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Quality Control on Herbal Medicine and its Application

Lead Guest Editor: Gallant K. L. Chan
Guest Editors: Rentian Wu, Vicky P. Chen, Kevin Y. Zhu, and Ying Q. Du

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Editorial Board

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Editorial

Quality Control on Herbal Medicine and Its Application

Gallant Kar-Lun Chan,[1] **Rentian Wu**,[2] **Vicky Ping Chen**,[2] **Kevin Yue Zhu**,[3] **and Yingqing Du**[4]

[1]*Division of Life Science and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Clear Water Bay Road, Clear Water Bay, Hong Kong*
[2]*Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN 55905, USA*
[3]*Jiangsu Key Laboratory for High Technology Research of TCM Formulae and Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, State Key Laboratory Cultivation Base for TCM Quality and Efficacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China*
[4]*Hanshan Normal University, Chaozhou, Guangdong 521041, China*

Correspondence should be addressed to Gallant Kar-Lun Chan; gallant@ust.hk

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In this issue, you will see how researchers applied systematic biological method to illustrate the importance of genuineness, so-called "Daodi," of herbal medicinal material. Metabolomics study on herbal medicine as well as their interactions with commonly used compound medicine, such as Aspirin, will also be demonstrated. Curcumin was known to be a chemical compound with many strong biological functions. We will show you an advanced extraction methods which helps establishing quality control standards for herbal medicine with Curcumin. Moreover, the detailed descriptions of clinical effect and safety of herbal medicinal formulation will be covered in this issue. Last but not least, a fast and innovative drug screening platform, namely HerboChips, will be introduced. The paper describes how HerboChips makes drug screening down to molecular level from herbal medicine become feasible.

Although the background of the authors varies, they share one goal—to unmask the beauty of herbal medicine, a system which support our health and population for more than several thousands of years in human history.

Acknowledgments

Here, we would like to thank the editorial board of eCAM who provided us with the chance to manage this special issue on promoting herbal medicine. The professional support team and user-friendly system make our editorial works with ease. Special thanks should be given to all authors who contributed their papers including those that have not been published in this special issue. The acceptance rate of this special issue (6 out of 20) matches the journal acceptance rate (i.e., 29%), implying that all the submitted works are in high quality. We wish you all enjoy this quality controlled special issue.

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Research Article

Quercetin Potentiates the NGF-Induced Effects in Cultured PC 12 Cells: Identification by HerboChips Showing a Binding with NGF

Gallant K. L. Chan,[1,2] **Winnie W. H. Hu**,[1,2] **Zoey X. Zheng**,[1,2] **M. Huang**,[3] **Yan X. Y. Lin**,[1,2] **Caroline Y. Wang**,[1,2] **Amy G. W. Gong**,[1,2] **X. Y. Yang**,[3] **Karl W. K. Tsim**,[1,2] **and Tina T. X. Dong**[1,2]

[1]*Shenzhen Research Institute, The Hong Kong University of Science and Technology, Shenzhen 518057, China*
[2]*Division of Life Science and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong*
[3]*YNBY Lab Inc., No. 51, Xi-Ba Rd., Kunming, Yunnan Province 650032, China*

Correspondence should be addressed to Tina T. X. Dong; botina@ust.hk

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Dementia is a persistent disorder of the mental processes and is strongly related to depression. However, the performance of current antidepression medicine is far from satisfactory. Herbal extract provides an excellent source to identify compounds for possible drug development against depression. Here, HerboChips were employed to search herbal compounds that could bind nerve growth factor (NGF). By screening over 500 types of herbal extracts, the water extract of Ginkgo Folium, the leaf of *Ginkgo biloba*, showed a strong binding to NGF. The herbal fractions showing NGF binding were further isolated and enriched. By using LC-MS/MS analysis, one of the NGF binding fractions was enriched, which was further identified as quercetin, a major flavonoid in Ginkgo Folium. Quercetin, similar to Ginkgo Folium extract, could enhance the effect of NGF in cultured PC 12 cells, including potentiation of neurite outgrowth and phosphorylation of Erk-1/2. This is the first report of discovering an NGF binding compound by using HerboChips from herbal extracts, which could be further developed for antidepression application.

1. Introduction

Dementia is a commonly mental problem found in elderly population today [1]. The symptoms of dementia included difficulties in thinking, interacting, and communication [2]. Depression, named as major depressive disorder (MDD), is a psychological and mental problem usually found in the patient of dementia [3]. Moreover, depression is especially common among those over 65 years of age and increases in frequency with age beyond this age [4, 5]. The signature of depression includes low self-esteem, loss of interest in normally delighted activities, inactive, and pain without an obvious cause [6]. About 2–7% of adults with major depression could result in suicide [7]. Today, there is neither preventive medication nor highly effective drugs for major depression [8].

