio of 85:1 (96 pharmacies sampled from a total of 8,382 pharmacies) based on the proportions of registered pharmacy businesses in the various municipalities, counties, and cities (**Figure 2A**).

This study was conducted from October 2020 to April 2021. The research team visited TCM pharmacies across Taiwan for a field investigation, and purchased medicinal materials used in prescriptions for dysmenorrhea from select TCM pharmacies (**Figure 2B**).

2.4 Identification of Botanical Origin of Medicinal Materials

The purchased medicinal materials were disassembled (**Figure 2C**) for the identification of the origin, plant parts, and processing methods of the materials using the five senses identification method. We also photographed the materials and recorded the weight of each material. Finally, the materials were numbered and preserved in the herbarium of China Medical University. The taxonomic ranks and scientific names of all materials were determined in accordance with the taxonomy and nomenclature adopted in The Plant List.

2.5 Data Collation and Analysis

The following information of the medicinal materials used in the prescriptions for dysmenorrhea collected from TCM pharmacies across Taiwan was collated:

1) Names of medicinal materials: Scientific names and local names were determined using The Plant List and the third edition of the Taiwan Herbal Pharmacopeia (Ministry of Health and Welfare Taiwan, 2019), respectively.
2) TCM property and flavor, traditional usages, and frequently used doses: Data were obtained from the third edition of the Taiwan Herbal Pharmacopeia (Ministry of Health and Welfare Taiwan and Taiwan Herbal, 2019).
3) Relative frequency of citation (RFC): The frequency of citation (FC) of each material was first determined by summing the number of times that the material was used in the collected prescriptions. Subsequently, FC was divided by the total number of prescriptions collected in the study to obtain the RFC value (Chao et al., 2021), as shown by the following formula:

RFC = FC/the total number of prescriptions

4) Modern pharmacological effects: Relevant pharmacological studies published during the last 5 years were searched on PubMed (https://pubmed.ncbi.nlm.nih.gov/) using the scientific names of the medicinal materials as search terms.

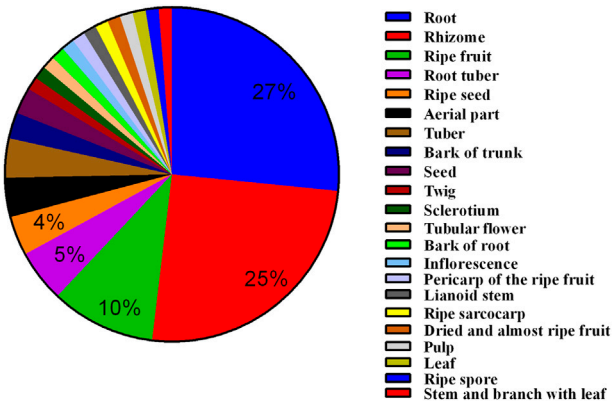

FIGURE 3 | Utilized plant parts in the 77 medicinal materials used in prescriptions for dysmenorrhea.

2.6 Network Analysis of Associations of Medicinal Materials

Analysis of the associations among the medicinal materials was performed using the Traditional Chinese Medicine Inheritance Support System (TCMISS) V2.5, with support and confidence score set as 50% and 0.95, respectively. Support indicates the frequency with which a medicinal material appears in all collected prescriptions (Miao et al., 2019), and confidence score refers to the association of two materials among the various medicinal combinations, e.g., the confidence score is 0.95 if the probability of material B appearing when material A appears is 95% (Zucheng, 2020). The frequently used medicinal combinations and association rules obtained from association analysis were used to plot a network diagram of associations among the various materials, so as to determine the core medicinal materials used in the prescriptions for dysmenorrhea.

3 RESULTS

3.1 Types and Taxonomic Characteristics of Medicinal Materials Used in Prescriptions for Dysmenorrhea Sold at TCM Pharmacies in Taiwan

A total of 99 prescriptions for dysmenorrhea were acquired from 96 TCM pharmacies during the field investigation. The

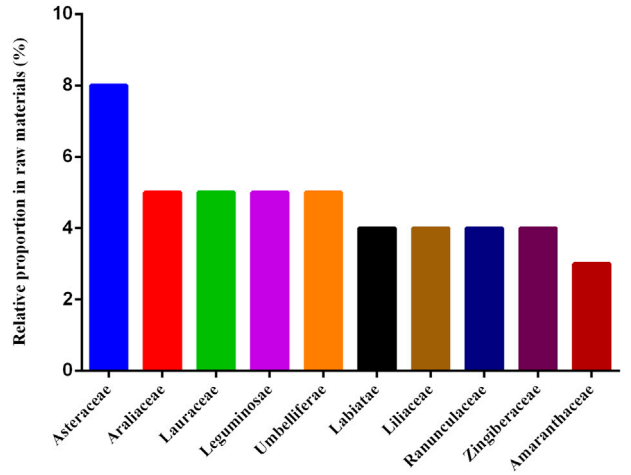

FIGURE 4 | Top 10 families with the highest proportions in the 77 medicinal materials used in prescriptions for dysmenorrhea.

prescriptions contained 77 different medicinal materials derived from organisms belonging to 45 families, with 73 materials derived from plants, 2 from fungi, and 2 from animals (**Supplementary Table S1**).

An analysis of the plant parts used in the 77 medicinal materials revealed that the root was the most frequently utilized plant part (27%), followed by the rhizome (25%), ripe fruit (10%), root tuber (5%), ripe seed (4%), dried aerial part (4%), and tuber (4%) (**Figure 3**). All 77 medicinal materials were dried materials, which included the following processed materials: steamed Di Huang (*Rehmannia glutinosa* Libosch., abbreviated as RG), stir-baked Bai Shao (*Paeonia lactiflora* Pall., abbreviated as PL), stir-baked Du Zhong (*Eucommia ulmoides* Oliv., abbreviated as EU), honey-roasted Gan Cao (*Glycyrrhiza uralensis* Fisch., abbreviated as GU), and soil stir-baked Bai Zhu (*Atractylodes macrocephala* Koidz., abbreviated as AM).

Members of the family Compositae accounted for the greatest proportion of the 77 medicinal materials (8%), followed by Araliaceae, Lauraceae, Leguminosae, and Umbelliferae (5% each), and Labiatae, Liliaceae, Ranunculaceae, and Zingiberaceae (4% each) (**Figure 4**).

3.2 Analysis of Frequently Used Medicinal Materials in Prescriptions for Dysmenorrhea

Among the 77 medicinal materials (**Supplementary Table S1**), 22 were frequently used in prescriptions for dysmenorrhea based on the criterion of RFC ≥0.1 (**Table 1**). The seven-most frequently used medicinal materials ranked in order of RFC were *Angelica sinensis* (Oliv.) Diels (Dang Gui, abbreviated as AS), *Ligusticum chuanxiong* Hort (Chuan Qiong, abbreviated as LiC), *Rehmannia glutinosa* Libosch. (Di Huang, abbreviated as RG), *Paeonia lactiflora* Pall. (Bai Shao, abbreviated as PL), *Hedysarum polybotrys* Hand.-Mazz (Hong Qi, abbreviated as HP), *Lycium chinense* Mill. (Gou Qi Zi, abbreviated as LyC), and *Cinnamomum cassia* (L.) J. Presl (Gui Zhi, abbreviated as CCT). The most common TCM flavor of the frequently used medicinal materials was sweet (73%), followed by pungent (41%). The most common TCM property was warm (45%), followed by neutral (27%). Among the various flavor-property combinations, sweet-warm was the most common (32%), followed by sweet-neutral (27%) and pungent-warm (23%) (**Figure 5**).

The most prevalent traditional effects of the frequently used medicinal materials were the blood-activating and stasis-dispelling effect (23%) and the qi-tonifying effect (23%), followed by the blood-tonifying effect, interior-warming effect, and heat-clearing and blood-cooling effect (9% each) (**Figure 5**).

As shown in **Figure 6**, the most commonly reported modern pharmacological effect of the frequently used medicinal materials was anti-inflammatory effect, which was reported for 16 of the 22 frequently used medicinal materials (73%). This was followed by antitumor (59%), antioxidant (20%), Antihyperglycemic (19%), immunomodulatory (17%), neuroprotective (15%), analgesic (12%), hepatoprotective (10%), antibacterial (8%), and antiviral (8%) effects (**Figure 6**).

Among the various medicinal materials used in the prescriptions, *Diospyros lotus* (Hei Zao, abbreviated as ZJH) had the highest average dose of 25.18 ± 8.48 g (**Supplementary Table S2**), and *Oroxylum indicum* (L.) Benth. ex Kurz (Mu Hu Die, abbreviated as OI) had the lowest average dose of 3.09 ± 2.04 g. DL (25.18 ± 8.48 g) and HP (15.12 ± 7.24 g) showed the largest dose differences across the various TCM pharmacies, whereas *Poria cocos* (Schwein.) F.A.Wolf (Fu Ling, abbreviated as PC), *Prunus persica* (L.) Batsch (Tao Ren, abbreviated as PP), *Zingiber officinale* Roscoe (Gan Jiang, abbreviated as ZO), and *Cyperus rotundus* L. (Xiang Fu, abbreviated as CR) exhibited the smallest dose differences across various TCM pharmacies (**Figure 7**).

3.3 Association and Network Analysis of Medicinal Materials Used in Prescriptions for Dysmenorrhea

Analysis of the associations among the 77 medicinal materials used in prescriptions for dysmenorrhea was performed using TCMISS with the support and confidence scores set as >50% and >0.95, respectively. A total of 58 frequently used combinations were obtained (**Supplementary Table S3**), with 19 being two-material combinations, 22 being three-material combinations, 13 being four-material combinations, and three being five-material combinations.

Network analysis performed on the associations of these medicinal combinations revealed a total of seven core medicinal materials used in the prescriptions for dysmenorrhea (**Figure 8**). The core materials ranked in order of FC were AS, LiC, RG, PL, HP, LyC, and CCT (**Figure 8**). Two-core material combinations with the highest FC were LiC-AS (92), AS-RG (75), and LiC-RG (73); three-core material combinations with the highest FC were LiC-AS-RG (73), LiC-PL-AS (69), and PL-AS-RG (67); four-core material combinations with the highest FC were LiC-PL-AS-RG (66), LiC-LyC-AS-RG (60), and LiC-AS-RG-HP (56). The five-core material combination with the highest FC was LiC-PL-LyC-AS-RG (55), i.e., 55 of the 99 acquired prescriptions for dysmenorrhea contained these five core medicinal materials.

TABLE 1 | Traditional effects and modern pharmacological effects of the 22 medicinal materials frequently used in prescriptions for dysmenorrhea (RFC ≥ 0.1).

No	Scientific name	Abbreviation	Family	Local name	Parts used	Dosage	Traditional usage	Property and flavor	Pharmacological effects	RFC
1	*Angelica sinensis* (Oliv.) Diels	AS	Umbelliferae	Dang Gui (當歸)	Roots	5–15 g	Tonifying and replenishing medicinal (blood-tonifying medicinal)	Warm; sweet and pungent	Antianemic and menstrual-pain-relieving effects (Li et al., 2015); anti-inflammatory and analgesic effects (Nie et al., 2009); antioxidant and anti-inflammatory effects (Yang et al., 2017; Cao et al., 2014)	0.96
2	*Ligusticum chuanxiong* Hort	LiC	Umbelliferae	Chuan Qiong (川芎)	Rhizome	3–10 g	Blood-regulating medicinal (blood-activating and stasis-dispelling medicine)	Warm; pungent	Anti-inflammatory and antioxidant effects (Shi et al., 2020)	0.95
3	*Rehmannia glutinosa* Libosch	RG	Scrophulariaceae	Di Huang (地黃)	Roots	9–30 g	Heat-clearing medicinal (heat-clearing and blood-cooling medicine)	Cold; sweet and bitter	Anti-inflammatory, antioxidant, and hypoglycemic effects. Kim et al. (2017)	0.79
4	*Paeonia lactiflora* Pall	PL	Ranunculaceae	Bai Shao (白芍)	Roots	6–15 g	Tonifying and replenishing medicinal (blood tonifying medicinal)	Mild cold; bitter and sour	Anti-inflammatory, antioxidant, antithrombotic, anticonvulsant, analgesic, cardioprotective, neuroprotective, hepatoprotective, antidepressant-like, antitumor, and immunoregulatory effects (Zhou et al., 2020)	0.71
5	*Hedysarum polybotrys* Hand.-Mazz	HP	Leguminosae	Hong Qi (紅耆)	Roots	9–30 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Mild warm; sweet	Anti-gastric-ulcer effects (Yang et al., 2019)	0.68
6	*Lycium chinense* Mill	LyC	Solanaceae	Gou Qi Zi (枸杞子)	Ripe fruit	6–15 g	Tonifying and replenishing medicinal (Yin tonifying medicinal)	Neutral; sweet	Antioxidant, immunomodulatory, antitumor, neuroprotective, and hepatoprotective effects (Tian et al., 2019)	0.64
7	*Cinnamomum cassia* (L.) J.Presl	CCT	Lauraceae	Gui Zhi (桂枝)	Twig	3–10 g	Exterior-releasing medicinal (pungent-warm exterior-releasing medicinal)	Warm; pungent and sweet	Antitumor, anti-inflammatory, analgesic, antidiabetic, anti-obesity, antibacterial, antiviral, cardiovascular protective, cytoprotective, neuroprotective, immunoregulatory, and anti-tyrosinase activities Zhang et al. (2019)	0.59

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TABLE 1 | (*Continued*) Traditional effects and modern pharmacological effects of the 22 medicinal materials frequently used in prescriptions for dysmenorrhea (RFC ≥ 0.1).

No	Scientific name	Abbreviation	Family	Local name	Parts used	Dosage	Traditional usage	Property and flavor	Pharmacological effects	RFC
8	*Glycyrrhiza uralensis* Fisch	GU	Leguminosae	Gan Cao (甘草)	Roots Rhizome	2–11.5 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Neutral; sweet	Antiulcer, antimycobacterial, uterine relaxant, analgesic, antioxidant, memory-enhancing, corticosteroidal, antiallergic, hepatoprotective, anti-inflammatory, anticancer, antimalarial, antiviral, antihyperglycemic, antitussive, immunostimulatory, anti-HIV, muscle relaxant, and antimicrobial activities (Batiha et al., 2020)	0.45
9	*Codonopsis pilosula* (Franch.) Nannf.	CP	Campanulaceae	Dang Shen (黨參)	Roots	9–30 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Neutral; sweet	Immunomodulatory, antitumor, antioxidant, neuroprotective, antiviral, anti-inflammatory, anti-fatigue, hypoglycemic, anti-hypoxia, renoprotective, gastroprotective, hepatoprotective, and prebiotic effects (Luan et al., 2021)	0.4
10	*Eucommia ulmoides* Oliv	EU	Eucommiaceae	Du Zhong (杜仲)	Bark of trunk	6–15 g	Tonifying and replenishing medicinal (Yang tonifying medicinal)	Warm; sweet	Antihypertensive, antihyperglycemic, antihyperlipidemic, antioxidant, anti-osteoporosis, antitumor, immunomodulatory, and neuroprotective activities (Wang et al., 2019)	0.38
11	*Ziziphus jujuba* Mill	ZJ	Rhamnaceae	Da Zao (紅棗)	Ripe fruit	6–30 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Warm; sweet	Immunomodulatory, antioxidant, antitumor, hepatoprotective, and hypoglycemic activities, and gastrointestinal-protective effects. (Ji et al., 2017)	0.36
12	*Oroxylum indicum* (L.) Benth. ex Kurz	OI	Bignoniaceae	Mu Hu Dieh (木蝴蝶)	Seeds	1–4 g	Heat-clearing medicinal (heat-clearing and detoxicating medicinal)	Cool; bitter and sweet	Anticancer, antibacterial, hypoglycemic, cardioprotective, anti-adipogenesis, anti-inflammatory, and wound-healing effects (Nik Salleh et al., 2020)	0.31
13	*Ziziphus jujuba* Mill	ZJH	Rhamnaceae	Hei Zao (黑棗)	Ripe fruit	6–30 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Warm; sweet	Immunomodulatory, antioxidant, antitumor, hepatoprotective, and hypoglycemic activities, and gastrointestinal-protective effects. (Ji et al., 2017)	0.3

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TABLE 1 | (*Continued*) Traditional effects and modern pharmacological effects of the 22 medicinal materials frequently used in prescriptions for dysmenorrhea (RFC ≥ 0.1).

No	Scientific name	Abbreviation	Family	Local name	Parts used	Dosage	Traditional usage	Property and flavor	Pharmacological effects	RFC
14	*Cinnamomum cassia* (L.) J.Presl	CCB	Lauraceae	Rou Gui (肉桂)	Bark of trunk	1–5 g	Interior-warming medicinal	Highly hot; pungent and sweet	Antitumor, anti-inflammatory, analgesic, antidiabetic, anti-obesity, antibacterial, antiviral, cardiovascular protective, cytoprotective, neuroprotective, immunoregulatory, and anti-tyrosinase effects Zhang et al. (2019)	0.28
15	*Poria cocos* (Schwein.) F.A.Wolf	PC	Polyporaceae	Fu Ling (茯苓)	Sclerotium	9–30 g	Dampness-dispelling medicinal (dampness-draining, diuretic medicinal)	Neutral; sweet and bland	Antitumor, immunomodulatory, anti-inflammatory, antioxidant, antiaging, anti-hepatitis, antidiabetic, and anti-hemorrhagic-fever effects Li et al. (2019)	0.27
16	*Atractylodes macrocephala* Koidz	AM	Compositae	Bai Zhu (白朮)	Rhizome	6–15 g	Tonifying and replenishing medicinal (Qi tonifying medicinal)	Warm; bitter and sweet	Antitumor, neuroprotective, anti-hepatotoxicity, and anti-inflammatory effects (Ruqiao et al., 2020)	0.25
17	*Prunus persica* (L.) Batsch	PP	Rosaceae	Tao Ren (桃仁)	Ripe seed	4.5–10 g	Blood-regulating medicinal (blood-activating and stasis-dispelling medicinal)	Neutral; bitter and sweet	Anti-obesity effect (Song et al., 2019); anti-inflammatory, antinociceptive, and antipyretic effects (Elshamy et al., 2019)	0.21
18	*Zingiber officinale* Roscoe	ZO	Zingiberaceae	Gan Jiang (乾薑)	Rhizome	3–9 g	Interior-warming medicinal	Hot; pungent	Antiemetic, antibacterial, antitumor, anti-inflammatory, and antioxidant effects Li et al. (2021)	0.17
19	*Cyperus rotundus* L	CR	Cyperaceae	Xiang Fu (香附)	Rhizome	6–11.5 g	Qi-regulating medicinal	Neutral; pungent, mild bitter and mild sweet.	Analgesic, anti-allergic, anti-arthritic, anti-candida, anticariogenic, anticonvulsant, antidiarrheal, antiemetic, antihelminthic, antihistamine, antihyperglycemic, antihypertensive, anti-inflammatory, antimalarial, anti-obesity, antioxidant, antiplatelet, antipyretic, anti-ulcer, antiviral, cardioprotective, cytoprotective, cytotoxic, gastroprotective, hepatoprotective, neuroprotective, ovicidal,	0.15

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TABLE 1 | (*Continued*) Traditional effects and modern pharmacological effects of the 22 medicinal materials frequently used in prescriptions for dysmenorrhea (RFC ≥ 0.1).