Chinese medicine (CM) is renounced for treating diseases and relieving unpleasant symptoms especially for those related to aging problems [9–11]. However, the correlations between active ingredients and efficacies of those CM are poorly understood. In our previous reports, we have demonstrated a high-throughput screening platform, namely, HerboChips [12]. In brief, the extracts from different CM were separately by HPLC system: those separated fractions were then printed on an activated silico/plastic surface. On the other hand, the key regulator/signature for depression (e.g., nerve growth factor (NGF)) was labelled with biotin. After the reactions between the biotinylated regulator and

different surfaces with immobilized fractions of CM extracts, a molecular reporter constructed by binding streptavidin with a fluorescence dye (e.g., Cy3 or Cy5) together was applied. Binding affinity between biotinylated NGF on specific CM was expected whenever a positive signal was shown after scanning. The CM with positive scanning result was submitted for biological evaluation using different cell assays. Eventual, the platforms successfully sought out potential CM candidates for possible development of antidepression drugs [12].

The extract of *Ginkgo biloba* leave, that is, Ginkgo Folium, was one of those many CM's extracts found with binding affinity to NGF as well as efficacies in antidepression functions [12]. Ginkgo Folium is one of the most well studied herbs in CM. The extract of Ginkgo Folium was rich in various bioactive compounds, including phenolic acids, proanthocyanidins, flavonoid glycosides, for example, myricetin, kaempferol, isorhamnetin, quercetin, and terpene trilactones, ginkgolides, and bilobalides [13, 14]. The identities of those bioactive compounds in Ginkgo Folium were well established. However, the NGF binding compounds have not been identified. Here, we demonstrated an application of HerboChips in searching NGF binding compounds from CM extracts, and Ginkgo Folium was shown to contain such a binding.

2. Materials and Methods

2.1. Chemicals and Herbal Materials. Quercetin with purity >98% was provided from TLCM (HKUST, Hong Kong). Ginkgo Folium was purchased from commercial sources and morphologically authenticated and qualified by Dr. X. Y. Yang at the Yunnan Institute of Materia Medica according to the Chinese Pharmacopoeia 2015 [15]. Voucher specimens of the herbs were sent to Yannanbaiyao (YNBY) Group Tianzihong Pharmaceutical Co. Ltd. (Kunming, Yunnan, China) to be cataloged.

2.2. Preparation of HerboChips. HerboChips were purchased from YNBY Group Tianzihong Pharmaceutical Co. Ltd. Herbal extracts were prepared by extracting 50 g of herb powder with 1 L of ethanol for 3 days, and the ethanoic extract was concentrated to 30 mL. The herbal extracts were then lyophilized and stored in vacuum. Extracts were resuspended in 50/50 water/ethanol and fractionated by a standardized HPLC method in gradient mode with a duration of 96 min (0 min; 0% acetonitrile (ACN) → 96 min; 100% ACN). Fractions were collected according to retention time with each fraction corresponding to 1 min. The chip surfaces were activated, and the epoxy groups were exposed before dotting. The herbal fractions were dotted and fixed on the surface of activated chips by an automatic arrayer (Biodot A101, Shuai Ran Precision, Taiwan). The lyophilized standards were redissolved with DMSO at a concentration of 100 mg/mL as a stock for cell culture studies.

2.3. Screening of HerboChips. Biotinylated NGF was prepared using an EZ-Link™ NHS-PEG4-biotinylation kit (Thermo Fisher Scientific, Rockford, IL). About 0.5 mg of NGF (Alomone Labs, Israel) was dissolved in 0.5 mL of phosphate-buffered saline (PBS), mixed with 39 μL of 20 mM biotin solution, and incubated at room temperature for 1 hour. Excess biotin reagent was removed by a desalting column. The labelled NGF was stored at −80°C. About 500 herbal extracts including Ginkgo Folium were selected and screened with the biotinylated NGF probe on the chips. The biotinylated NGF was incubated with HerboChips dotted with different herbal fractions. Streptavidin-Cy5™ (Invitrogen Life Technologies, Carlsbad, CA) was used to detect biotinylated NGF on the HerboChips arrays. The fluorescence signal of streptavidin-Cy5 was measured at 535 nm by a fluorophore microarray scanner (GenePix 4100A, Molecular Devices Corp., Sunnyvale, CA). The fluorescence results were analyzed with GenePix Pro 7 (ver. 7.1.16) software provided by the manufacturer of microarray scanner. Fluorescence intensity higher than 600 was counted as positive. The validation of biotinylated NGF probe using western blat analysis and fluorescence signal imager was performed. Twenty ng of biotinylated NGF was applied to each incubation chamber for 16 hours at room temperature followed by probing with streptavidin-Cy5 for 1 hour at room temperature. Unbound biotinylated NGF probe and streptavidin-Cy5 were removed by repeated rinsing with TBS solution with 0.1% Tween 20.