No	Scientific name	Abbreviation	Family	Local name	Parts used	Dosage	Traditional usage	Property and flavor	Pharmacological effects	RFC
									larvicidal, and wound healing effects as well as inhibitory effect on Na^+ K^+ ATPase activities in the brain (Kamala et al., 2018)	
20	*Leonurus japonicus* Houtt	LJ	Labiatae	Yi Mu Cao (益母草)	Aerial part	9–30 g	Blood-regulating medicinal (blood-activating and stasis-dispelling medicinal)	Mild cold; bitter and pungent	Antioxidant, anti-inflammatory, and antiapoptotic effects (Huang et al., 2021)	0.14
21	*Carthamus tinctorius* L	CaT	Compositae	Hong Hua (紅花)	Tubular flower	3–10 g	Blood-regulating medicinal (blood-activating and stasis-dispelling medicinal)	Warm; pungent	Cardioprotective, neuroprotective, anticancer, and anticoagulant effects (Orgah et al., 2020)	0.1
22	*Corydalis yanhusuo* W.T. Wang	CY	Papaveraceae	Yan Hu Su (延胡索)	Tuber	3–12 g	Blood-regulating medicinal (blood-activating and stasis-dispelling medicinal)	Warm; pungent and bitter	Antianxiety, hypnosis-inducing effect, analgesic, anti-arrhythmic, anti-ulcer, and anti-myocardial-ischemia effects (Tian et al., 2020)	0.1

Results of association rule analysis showed the presence of 107 association rules (**Supplementary Table S4**). Two-material associations with a confidence value of 1 included CCT → LiC, LyC → LiC, PL→AS, and RG → AS. In other words, when support >50%, the probability that the medicinal material on the right side of the arrow appeared in the same prescription as the medicinal material on the left side of the arrow was 100%.

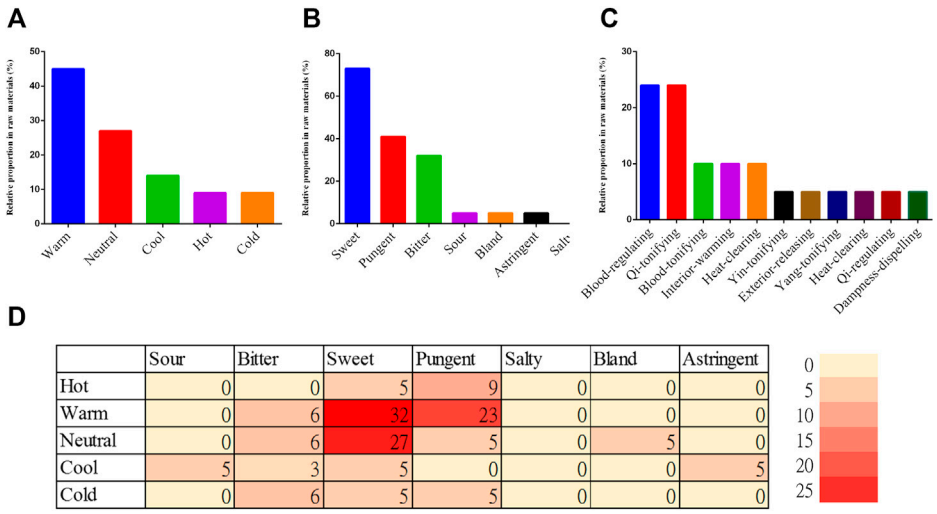
4 DISCUSSION

4.1 Ethnopharmacological Investigation Sites

The remaining TCM pharmacies in Taiwan are an invaluable asset to ethnopharmacology because a considerable amount of knowledge regarding Chinese herbal medicines, TCM formulations, and medicinal material processing techniques is contained exclusively within these age-old pharmacies. However, such pharmacies have gradually become a sunset industry with the gradual westernization of the Taiwanese society (Kuo, 2021). This has resulted in an urgent need for the systemic retention and documentation of valuable ethnopharmacological knowledge (Chao et al., 2021). Thus, in this study, we surveyed and collated prescriptions for dysmenorrhea treatment from TCM pharmacies across Taiwan.

4.2 Taxonomic Characteristics of Medicinal Materials Used in Prescriptions for Dysmenorrhea

Members of the Asteraceae (Compositae) family accounted for the greatest proportion of the medicinal materials used for dysmenorrhea. This finding echoes a previous study that concluded that plants belonging to the Compositae family constitute the major source of TCM herbs in Taiwan (Huang et al., 2020). The properties and the flavors of Compositae are often cool and bitter, and there are not many medicinal materials of Compositae family are warm (Wei, 2014). However, the present investigation found that medicinal materials of Compositae used in dysmenorrhea are mostly warm, such as AM, *Carthamus tinctorius* L. (Hong Hua), *Artemisia argyi* H. Lév. and Vaniot (Ai Ye), etc. Compositae plants used in dysmenorrhea are very different from those used in other diseases. The family Leguminosae is also a main source of medicinal materials



D

	Sour	Bitter	Sweet	Pungent	Salty	Bland	Astringent
Hot	0	0	5	9	0	0	0
Warm	0	6	32	23	0	0	0
Neutral	0	6	27	5	0	5	0
Cool	5	3	5	0	0	0	5
Cold	0	6	5	5	0	0	0

0 5 10 15 20 25

FIGURE 5 | TCM property, flavor, and traditional effects of medicinal materials used in prescriptions for dysmenorrhea (**A**) TCM property; (**B**) TCM flavor; (**C**) Traditional effects; (**D**) Analysis of TCM flavor-property combinations; each number represents the proportion (%) of medicinal materials with the flavor-property combination among the 22 frequently used medicinal materials.

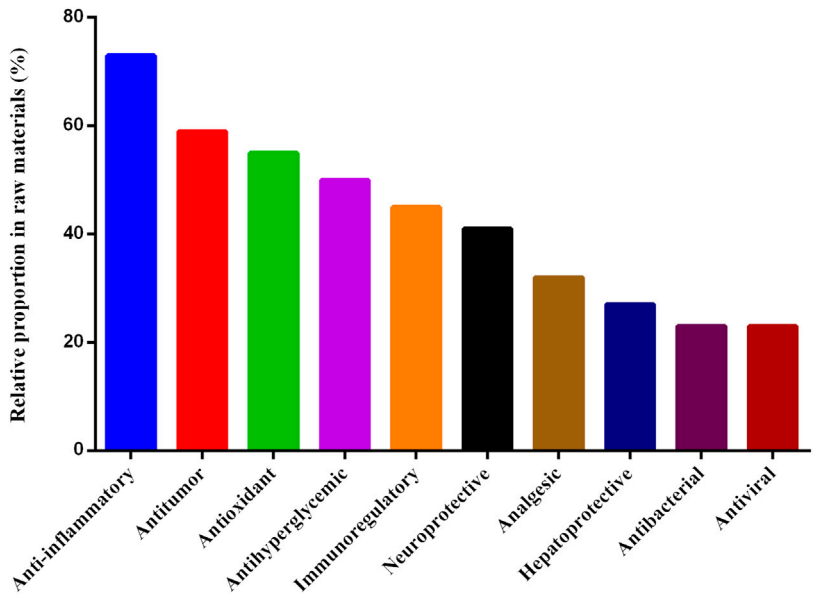


FIGURE 6 | Modern pharmacological effects of the frequently used medicinal materials.

frequently used in prescriptions for dysmenorrhea, which include *Astragalus membranaceus* (Fisch.) Bge (Huang Qi), HP, and GU. Leguminosae plants have also been used in the treatment of infertility and menopausal complaints in Cameroon (Ateba et al., 2013) and as galactagogues in Taiwan (Chao et al., 2021). Other plant families frequently used as medicinal materials in prescriptions for dysmenorrhea include Araliaceae (e.g., *Panax ginseng* and *Panax quinquefolius*), Lauraceae (e.g., *Cinnamomum cassia* (L.) J. Presl (Rou Gui, abbreviated as CCB) and CCT), and Umbelliferae (e.g., AS, LiC, and *Radix Bupleuri* (Chai Hu)).