2.4. Enrichment of Identified Fractions. For enrichment of specific fraction, HPLC system with semipreparative column (Dikma, Diamonsil, C18 column, 10.0 × 250 mm^2, 5 μm) was applied. The injection volume was 20 μL. As soon as the target peak appeared, the peak fraction was collected into another flask separately until the collection wascompleted. Repeating the above procedure described above, the target peaks were completely fished from the total components peaks. The collected fractions were evaporated and redissolved in 1 mL DMSO. A dilution of 1 : 1,000 using culture medium or water was applied for cell assay and analytic measurement.

2.5. Chemical Identification by NMR. After enrichment, the unknown fraction, named as D1, was submitted for liquid chromatography with tandem mass spectrometric (LC-MS/MS) analysis and nuclear magnetic resonance (NMR) examination. For LC-MS/MS, the liquid chromatograph is equipped with an Agilent 6410 Triple Quad MS/MS (Agilent, Waldbronn, Germany) and an Eclipse XDBC18 column (2.1 × 100 mm; 3.5 μm particle size). The injection volume was 2 μL. A 20 min linear gradient at flow rate of 0.3 mL/min between solvent A (Milli-Q water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid) was used. Starting from 30% B, after reaching 70% B, the system returned to 70% A. Retention time of D1 was at 10.5–11.0 min. The MS was operated in negative electron spray ionization mode. A capillary voltage of 3.5 kV and a cone voltage of 10 V were applied. Total ion scanning spectra from m/z 100 to m/z 1000 were recorded. For NMR examination, 3 mg of the dried extract was dissolved in 1 mL of methyl sulfoxide-d6 from which 550 μL was drawn and mixed with 50 μL of a D2O solution with 0.2% TSP-d4 and 3 mM sodium azide. TSP-d4 served as an internal standard for NMR, and sodium azide inhibited microbial growth. All particulate materials were removed by centrifugation at 13,000 ×g for 1 min, and the supernatant was transferred to a standard 5 mm NMR tube. NMR spectra

were acquired on a Bruker AV 400 MHz NMR spectrometer with a 5 mm PA BBO 400SB BBFO-H-D05 Z-gradient BB observe probe head, operating at 400.13 MHz -NMR frequency at 298 K. Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisition. The NMR spectra of the samples were acquired using a 1D CPMG pulse sequence (RD-90°-*t*1-90°-*tm*-90°-acquire) to generate a spectrum with a reduced residual solvent peak. The experimental time for each sample was around 10 min. All spectra were Fourier-transformed, phase-corrected, and baseline-corrected manually. NMR data was analyzed using MestReNova software.

2.6. HPLC Conditions for Ginkgo Folium. The standard solutions of quercetin (1 mM) and extract of Ginkgo Folium, spiked with 0.5 mM quercetin, were prepared. Liquid chromatography was performed on an Agilent 1200 series system (Agilent, Waldbronn, Germany), which was equipped with a degasser, a binary pump, an autosampler, a DAD, and a thermostated column compartment. The herbal extract was separated on a Grace Prevail C-18 column (5 μm id, 250 mm × 4.6 mm). The mobile phase was composed of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) using the following gradient program: 0–90 min, linear gradient 25.0–60.0% (A); 90–95 min, linear gradient 60.0–75.0% (A); 95–120 min, linear gradient 75.0–95.0% (A). A preequilibration period of 4 min was used between each run. The flow rate was 0.75 mL/min. The column temperature was 30°C. The injection volume was 100 μL. The UV detector wavelength was set to 330 nm.

2.7. Neurite Outgrowth of PC12 Cells. Pheochromocytoma PC12 cells, a cell line derived from rat adrenal medulla, were obtained from American Type Culture Collection (ATCC® CRL-1721™). PC 12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal calf serum, 6% horse serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen Life Technologies) in a humidified CO_2 (7.5%) incubator at 37°C. Culturing media were renewed every 2 to 3 days. Cultured PC12 cells were treated with quercetin or the isolated herbal fraction (D1) and/or NGF for 72 hours, with fresh medium and reagents supplied every 24 hours. A light microscope (Diagnostic Instruments, Sterling Heights, MI) equipped with a phase-contrast condenser, 10x objective lens, and a digital camera (Diagnostic Instruments) was used to capture the images with manual setting. For analyzing the number and length of neurite, approximately 100 cells were counted from at least 10 randomly chosen visual fields for each culture. Using the SPOT software, the cells were then analyzed for number and length of neurite. The cells were scored as differentiated if one or more neurites were longer than the diameter of cell body (i.e., ~ 30 μm).

2.8. Phosphorylation of Erk-1/2. Cultured PC 12 cells were starved for 5 hours and then challenged with NGF, quercetin, and extract from Ginkgo Folium for 0, 5, or 10 min. Cells were solubilized in lysis buffer containing 125 mM Tris-hydrochloride (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 2% 2-mercaptoethanol and stored frozen at −20°C. Lysates were separated on 8% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and then incubated overnight with anti-phospho-Erk-1/2 primary antibodies (1 : 1,000; Cellular Signaling Technology, Danvers, MA), followed by anti-rabbit secondary antibodies (1 : 5,000; Invitrogen Life Technologies) for an hour. The immune complexes were visualized by the enhanced chemiluminescence (ECL) method (GE Healthcare, Chicago, IL). The samples were run on the same gel under strict standardized ECL conditions and the intensities of the bands were compared using an image analyzer and ImageJ 1.48 v software [16].