4.3 Traditional Effects and Modern Pharmacological Effects of Medicinal Materials Frequently Used in Prescriptions for Dysmenorrhea

4.3.1 TCM Properties, Flavors, and Traditional Effects of Prescriptions for Dysmenorrhea

Traditional Chinese medicine has a unique theoretical framework, including four characteristics, five flavors and so on. Cold, hot, warm, cool and neutral represent different

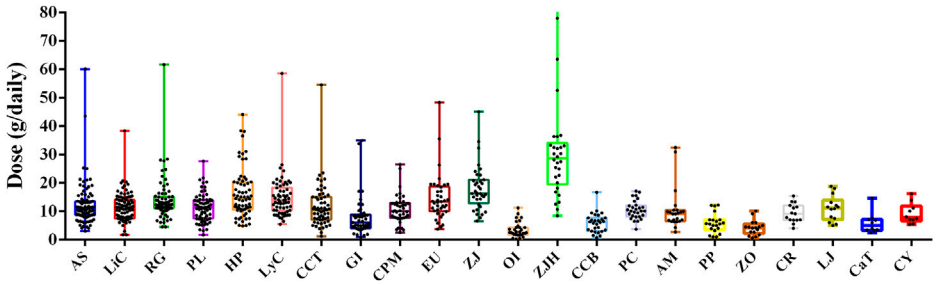
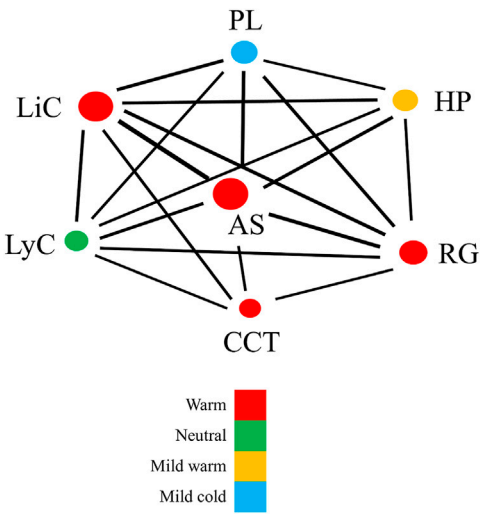


FIGURE 7 | Box plot of dose ranges of frequently used medicinal materials (top line denotes the maximum value, and bottom line denotes the minimum value; the top edge of each box represents the third quartile (Q3), middle line represents the second quartile (Q2), and bottom edge represents the first quartile (Q1). Black dots represent the doses in the collected samples.; AS, *Angelica sinensis* (Oliv.) Diels; AM, *Atractylodes macrocephala* Koidz.; CCT, *Cinnamomum cassia* (L.) J. Presl; CPM, *Codonopsis pilosula* (Franch.) Nannf. var. *modesta* (Nannf.) L.T.Shen; CCB, *Cinnamomum cassia* (L.) J. Presl; CR, *Cyperus rotundus* L.; CaT, *Carthamus tinctorius* L.; CY, *Corydalis yanhusuo* W.T.Wang.; DL, *Diospyros lotus*; EU, *Eucommia ulmoides* Oliv.; GI, *Glycyrrhiza inflata* Batalin; HP, *Hedysarum polybotrys* Hand.-Mazz.; LiC, *Ligusticum chuanxiong* Hort; LyC, *Lycium chinense* Mill.; LJ, *Leonurus japonicus* Houtt.; OI, *Oroxylum indicum* (L.) Benth. ex Kurz; PL, *Paeonia lactiflora* Pall.; PC, *Poria cocos* (Schwein.) F.A.Wolf; PP, *Prunus persica* (L.) Batsch; RG, *Rehmannia glutinosa* Libosch.; ZJ, *Ziziphus jujuba* Mill.; ZO, *Zingiber officinale* Roscoe.



	Couplet medicine	Times
1	LiC - AS	92
2	AS - RG	75
3	LiC - RG	73
4	PL - AS	70
5	LiC - PL	69
6	PL - RG	67
7	AS - HP	66
8	LiC - HP	65
9	LiC - LyC	63
10	LyC - AS	62
11	LyC - RG	60
12	LiC - CCT	58
13	CCT-AS	57
14	RG - HP	57
15	LyC -HP	56
16	PL - LyC	55
17	PL - HP	52
18	LyC - CCT	50
19	CCT - RG	50

FIGURE 8 | Network diagram of core medicinal materials used in prescriptions for dysmenorrhea. Different colors represent different TCM properties. Circle sizes are proportional to the respective FC values, and line thicknesses are proportional to the respective frequencies of two-material combinations. AS, *Angelica sinensis* (Oliv.) Diels; CCT, *Cinnamomum cassia* (L.) J. Presl; HP, *Hedysarum polybotrys* Hand.-Mazz.; LiC, *Ligusticum chuanxiong* Hort; LyC, *Lycium chinense* Mill.; PL, *Paeonia lactiflora* Pall.; RG, *Rehmannia glutinosa* Libosch.

medicinal properties (Guan et al., 2009). The ^{1}H-NMR spectrum results show that there are obvious differences in the chemical composition of Chinese medicines with different medicinal properties (Zhang et al., 2020). Moreover, "cold" nature-related drugs have more fatty rings, while "hot" nature-related drugs have lower average molecular weight and more aromatic ring systems. "Neutral" nature-related drugs have more cyclohexene (Fu et al., 2017).

Among the various flavor-property combinations, sweet-warm, sweet-neutral, and pungent-warm were the most common in the prescriptions for dysmenorrhea. In TCM theory, sweet medicinal materials are regarded as having tonifying and replenishing effects and being capable of relaxing tension and relieving pain; warm and hot materials are used for the treatment of cold-type diseases, such as aversion to cold; pungent materials can promote qi and blood flow, and are used to treat symptoms of poor blood circulation (Wei, 2019). Warm and hot materials are also related to enhance fertility, sexual function, and endocrine, nutrition and metabolic state (Fu et al., 2017).

The most prevalent traditional effects of the frequently used medicinal materials were the blood-activating and statis-dispelling effect as well as the qi-tonifying effect, which were each reported in five materials. This was followed by the blood-tonifying effect, interior-warming effect, and heat-clearing and blood-cooling effect (9%

each) (**Table 1**). The most common TCM syndrome type of primary dysmenorrhea is Qi stagnation with blood stasis syndrome, indicating the interruption of blood flow would cause menstrual pain (Park et al., 2012; Kim et al., 2017). The high prevalence of the blood-activating, blood-tonifying, qi-tonifying, and interior-warming effects among the frequently used medicinal materials is consistent with the principles of dysmenorrhea treatment in TCM (Xin, 2021).

4.3.2 Modern Pharmacology and Dysmenorrhea

Among the seven core medicinal materials that are frequently used for dysmenorrhea (**Figure 8**), AS showed the highest RFC among the prescriptions. It is also an edible traditional medicinal plant in China, Japan, and South Korea. The organic acids contained in AS has been shown to reduce inflammation by enhancing the autophagy flux of damaged endothelial cells (Li et al., 2020). In clinical practice, AS is used for blood replenishment and the treatment of irregular menstruation and dysmenorrhea (Wei et al., 2016). Ferulic acid, a component of AS, reduces the secretion of expression of interleukin-8 and vascular endothelial growth factor in endometriotic tissues, which explains the therapeutic effect of dysmenorrhea (Takeuchi et al., 2020). LiC has been widely used in the treatment of thrombotic diseases and can reduce the levels of pro-inflammatory cytokines (IL-1β, TNF-α) (Wang et al., 2020). Research has shown that the main active constituents of PL are monoterpene glucosides, which possess antioxidant and anti-inflammatory effects (Li et al., 2021). In particular, paeoniflorin, which is one of the monoterpene glucosides and a major active compound of PL, improves endometrial receptivity by inducing the expression of leukemia inhibitory factors, thereby enhancing the embryo implantation rate (Park et al., 2021). PL may also provide beneficial effects towards ovarian function and oocyte quality, possibly by stimulating ovarian angiogenesis and follicular development (Park et al., 2020). Consequently, PL has been used as an important herbal remedy for the treatment of dysmenorrhea in many ethnomedical medical systems around the world (Li et al., 2021). The main constituents of CCT are terpenoids, phenylpropanoids, and glycosides, and modern studies have confirmed that CCT possesses a wide range of pharmacological effects, including anti-inflammatory and analgesic effects (Zhang et al., 2019). *Trans*-cinnamaldehyde, a bioactive component found in CCT, exhibited good anti-inflammatory effects in a lipopolysaccharide-induced zebrafish inflammation model and rat experiments (Lee and Lim, 2021; Park et al., 2021). *Lycium barbarum* polysaccharides, which are the active component of LyC, exerted protective effects against ovarian injury in rats by reducing oxidative stress and activating the Nrf2/ARE-signaling pathway (Yang et al., 2017). Therefore, the treatment of dysmenorrhea by the aforementioned medicinal materials may be related to their anti-inflammatory, antioxidant, and analgesic effects. Further research will be required for the validation of this conjecture.