2.9. Statistical Analysis. The protein concentration was measured using the Bradford's method (Bio-Rad Laboratories, Hercules, CA). All data were analyzed using one-way analysis of variance followed by Student's *t*-test (GraphPad Prism 5 (ver 5.01), GraphPad Software, La Jolla CA). Results were classed into three levels of statistical significance: $*$ where $P < 0.05$; $**$ where $P < 0.01$, and $***$ where $P < 0.001$.

3. Results

Around 500 herbal extracts were selected for HerboChips screening. All herbal extracts were screened by HerboChips under standard workflow, as described in our previous report (Figure 1S). The screening probe, NGF, was labelled with biotin. The biotinylated NGF probe was validated by western blot analysis and fluorescence imaging (Figure 2S). The format of array on HerboChips was shown in Figure 1(a), which was adopted from Huang et al. (2015) [17]. From the NGF binding screening, 26 herbal extracts showed positive signals, which was reported by Lee et al. (2016) [12]; however, the identity of NGF binding compounds in those extracts was not revealed. Among the 26 positive-hit herbs, the extract from Ginkgo Folium was the one showing the strongest signaling. A positive signal implied that there was a direct binding between the probe (i.e., NGF) and fractions from extracts. The scanning result was shown for Ginkgo Folium and the blank control (Figure 1(b)). Apart from the control spots dotted with known amount of biotin and SA-Cy5, no positive signal was found in the blank control. As shown in Figure 1, more than one fraction of Ginkgo Folium extracts showed binding with NGF. There were at least two groups of fractions, one from positions 24 to 60 (i.e., elution time 24 min to 60 min after injection of Ginkgo Folium extract) and another one from positions 65 to 70 (i.e., elution time 65 min to 70 min after sample injection). Position 39 and 40 showed the strongest binding signal, as compared to others. The binding signal was quantified by GenePix Pro 7 (ver. 7.1.16) software, and the signal intensity (dotted line) was superimposed with the HPLC chromatograph (solid line) of the extract of Ginkgo Folium (Figure 1(c)). A well-resolved peak (namely D1) was colocalized from both the binding signal and HPLC chromatograph. The fraction D1 was then isolated, enriched, and submitted for further chemical analysis.

The herbal fraction D1 was then submitted for chemical identification. By using LC-MS/MS, D1 fraction was composed of only a single mass at 301.1 m/z (Figure 2(a)). The compound then underwent ^{1}H and ^{13}C NMR analysis (Figures 2(b) and 2(c)), and the profiles of NMR were then characterized by submitting to Biological Magnetic Resonance