4.4 Combinations, Doses, and Processing of Medicinal Materials Used in Prescriptions for Dysmenorrhea

The seven core medicinal materials result from network analysis includes warm (AS, LiC, RH, CCT, and HP), neutral (LyC), and cool herbs (PL). The four materials with highest RFC, including LiC, AS, RG, and PL, is exactly a traditional formula, the Si Wu (four-substance) Decoction (Dan 2020). Association analysis showed that the four materials are the most frequently combined materials in the prescriptions. The daily doses of the four medicinal materials in the prescriptions for dysmenorrhea were almost the same, i.e. 11 g per day. This gives a dose ratio of close to 1:1:1:1, which is almost identical to the dose ratio used in the formula for the Si Wu decoction in the original use (Lai et al., 2020). In a study by Li et al., a network of "compound-target-pathway-disease" of the Si Wu Decoction was constructed, and network analysis showed that 16 components, 16 target proteins, and 24 pathways of the decoction were related to primary dysmenorrhea (Li et al., 2019). The four medicinal materials may play a role in treating dysmenorrhea by acting on protein targets and pathways related to hormone regulation, analgesia, spasmolysis, inflammation, and immunity (Li et al., 2019). In the present study, the fifth-most frequently used core medicinal material used in the prescriptions for dysmenorrhea was HP, which has long been used as an alternative to *Astragalus membranaceus* (Fisch.) Bge (Huang Qi) in Taiwan (Chao et al., 2020). LyC and CCT, with respective RFC values of 0.64 and 0.59, were also frequently used in prescriptions for dysmenorrhea.

The purpose of medicinal materials processing is to enhance the therapeutic efficacy and reduce the toxicity of original medicinal materials, by using vinegar, wine, honey, brine and other auxiliary materials. Scientific reports show that processing has a synergistic effect on the chemistry, pharmacology and pharmacokinetics with the active ingredients of medicinal materials (Chen et al., 2018). Processed medicinal materials in the prescriptions for dysmenorrhea included steamed RG, stir-baked PL, stir-baked EU, honey-roasted GU, and soil stir-baked AM. Steaming increases the anti-inflammatory and hematopoietic effects of RG, which significantly improves hematopoiesis in the body after consumption (Wang et al., 2018). The extracts of stir-baked PL promote the synthesis and release of IL-4 and IL-10 and inhibit the expression of IL-1β, TNF-α, and high mobility group box 1 protein (HMGB1), thereby providing anti-inflammatory and analgesic effects (Xian-wen, 2020). It has been found that the alcohol extract of stir-baked EU has a significantly higher EU content than the alcohol extract of raw EU, which enhances its inhibitory effects on voluntary uterine contractions and antagonistic effects on acetylcholine-induced spasmodic uterine contractions, thus alleviating spasmodic contractions of the uterus (He et al., 2021). Honey-roasted GU improves blood circulation, boosts immunity, and enhances the palatability of medications (Xu YL. et al., 2020). Stir-baking increases the content of

polysaccharides in AM, which form the material basis for the spleen-fortifying and diarrhea-relieving effects of AM and enhance nutrient absorption by the digestive system (Haoyu, 2019).

Among the medicinal materials in the prescriptions for dysmenorrhea, ZJH (Hei Zao, abbreviated as ZJH) and ZJ (Da Zao, abbreviated as ZJ) showed the highest daily doses. There were also considerable differences in the dose level of ZJH and ZJ across different TCM pharmacies, with the adopted dose ranging from 8 to 94 g for ZJH and 6–45 for ZJ. This may be attributed to the fact that both ZJH and ZJ are sweet-tasting, fruit-derived medicinal materials with a lack of toxicity and strict dose limits. Therefore, the dose levels of these materials are largely determined by the preferences of the various TCM pharmacies. Our results also showed that the dose level of HP (15.12 g/day) in the prescriptions for dysmenorrhea was higher than that of the other medicinal materials, but still within the reasonable dose range of 9–30 g as stated in TCM-related pharmacopeias.

5 LIMITATIONS AND FUTURE WORKS

The study was a field investigation that only collected prescriptions currently sold by TCM pharmacies. The first limitation was that the therapeutic efficacy for these prescriptions was not surveyed. The study only identified the core medicinal materials used for dysmenorrhea, with their doses and processing methods of common usage. The study did not evaluate the activity and efficacy for these medicinal materials. The second limitation was that the network diagram only shows the prescription relationship between the medicinal materials, but not the pharmacological interaction between them. In future works, we will clarify the therapeutic effects of prescriptions for dysmenorrhea by both interviewing the customers and performing clinical trials. The core medicinal materials can be combined to form a new formula based on the data generated by the study. This new formula could become a new product, but its efficacy and safety need to be tested.

6 CONCLUSION

In the present study, an ethnopharmacological survey of prescriptions for dysmenorrhea from TCM pharmacies across Taiwan was performed for the first time. Our results will be beneficial towards the preservation of important knowledge regarding prescriptions for dysmenorrhea in Taiwan. Although the modern pharmacological effects and the processing methods of the component materials have been collated and documented in this study, further in-depth research remains necessary. The results of this study may also serve as reference for the development of naturally derived medications for the treatment of dysmenorrhea. Given that TCM pharmacies may completely disappear in the near future, it is imperative to hasten our efforts in documenting traditional medical knowledge and adopting the necessary measures to preserve the techniques and knowledge passed down in these pharmacies.

7 CONTRIBUTIONS OF THIS STUDY

TCM pharmacies are among the most iconic traditional medical settings of Taiwan. Treatment experiences passed down from one generation to another in these pharmacies have contributed to a wealth of prescriptions for dysmenorrhea, which have achieved significant therapeutic effects in countless Taiwanese women. The present study is the first to report an ethnopharmacological survey of prescriptions for dysmenorrhea of TCM pharmacies in Taiwan, and our results can contribute to the documentation, analysis, and retention of medical knowledge related to dysmenorrhea. In addition to collating data on the frequently used medicinal material combinations, TCM property and flavor, and traditional effects of the materials, we also performed a literature search of relevant pharmacological studies to determine the modern pharmacological effects of these materials related to the relief of dysmenorrhea. The doses of the frequently used medicinal materials have also been recorded in this study, which may serve as a reference for the clinical use of these materials by TCM practitioners. Moreover, the frequently used core medicinal material pairs in prescriptions for dysmenorrhea were determined, and a network analysis was performed to provide a network of core medicinal materials used in TCM pharmacy prescriptions for dysmenorrhea. Therefore, the present study can contribute to the documentation and passing down of traditional medical knowledge related to TCM pharmacy prescriptions for dysmenorrhea.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CRREC-109-125. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

K-HS, S-YS, Y-CC, S-SH, and JC designed the study. K-HS, S-YS, C-YK, S-SH, and JC conducted the field work. K-HS, S-YS, C-YK,Y-CC, S-SH, and JC performed the data analysis. K-HS, S-YS, Y-CC, S-SH, and JC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.746777/full#supplementary-material

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Use of Herbal Medicines Among Breastfeeding Mothers in Tanzania: A Cross-Sectional Study

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Background: There are limited data on the use of herbal medicines (HM) among breastfeeding mothers, despite the fact that knowledge of the possible benefits or harms of HM use has a direct relationship with the health of infants, especially in resource-limited countries. The study aims to determine the prevalence and factors associated with HM use among breastfeeding mothers in Tanzania.

Methods: The study followed a cross-sectional design using a structured survey questionnaire. Survey participants were recruited from the reproductive and child health clinic at Uhuru health center in Morogoro, Tanzania. The survey instrument comprised of 34 questions, including demographic information, the pattern of HM use during breastfeeding, and women's perceptions of HM. Chi-square test and logistic regression were used for data analysis using SPSS ver. 24.0.

Results: The majority of the respondents (53.8%) used HM during breastfeeding. The most commonly used HM was black pepper (*Piper nigrum* L.; 80.0%), followed by pumpkin seeds (*Cucurbita pepo* L.; 18.0%). About one-third (27.5%) of HM users discussed their use with their healthcare providers. In addition, higher education levels and low breastmilk supply were identified as potential predictors of HM use.

Conclusion: The practice of HM use among breastfeeding mothers in Tanzania is popular to ease breastfeeding difficulties. However, the issue of the safety or effectiveness of HM is still an unknown agenda. This awakens the need to evaluate HM's safety, efficacy, and quality through pharmacological studies for scientific evidence. Lastly, a clinical guideline should be developed in healthcare settings to promote open dialogues between the healthcare providers and mothers to ensure the safe use of HM.

Keywords: herbal medicine, breastfeeding, health behavior, tanzania, cross-sectional study

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Abbreviations: HM, herbal medicine; WHO, world health organization; EBF, exclusive breastfeeding.

INTRODUCTION

Globally, there is an increasing number of studies on women's lifelong use of herbal medicines (HM). Several studies have documented women's HM use in various life stages, starting from menstruation, pregnancy, childbirth, postpartum, and menopause (Ngoma and Siachapa, 2017; Wang J. et al., 2018; Moini Jazani et al., 2018; Zheng et al., 2019; Adane et al., 2020; Sumankuuro et al., 2020). Especially during some of these life stages, such as pregnancy, delivery, and the postpartum period, women's HM use should be paid close attention to, as seemingly naive HM that has no negative impact on non-pregnant women can have a detrimental effect on the child and pregnant woman (Budzynska et al., 2012; Ahmed et al., 2017).