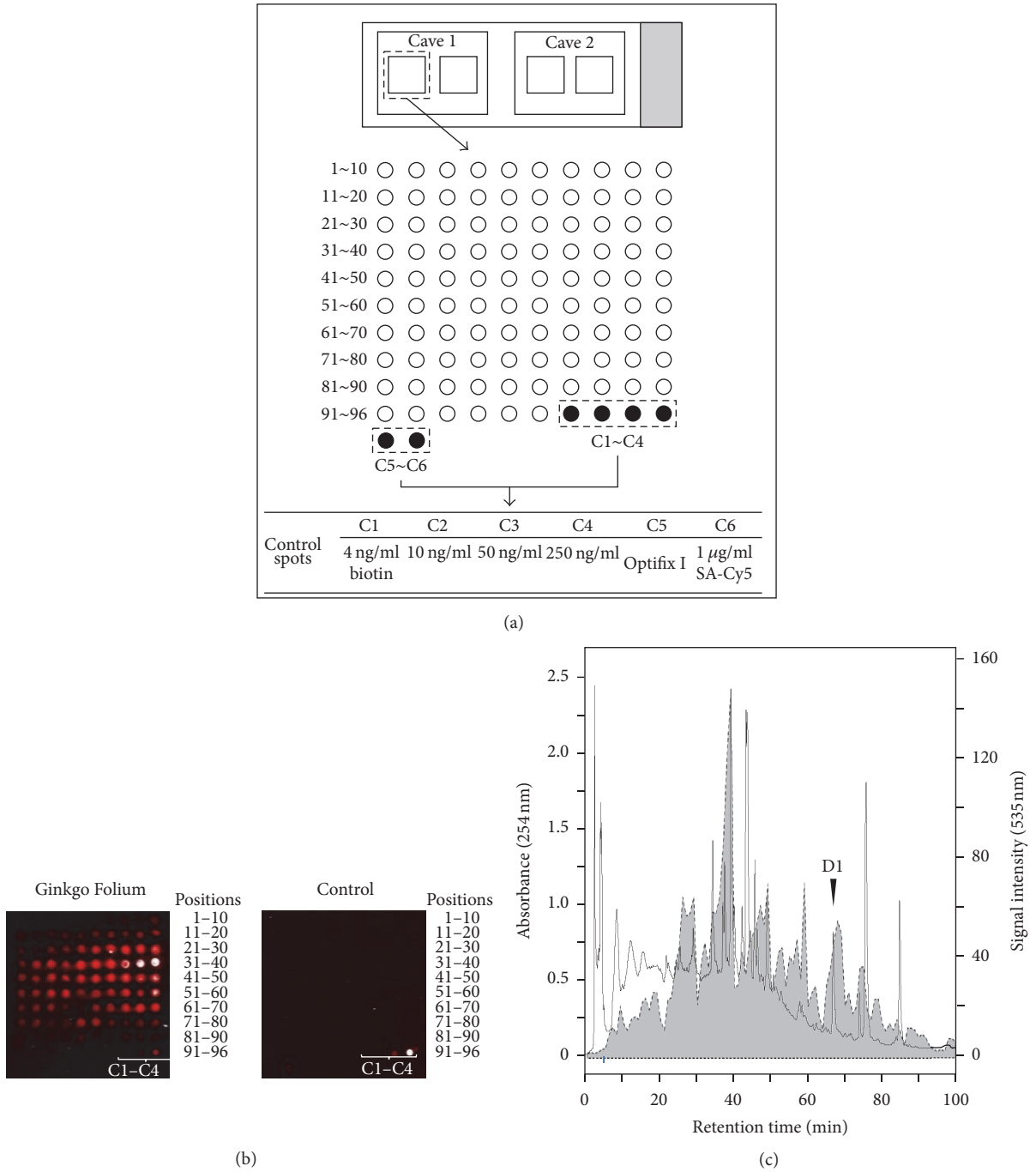

Figure 1: Extract of Ginkgo Folium was fractionated by HPLC and then dotted and fixed on a chip. The chip was incubated with biotinylated protein target (NGF) and then probed with streptavidin-Cy5 before fluorescence detection. (a) Adopted from Huang et al., 2015, showing the design of HerboChip, the quantifier and qualifier controls were included. (b) Scanning result of HerboChip using Ginkgo Folium extract and probed with biotinylated NGF (left) and probed with incubation buffer only (right). Representative scanned images were shown, $n = 5$. (c) Superimposition of the read-out of HerboChip screening and HPLC chromatogram. HPLC chromatograms (solid line) and quantified HerboChip screening read-out (dotted line with shaded area) of the extract of Ginkgo Folium were superimposed. A will resolved peak at retention time ~67 min was labelled as D1 and submitted for further analysis.

Data Bank (http://www.bmrb.wisc.edu). After analysis, the fraction D1 was identified as quercetin (Figure 2).

The role of quercetin, as well as Ginkgo Folium extract, in NGF effect was determined here. Figure 3(a) shows the control and differentiated PC12 cells in cultures. Having the binding to NGF, we hypothesized that quercetin could interfere NGF normal functions. As a positive control, application of NGF at 50 ng/mL induced robustly PC12 cell to

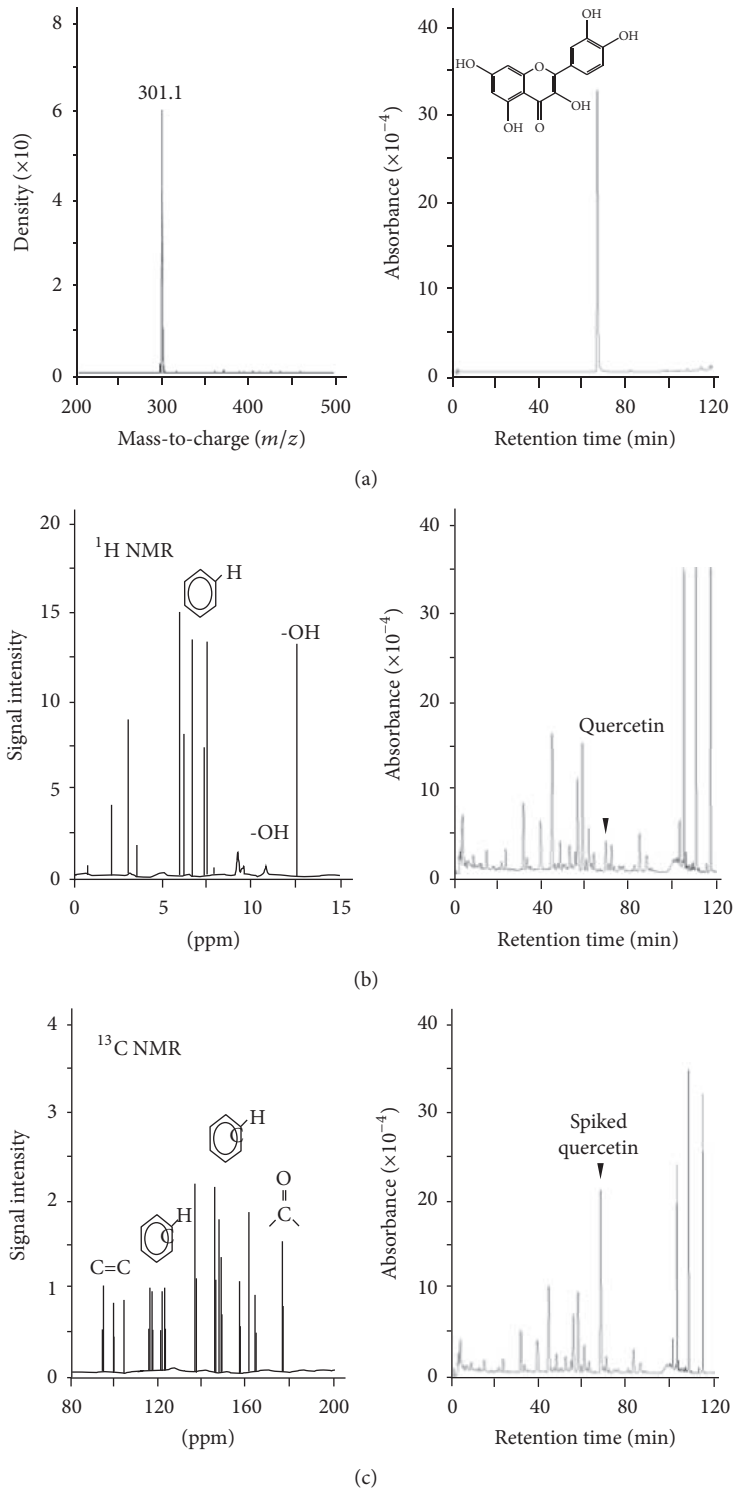

Figure 2: (a) Under full scanning mode, the molecular weight of the enriched fraction D1 was shown by LC-MS/MS. (b) ^{1}H-NMR spectra of enriched fraction D1. (c) ^{13}C-NMR spectra of enriched fraction D1. Corresponding chemical structures were indicated according to their ppm in different NMR spectrum. The spectrum was submitted to the database, Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) for characterization. (a) HPLC chromatograms using the HPLC condition described in the section of Materials and Methods were shown: quercetin at 1 mM. Chemical structure of the enriched fraction from the extract of Ginkgo Folium (D1), that is, quercetin, was shown. (b) Extract of Ginkgo Folium (100 mg/mL) and (c) extract of Ginkgo Folium (50 mg/mL) spiked with 0.5 mM quercetin. The position of quercetin was being indicated in (b) and (c).