According to the World Health Organization, exclusive breastfeeding (EBF) is an essential practice during the postpartum period for maternal and child health (WHO, 2015), as it is one of the most effective ways to promote both mothers' and newborns' physiological and psychological health (Ebina and Kashiwakura, 2012; Antonakou et al., 2013; Al Sabati and Mousa, 2019; Mikšić et al., 2020). Breastmilk is more natural and cost-effective than bottle feeding and ensures ideal nutrition for newborns, thus promoting infant health, providing essential bioactive nutrients, and lowering the risks for various infections and hospitalizations (Oguchi et al., 1997; Butte et al., 2002; Hauck et al., 2011; Ajetunmobi et al., 2015). In addition, breastfeeding facilitates mother and child bonding by promoting physical intimacy between the mother and child (Johnson, 2013; Liu et al., 2013). However, up to 92% of nursing women encounter various difficulties during their breastfeeding journeys (Wagner et al., 2013). Some mothers seek an alternative mode of care, such as HM, to alleviate the problems due to the lack of access to conventional healthcare services and the belief that HM is more natural and safe to use than conventional medicine (Augustino and Gillah, 2005; Sim et al., 2013; Aleandri et al., 2014; Zheng et al., 2020). Some of the commonly used HM among breastfeeding mothers include fenugreek (*Trigonella foenum-graecum* L.), ginger (*Zingiber officinale* Roscoe), fennel (*Foeniculum vulgare* Mill.), shatavari (*Asparagus racemosus* Willd.), and aniseed (*Pimpinella anisum* L.). Moreover, various phytochemical compounds (e.g., glycosides, alkaloids, flavonoids, and minerals) found in medicinal plants modulate hormone levels and act as lactogenic agents (Bnouham, 2010; Dandotiya et al., 2013; Sim et al., 2013).

Nevertheless, there is much debate about whether the existing evidence related to breastfeeding mother's HM use supports their safety and efficacy (Sim et al., 2013; Bettiol et al., 2018a) as previous studies show inconclusive evidence explaining the mechanism behind herbs as galactagogues, requiring further research to determine HM's clinical efficacy (Anderson, 2017; Bowman, 2017; Wang S. et al., 2018; Sibeko et al., 2021). Furthermore, inappropriate use of certain HM during breastfeeding was found to cause allergic reactions and neurotoxic effects on newborns (Bnouham, 2010). Yet, irrespective of the lack of scientific evidence and potential harm to newborns, as large as 97% of breastfeeding women use HM to overcome challenges with breastfeeding, and concerns are rising about the level of knowledge among healthcare providers on breastfeeding women's HM use (Sim et al., 2013; Bettiol et al., 2018b; James et al., 2019; Zheng et al., 2020).

Thus, several studies have examined breastfeeding mothers' HM use in various cultural settings (Bnouham, 2010; Sim et al., 2013; Aleandri et al., 2014; Bettiol et al., 2018b; James et al., 2019; Orabi et al., 2020; Zheng et al., 2020). However, limited data is available regarding the HM use in East African countries like Tanzania even though HM use is widely practiced to treat various physical conditions such as women's complications as well as infectious and chronic diseases (Augustino and Gillah, 2005; Moshi et al., 2009; Amri and Kisangau, 2012; Kacholi and Amir, 2022). Consequently, breastfeeding mothers' self-care practices must be examined to establish appropriate policy measures and clinical protocols to ensure the health of the mother and the child. Therefore, this study aims to examine the pattern and factors associated with HM use among breastfeeding mothers in Tanzania. Furthermore, this study emphasizes the importance of informing policymakers for developing a clinical guideline that could improve the self-care behaviors of women during breastfeeding.

MATERIALS AND METHODS

Study Design

A descriptive cross-sectional study was conducted to identify HM use among breastfeeding mothers in Morogoro, Tanzania.

Study Setting and Participants

The eligible participants included all breastfeeding women but are not limited to exclusive breastfeeding women between the ages of 18–49 years old with a child below 2 years who visited the reproductive and child health clinic at Uhuru health center in Morogoro, Tanzania. On average, approximately 160 pregnant women visit the Uhuru health center per month, and about 20% of those women visit the center for the reproductive and child health services. Exclusion criteria were those women attending the clinic for services apart from reproductive and child healthcare services and those who did not consent to participate in the survey.

Study Size

The sample size determination formula, based on the confidence interval (CI) was used to calculate the required sample size: $n = z^2_{\alpha/2} \cdot \frac{pq}{d^2}$. In this equation, n is the required sample size, $Z_{\alpha/2}$ is 1.96 with a confidence interval of 95%, d is the margin of error set on 0.05, p is the expected proportion based on an average prevalence of HM use among breastfeeding mothers (72.3%) (Sim et al., 2013; Aleandri et al., 2014), and q is the proportion of women that do not use HM during breastfeeding (1-p). The required sample size for adequate power was 308, and a total of 400 survey questionnaires were distributed assuming a 30% non-response rate.

Data Collection

A face-to-face interview using a structured questionnaire was conducted from August 2017 to November 2017. The survey was conducted by three trained surveyors from Tanzania. All

participants were informed about the confidentiality of the study and were given verbal information and instructions regarding the research. Participants who completed the IRB-approved consent form were able to participate in the study. A total of 400 questionnaires were distributed, 28 questionnaires were incomplete (response rate: 93.0%); therefore, the data of 372 respondents were included in the analysis.

Survey Instrument

The questionnaire was first developed in English based on previous studies investigating HM use among breastfeeding women (Sim et al., 2013). In order to measure the content and face validity of the survey instrument, the questionnaire was reviewed by three experts (two researchers who were maternal and child health experts in Korea and one researcher from Uhuru health center, Tanzania). The questionnaire was then translated into Swahili, which was translated back into English by a third party to confirm its accuracy. The questionnaire was pilot-tested on a sample of 20 participants to evaluate the clarity and reliability of the questions.

The final version of the questionnaire consisted of three sections with 34 items, including multiple-choice and open-ended questions. The first section included eight questions on the health-related characteristics of the breastfeeding mothers, such as general health status (1 = very good, 5 = very poor), number of children, age of the youngest child, duration of breastfeeding of the youngest child, uptake of counseling on breastfeeding, and problems/difficulties experienced during breastfeeding. The second section included 18 questions regarding HM use (i.e., types and frequency of HM used, age of the child when HM was first used, indications and reasons for HM use, source of information and recommendation of HM, the experience of side effects, perceived safety and efficacy of HM, intention to recommend HM to other women, and disclosure and non-disclosure of HM use to health physicians and its reasons). Lastly, the final section consisted of eight questions on the participants' sociodemographic characteristics such as age, level of education, occupation, number of family members in the household, household income, health insurance status, and marital status.

Statistical Analysis

In this study, the collected data of 372 participants were analyzed using Statistical Package for Social Sciences (SPSS) version 24.0. The descriptive statistics were used to examine the sociodemographic characteristics of the participants (i.e., age, education level, family size, occupation, marital status, number of assets, health insurance status, perceived health status, number of children, exclusive breastfeeding, and the types of breastfeeding problems). A Chi-square test was used to analyze the differences in sociodemographic characteristics between the users of HM during breastfeeding and the non-users. Only the statistically significant variables (i.e., education level, number of children, and low breastmilk supply) from previous chi-square analysis were analyzed using multivariate logistic regression analysis to identify the potential predictors of HM use among breastfeeding mothers.

Ethical Clearance

The study was approved by the Institutional Review Board on Human Subjects Research and Ethics Committees at Hanyang University Seoul, Korea (HYI-17-134-2). Formal permission was also obtained from Morogoro Municipal Council in Tanzania to carry out the study (E10/MMC-138/VOLIV/100).

RESULTS

Sociodemographic Characteristics of Study Participants

The details of the sociodemographic characteristics of respondents are shown in **Table 1** The mean age of breastfeeding mothers was 27.4 ± 5.3 years. Most participants were secondary school graduates (31.7%), or had a certificate/diploma and above (19.9%), were married (50.3%), and did not have health insurance (75.8%). Mothers had one (41.4%) or two (30.4%) children, and 54.8% of respondents exclusively breastfed their children. Among the respondents, 23.7% reported having problems with breastfeeding, and low breastmilk supply (14.8%) was the most commonly reported problem. More than half (53.8%) of the respondents have used at least one of the HM modalities listed during breastfeeding. Significant differences found between HM users and non-users were education level ($p = 0.001$), number of children ($p = 0.008$), having breastfeeding problem ($p < 0.001$), and low breastmilk supply ($p < 0.001$).

Types of HM Modalities for Breastfeeding

As presented in **Table 2**, the most commonly used HM modality was *Piper nigrum* L. (80.0%), followed by *Cucurbita pepo* L. (18.0%) and *Arachis hypogaea* L. (12.0%).

Source of Information on HM

The primary information sources of HM use were family members and friends (67.5%) and other breastfeeding mothers (31.5%), 26.5% from healthcare professionals, and 1.0% from herbalists (**Table 3**).

Patterns of HM Use Among Participants

The experience and pattern of HM use during breastfeeding are presented in **Table 3**. The majority of HM users (89.5%) used the herbal products daily, and most women received recommendations on HM use from their family members and friends (70.0%). Among the HM users, 9.0% reported to have experienced side effects such as nausea, abdominal pain, and diarrhea. The most frequently reported reason for using HM during breastfeeding was the belief in improving low breastmilk supply (92.0%). A smaller proportion of HM users believed that HM does not cause side effects nor contains chemicals (10.0%). Among 200 participants using HM, 145 (72.5%) did not disclose their use to physicians, and the most common reasons for non-disclose were lack of physician inquiry (64.8%) and women's fear of the doctor's response (32.4%).