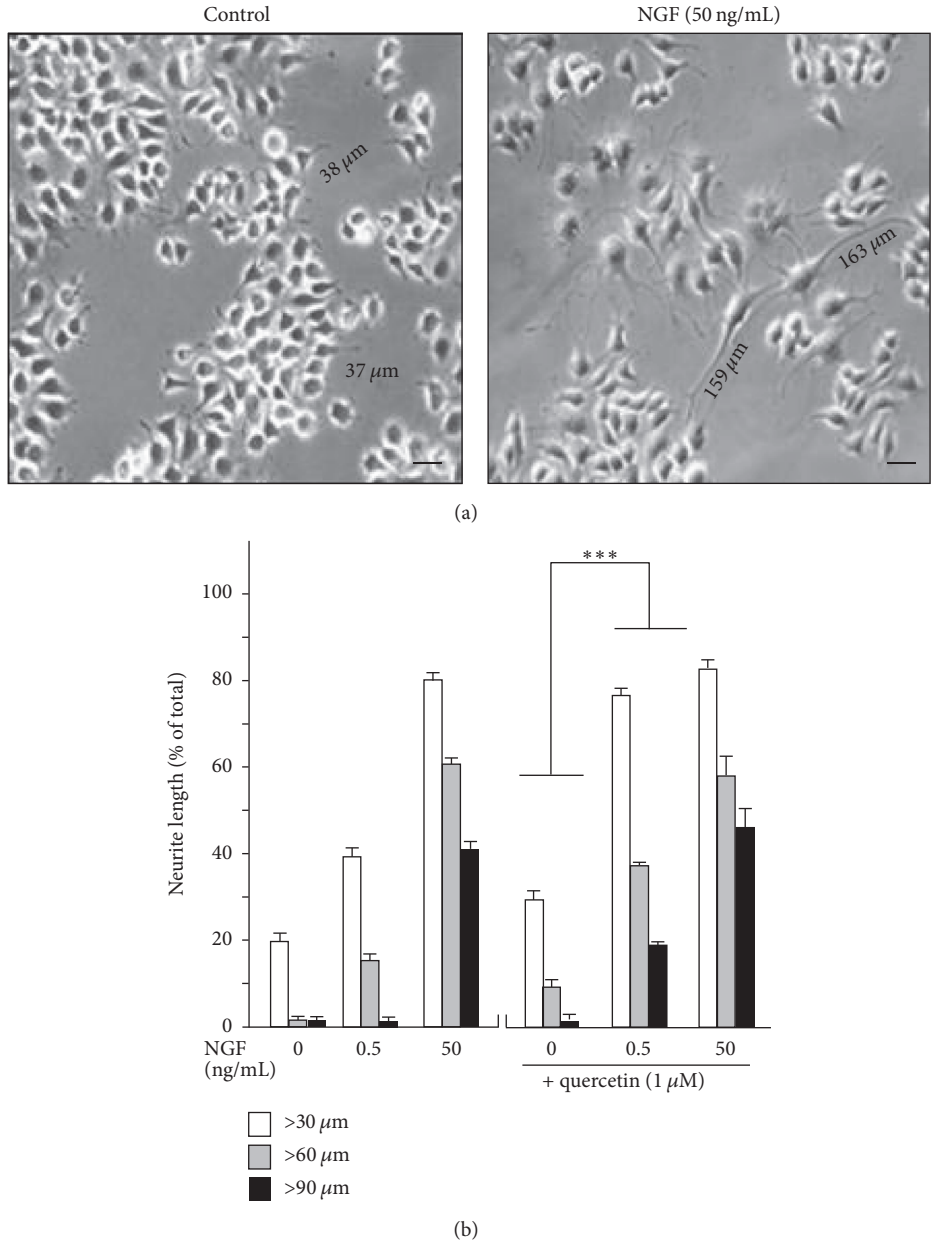

Figure 3: Cultured PC12 cells were treated with control, NGF (0.5 ng/mL or 50 ng/mL), and enriched fraction from the extract of Ginkgo Folium (quercetin) with or without NGF at 0.5 ng/mL for 48 h, and then the neurite outgrowth was examined under microscope. (a) Microscopic image of PC12 cells treated with buffer alone or treated with NGF in 50 ng/mL. Representative images were shown, $n = 3$. Bar = 50 μm. (b) To quantify the differentiation effect, the percentage of differentiated cell numbers and length of neurites were counted by the methods described in Materials and Methods section. Data were expressed as % of cells in 100 counted cells, mean ± SEM, $n = 3$. $^{***}P < 0.001$ verse control.

a full differentiated stage (Figures 3(a) and 3(b)). Inclusion of quercetin did not inhibit the NGF-induced neurite outgrowth instead which could slightly increase the neurite growth (Figure 3(b)). To ensure the effect of quercetin could be determined, a low dose of NGF at 0.5 ng/mL not able to induce any growth of neurite was used (Figure 3(b)). Quercetin alone did not increase the neurite out growth significantly; only those having the length of >30 μm were slightly increased. The coapplication of quercetin and low dose of NGF markedly induced the neurite outgrowth (Figure 3(b)), which suggested a potentiation effect of quercetin in NGF function. By counting the induced neurite of >90 μm in cultured PC 12 cells, quercetin together with low dose of NGF could induce the neurite outgrowth in a dose-dependent manner, and this effect similarly was revealed by using Ginkgo Folium extract (Figure 4).

The phosphorylation of Erk-1/2 at 42 and 44 kDa, a downstream effector of NGF signaling, was coherent with neurite

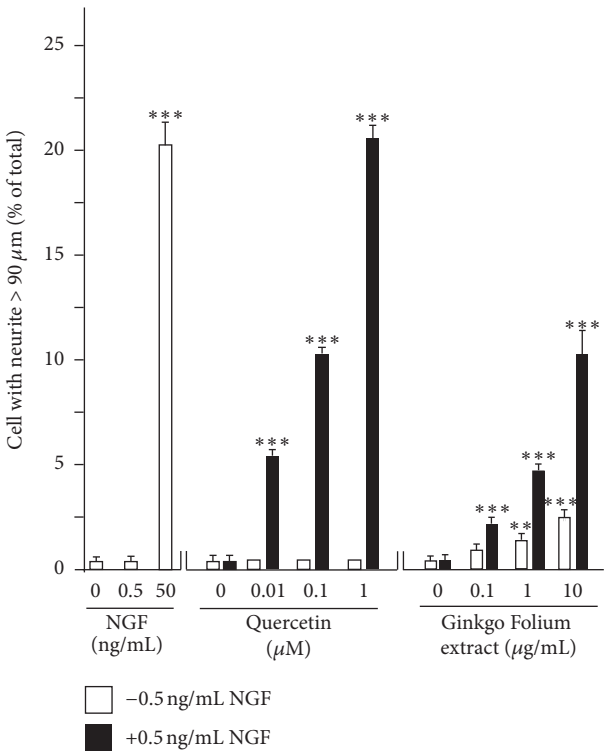

Figure 4: Serum-starved cultured PC12 cells were treated with control, NGF (0.5 ng/mL or 50 ng/mL), or quercetin (0.01–1.0 μM) with or without NGF at 0.5 ng/mL, or the extract of Ginkgo Folium (0.1–10 μg/mL) with or without NGF at 0.5 ng/mL for 48 hours, and then the neurite outgrowth was examined under microscope. To quantify the differentiation effect, the percentage of differentiated cell numbers and length of neurites were counted by the methods described in Material and Methods section. Data were expressed as % of cells in 100 counted cells, Mean ± SEM, $n = 3$. $^{***}P < 0.001$ verse control.