Potential Predictors of HM Use During Breastfeeding

The findings from multivariate logistic regression analysis are presented in **Table 4**. An education level of certificate/diploma

TABLE 1 | Socio-demographic characteristics of participants (N =372).

Variables		Total	HM users	Non-users	*P*-value
		N=372 (%)	N=200 (%)	N=172 (%)	
Age (Mean± SD)		27.40 ± 5.33	27.30 ± 5.32	27.51 ± 5.35	—
≤ 24		119 (32.0)	67 (33.5)	52 (30.2)	0.707
25 ~ 29		139 (37.4)	75 (37.5)	64 (37.2)	—
≥ 30		114 (30.6)	58 (29.0)	56 (32.6)	—
Education level		—	—	—	0.001
Primary education level and below		29 (7.8)	9 (4.5)	20 (11.6)	—
Primary education level		151 (40.6)	69 (34.5)	82 (47.7)	—
Secondary education level		118 (31.7)	74 (25.6)	44 (25.6)	—
Certificate/Diploma and above		74 (19.9)	48 (24.0)	26 (15.1)	—
Family Size		—	—	—	0.793
≤ 3		120 (32.3)	64 (32.0)	56 (32.6)	—
4 ~ 5		175 (47.0)	92 (46.0)	83 (48.3)	—
≥ 6		77 (20.7)	44 (22.0)	33 (19.2)	—
Occupation		—	—	—	0.650
Employed		91 (24.5)	54 (27.0)	37 (21.5)	—
Street-vendor		113 (30.4)	59 (29.5)	54 (31.4)	—
Self-employed		75 (20.2)	40 (20.0)	35 (20.3)	—
Housewife		93 (25.0)	47 (23.5)	46 (26.7)	—
Marital status		—	—	—	0.863
Without spouse		63 (16.9)	32 (16.0)	31 (18.0)	—
Married		187 (50.3)	101 (50.5)	86 (50.0)	—
Cohabiting		122 (32.8)	67 (33.5)	55 (32.0)	—
Number of assets [†]		—	—	—	0.171
≤ 3		139 (37.4)	69 (34.5)	70 (40.7)	—
4—6		141 (37.9)	74 (37.0)	67 (39.0)	—
≥ 7		92 (24.7)	57 (28.5)	35 (20.3)	—
Health insurance		—	—	—	0.882
No		282 (75.8)	151(75.5)	131(76.2)	—
Health insurance		90 (24.2)	49(24.5)	41(23.8)	—
Perceived health status		—	—	—	0.750
Excellent		101 (27.2)	53 (26.5)	48 (27.9)	—
Very good		240 (64.5)	132 (66.0)	108 (62.8)	—
Fair		31 (8.3)	15 (7.5)	16 (9.3)	—
Number of Children		—	—	—	0.008
1		154 (41.4)	96 (48.0)	58 (33.7)	—
2		113 (30.4)	49 (24.5)	64 (37.2)	—
3		105 (28.8)	55 (27.5)	50 (29.1)	—
Exclusive Breastfeeding		—	—	—	0.782
Yes		204 (54.8)	111 (55.5)	93 (54.1)	—
No		168 (45.2)	89 (44.5)	79 (45.9)	—
Breastfeeding problem		—	—	—	< 0.001
No		284 (76.3)	131 (65.5)	153 (89.0)	—
Yes		88 (23.7)	69 (34.5)	19 (11.9)	—
Types of breastfeeding problems	—	—	—	—	—
Low breastmilk supply	Yes	55 (14.8)	50 (25.0)	5 (2.9)	< 0.001
	No	317 (85.2)	150 (75.0)	167 (97.1)	—
Swollen breasts, sores on breasts, Cracked nipples, mastitis	Yes	30 (8.1)	21 (10.5)	9 (5.2)	0.063
	No	342 (91.9)	179 (89.5)	163 (94.8)	—
Breast engorgements, pain	Yes	6 (1.6)	4 (2.0)	2 (1.2)	0.523
	No	336 (98.4)	196 (98.0)	170 (98.8)	—

Note: HM, Herbal medicine.

[a]*Type of assets were based on the Tanzania Bureau Statistics Survey 2012–2014 (Car, Bajaj, Motorcycle, Bicycle, Cart, Fridge, Electric or gas cooker, Television, Electric/charcoal iron, Cell phone, Radio, Plough, Charcoal / kerosene cooker, Livestock, Power tiller, House, Farm/Land).*

[b]*Columns do not add up to 100% due to the selection of multiple answers.*

and above (OR: 7.447, CI: 2.492–22.252, $p < 0.001$), secondary education level (OR: 5.524, CI: 1.943–15.711, $p = 0.001$), primary education level (OR: 3.348, CI: 1.197–9.361, $p = 0.021$), and having low breast milk supply (OR: 13.758, CI: 5.022–37.689, $p < 0.001$) were associated with HM use during breastfeeding.

DISCUSSION

This study explored the prevalence and determinants of HM use among breastfeeding mothers in Tanzania, and our results revealed the high popularity of HM use among nursing mothers. As previous studies found, globally, 37.0–97.3% of

TABLE 2 | Types of HM used by breastfeeding mothers (N = 200).

Common name(s)[a]	Binomial name(s)	N (%)
Black pepper	*Piper nigrum* L	160 (80.0)
Pumpkin seeds	*Cucurbita pepo* L	36 (18.0)
Raw groundnuts	*Arachis hypogaea* L	23 (12.0)
Ginger	*Zingiber officinale* Roscoe	19 (10.0)
Lemon	*Citrus limon* (L.) Osbeck	12 (6.0)
Cinnamon	*Cinnamomum verum* J. Presl	12 (6.0)
Garlic	*Allium sativum* L	10 (5.0)
Raw cassava roots	*Manihot esculenta* Crantz	7 (4.0)
Lemongrass	*Cymbopogon citratus* Stapf	7 (4.0)
Aloe Vera	*Aloe vera* L	7 (4.0)
Clove	*Syzygium aromaticum* (L.) Merr. and L.M. Perry	5 (3.0)
Neem tree leaves	*Azadirachta indica* A. Juss	2 (1.0)

[a]*Columns do not add up to 100% due to the selection of multiple answers.*

TABLE 3 | Breastfeeding mother's experience and patterns of HM use.

Variables	N	%
Conditions for HM use		
Low breastmilk supply	160	80.0
Swollen breasts, breast engorgement, sores, pain during breastfeeding	47	23.5
Body pain (backache, headache)	12	6.0
Any chronic illness (NCD's)	11	5.5
Frequency of HM use		
Daily	179	89.5
Weekly	21	10.5
Experience side effects after using HM		
No	182	91.0
Yes	18	9.0
Sources of Information[a]		
Family member and friends	135	67.5
Other breastfeeding mother	63	31.5
Health professional	53	26.5
Herbalists	2	1.0
Internet	2	1.0
TV, radio and newspaper	2	1.0
Recommendation[a]		
Family member and friends	140	70.0
Other breastfeeding mother	64	32.0
Health professional	51	25.5
Herbalists	2	1.0
Reasons for using HM[a] (N = 200)		
Herbal medicine can improve breastmilk production	184	92.0
Herbal medicine is cheap and easily accessible	44	22.0
Experience of using HM treatment in family	21	10.5
Herbal medicine have no side effects, nor chemicals	20	10.0
Herbal medicine can improve both my health and child health	15	7.5
Disclosure of HM use to physician		
Yes	55	27.5
No	145	72.5
Reason for non-disclosure[a] (N = 145)		
Doctor did not ask me	94	64.8
I was afraid to tell the doctor	47	32.4
I thought it was not important to tell the doctor	7	4.8
There was not an important reason	5	3.4

[a]*Columns do not add up to 100% due to the selection of multiple answers.*

mothers use HM to ease breastfeeding problems (Sim et al., 2013; Aleandri et al., 2014; Bettiol et al., 2018b; James et al., 2019; Zheng et al., 2020). Such variations in utilization rate can be attributed to differences in how HM is defined in each study, as well as the differences in the public acceptance and availability of HM due to cultural influences (Sim et al., 2013; James et al., 2019).

TABLE 4 | Potential predictors of HM use among breastfeeding mothers using multivariate logistic regression analysis.

Variables		OR	95% CI	*p*-value
Educational level	Primary education level and below	1	Ref	—
	Primary education level	2.980	1.058–8.396	0.039
	Secondary education level	5.248	1.833–15.024	0.002
	Certificate/Diploma and above	8.325	2.745–25.242	<0.001
Number of Children	1	1	Ref	—
	2 or more	0.803	0.498–1.296	0.369
Low breastmilk supply	No	1	Ref	—
	Yes	15.526	5.564–43.327	<0.001
Asset holdings	No	1	Ref	—
Livestock	Yes	2.195	1.285–3.747	0.004

Considering the low prevalence of exclusive breastfeeding and lack of appropriate breastfeeding knowledge among Tanzanian women (Hashim et al., 2016; Hasselberg et al., 2016; Kaaya et al., 2021) due to various individual, socio-cultural, and environmental barriers (Agho et al., 2019; Mundagowa et al., 2021), appropriate use of HM as galactagogues can potentially allow the mothers to engage in optimal breastfeeding practices and act as a safe alternative to synthetic galactagogues (Penagos-Tabares et al., 2014; Jyotsna and Sameet, 2020).

Similar to the broad range observed in the prevalence of HM use, wide varieties of HM were used by breastfeeding mothers in different countries. In Tanzania, the most commonly used herbal products were *P. nigrum* and *C. pepo*, whereas in Sierra Leone, *Cassia sieberiana* DC. and *Luffa acutangula* (L.) Roxb. were the most popular (James et al., 2019). *T. foenum-graecum* and *Z. officinale* use was the highest during lactation in Australia (Sim et al., 2013), while *Tetrapanax papyrifer* (Hook) K. Koch and *Vaccaria segetalis* (Neck.) Garcke consumption were most prevalent in Macau (Zheng et al., 2020). Such differences in preferred modalities can be due to different cultural traditions, as each traditional medicine is rooted in varying climate settings and religious backgrounds (Jaiswal and Williams, 2017; Tesfahuneygn and Gebreegziabher, 2019; Zhang and Dong, 2020). In addition, differences in the main indications for use reported among the studies may explain variations in HM modalities observed. For example, in our study, the most reported indication for HM was poor milk production, yet, in the Italian study, the main indications for HM use were dermatological conditions such as preventing stretch marks and preparing nipples for breastfeeding (Aleandri et al., 2014). Furthermore, among the Macau women, the primary indications for use were in relation to modulating the amount of breastmilk produced and managing breast and nipple pain (Zheng et al., 2020). These findings indicate that variations in the types of HM used can be expected depending on women's cultural background and main indications for use (Forinash et al., 2012; Budzynska et al., 2013).

The procedures for preparing herbal remedies also vary by cultural background. *P. nigrum*, used by 80% of mothers in this study, is considered a medicinal plant in various cultures (Jamal et al., 2011; Takooree et al., 2019) and is specifically used to promote breastmilk production and postpartum care in India, Indonesia, and Morocco (Ramarao et al., 2000; Buragohain, 2008; Bnouham, 2010; Dandotiya et al., 2013; Chellappandian et al., 2014; Goyal, 2017; Muslichah et al., 2021). Depending on the cultural tradition, *P. nigrum*'s fruits are often mixed with other herbs (i.e., *Terminalia chebula* Retz., *Piper longum* L., *Zingiber officinale* Rosc., *Curcuma aeruginosa* Roxb., and *Piper betel* L.) and prepared as herbal concoctions to be consumed as galactagogue (Jyotsna and Sameet, 2020; Muslichah et al., 2021). Although not native to Africa, *P. nigrum* is also cultivated in East, Central, and West African regions and is often used as food condiments, insecticides, and cold-remedy (Kuete, 2017). It is also considered a galactagogue in places, and lactating mothers are recommended to consume a porridge with *P. nigrum* to promote breastmilk production (Hasselberg et al., 2016; Makwetta, 2021).

Nevertheless, despite women's high dependency on HM to improve their breastfeeding due to the perception that HM is a natural and safe alternative to conventional medicine, HM's clinical efficacy and safety as galactagogues still remain inconclusive and need further research (Sim et al., 2013; Zheng et al., 2020). For example, in the case of *P. nigrum*, its reputation as a lactogenic agent have received limited attention and relies on anecdotal evidence rather than clinical (Khare, 2007; Takooree et al., 2019). This may be because modalities such as *P. nigrum* are considered a food additive rather than a product with pharmacological value in many cultures, the importance of examining the effectiveness and potential dangers of such modalities can be neglected (Heinrich, 2016).

Although *P. nigrum* is better recognized for its anti-bacterial and anti-inflammatory effects in the existing literature, previous findings suggest its potential indirect effect as a galactagogue (Takooree et al., 2019). For instance, piperine, the active alkaloid contained in *P. nigrum*, improves the digestive capacity and enhances the bioavailability of drugs by increasing absorption activity in the intestines (Srinivasan, 2007; Goyal, 2017; Haq, et al., 2021) Piperine also possesses antioxidant properties which offer protection against oxidative stress by removing free radicals, reactive oxygen species, and chemical carcinogens (Srinivasan, 2007; Haq, et al., 2021). Managing postpartum oxidative stress is important as prolonged stress is involved in the pathogenesis of various maternal and neonatal diseases and can potentially alter the quality of breastmilk (Ozsurekci & Aykac, 2016; Kuramoto & Kitagawa, 2017). As consumption of balanced meals and proper nutrition is crucial for successful postpartum recovery and breastfeeding, piperine's positive effect on the digestive tract, as well as the antioxidative effect may have helped the mothers to maintain good health and nutritional status to sustain optimal

breastfeeding (Mistry & Williams, 2011; Goyal, 2017; Haq, et al., 2021). Nevertheless, as lactating mothers' consumption of *P. nigrum* results in the transfer of piperine into the breastmilk, its potential effect on newborns warrants further investigation (N' Diaye et al., 2021).

Consumption of *C. pepo* was also prevalent among Tanzanian mothers. An animal study found that phytochemicals of *C. pepo* extract have a stimulatory effect on the pituitary gland, which stimulates prolactin secretion and increases the production of breast milk (Malgwi et al., 2013). However, further pharmacological research is required to ascertain their clinical efficacy in humans. As such, large-scale human clinical trials should be conducted for various traditional modalities to generate scientific evidence and demonstrate the safety and effectiveness of HM used among breastfeeding mothers (Jyotsna and Sameet, 2020).

In addition to the lack of scientific evidence to support HM's effectiveness as galactagogues, the potential toxicity of HM due to heavy metal contamination is also a significant problem (Okem et al., 2014; Zhou et al., 2019; Luo et al., 2020). Because heavy metal components can pass through the breastmilk, inappropriate use of HM can expose infants to toxic substances (Palmieri et al., 2019). In fact, a study from Taiwan found that mothers' consumption of Chinese herbal medicine was associated with the lead body burden in infants, posing potential health risks (Chien et al., 2006). The problems with contaminated HM have received considerable attention among academics and policymakers as HM is often obtained from unreliable sources such as local herbal shops and supermarkets; therefore, it is difficult to determine whether herbal products are manufactured and processed safely (Kunle et al., 2012; Sim et al., 2013; Aleandri et al., 2014). Thus, regulatory measures should be established to properly monitor the production of herbal products.

Lack of communication on HM use between the healthcare providers and nursing mothers is also a growing issue as poor communication can inhibit proper physician intervention when potential harm arises from inappropriate HM use (Foley et al., 2019). Congruent with previous findings, more than half of breastfeeding mothers in this study did not disclose their HM use to the doctors because the doctors did not ask and the women feared the doctors' negative response (Foley et al., 2019; James et al., 2019). This implies that to prevent the potential risk of HM associated with inadequate patient-doctor communication, the physicians should actively inquire the nursing mothers about HM use and provide appropriate feedback to protect the health of the mothers and children.

Lastly, potential predictors of HM use identified in our sample include having low breastmilk supply and higher education attainment. Higher education level was frequently associated with the use of the non-conventional mode of care as people with higher education levels tend to have better health literacy, thus are more likely to practice patient autonomy and actively seek additional modes of care (Kemppainen et al., 2018; Fjær et al., 2020). Additionally, previous studies also found the age of the breastfeeding child, family income, ethnicity, previous use and positive attitudes towards non-conventional therapies, and the perceived accessibility of reliable information on HM use to be associated with HM use (Sim et al., 2013; Bettiol et al., 2018b; James et al., 2019; Zheng et al., 2020).

Several limitations can be found in this study. Because the study was conducted at a single health center, the views and opinions of study participants may not fully represent those of the other breastfeeding women. In addition, a recall bias can exist due to the retrospective design of the study. Lastly, cross-sectional data only provides information regarding the association between HM use and potential predictor variables. However, this is the first study to document the pattern of HM use among breastfeeding mothers in Tanzania and identify the factors that are significantly associated with HM use.

CONCLUSION

There is a high prevalence of HM use among breastfeeding mothers in Tanzania to overcome various problems with breastfeeding. Appropriate HM use can be used as one of the strategies to achieve EBF as it can help nursing mothers from resource-limited countries to tackle common breastfeeding challenges and practice optimal feeding behavior. However, the effectiveness and safety of the most commonly used HM among nursing mothers rely on anecdotal evidence rather than clinical, and this awakens the need to evaluate HM's safety and efficacy through pharmacological studies for scientific evidence. In addition, despite the popular use of HM among nursing mothers, no guideline has been established in clinical settings to promote open dialogues between the healthcare providers and mothers to ensure the safe use of HM. This puts emphasis on the development of public awareness program to encourage active patient-physician communication and facilitate proper HM use.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board on Human Subjects Research and Ethics Committees, Hanyang University (HYI-17-134-2); Morogoro Municipal Council in Tanzania (E10/MMC-138/VOLIV/100). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VM, JH, and DH were responsible for the study concept and design. HI, JH, SC, and DH analyzed the data and drafted the manuscript. VM, JH, and DH contributed to the designing of data collection tools and data collection. VM, HI, JH, SC, and DH

critically reviewed the manuscript and contributed intellectual content. All authors read and approved the final manuscript.

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