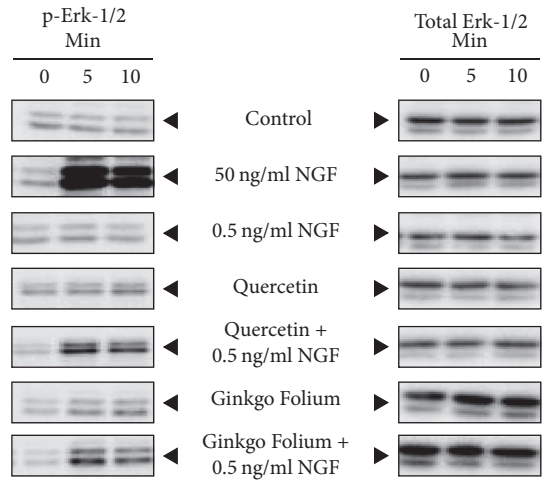

Figure 5: PC12 cells were serum-starved for 3 hours before treatment of control, 0.5 ng/mL, or 50 ng/mL of NGF and quercetin and cotreatment of quercetin with NGF at 0.5 ng/mL. Cell lysates (20 μg) were subjected to western blotting for phosphorylation analysis. Only 50 ng/mL NGF and the cotreated quercetin with 0.5 ng/mL NGF induced phosphorylation of Erk-1/2 (P-Erk-1/2 at ~42 and ~44 kDa) at 5 min after treatments, no notable changes in phosphorylation were observed in other groups, $n = 4$. The representative gels were shown here.

outgrowth in cultured PC12 cells in responding to NGF challenge (Figure 5). Notable increment in phosphorylation of Erk-1/2 was observed at 5 min after the cotreatment of 0.5 ng/mL NGF and quercetin. As expected, thers are no obvious changes after the treatment for low level NGF and quercetin alone (Figure 5). In all cases, the total Erk-1/2 did not change.

4. Discussion

Quercetin was named in 1857 according to its source, that is, quercetum (oak forest). Quercetin exists in a variety of plants, especially in fruit with color, for example, red onion, berries, and apple (Health remedy 2017). In Chinese medicine, quercetin is also commonly found as major ingredient, for example, *Panax notoginseng* [18] and *Apocynum venetum* [19]. As a dietary flavonoid, the average daily consumption of quercetin could be from 25 to 50 mg. Quercetin is a strong antioxidant and served as scavenger for free radicals. Quercetin can activate both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) [20, 21]. Several reports mentioned the beneficial functions of quercetin on nerve system [22]. Those beneficial functions included extension of survivability of neuron by suppressing inflammation [23] and oxidation stress [24, 25]. Here, we showed the potentiation effect of quercetin in NGF-induced neurite outgrowth in cultured PC 12 cells. Our results suggested quercetin might exhibit its potentiation ability via binding of NGF and trigger the downstream signaling in nervous system. Indeed, low expression of NGF is one of the causes for patients suffering from dementia or depression [26]. Thus, this study strengthens the possible usage of quercetin and Ginkgo Folium as health supplements for alleviating dementia or depression.

EGb 761 (Rökan, Tanakan) is a commercial health food product from Ginkgo Folium, which is standardized of having ~ 24% flavone glycosides (primarily quercetin at 5-6%, kaempferol, and isorhamnetin) and 6% terpene lactones (2.8–3.4% ginkgolides A, B, and C and 2.6–3.2% bilobalide). Different lines of evidence have supported the benefits of EGb761 for age-related dementia [27]. In addition to current result of quercetin in NGF function, isorhamnetin, another major flavonoid in Ginkgo Folium, was shown to induce neuronal differentiation [14], synapse formation [28], and secretion of neurotropic factors [29]. Interestingly, isorhamnetin shares similar chemical structure to quercetin.

Characterization of a NGF-bound quercetin from Ginkgo Folium was not the only successful case. HerboChips have also been applied to drug screening for CM that shows binding to tumor necrosis factor (TNF) alpha. The water extract of Andrographis Herba has ability to bind TNF alpha in HerboChips, and this herbal extract is being developed as a product for rheumatoid arthritis [17]. Similarly, our lab is on its way to search herbal extracts binding to vascular endothelial growth factor (VEGF), interleukin 17, β-amyloid, and

insulin. In view of these routine screening of herbal extracts, the current version of HerboChips was not ideal. A new version of HerboChips must be developed to reach a higher sensitivity. First, HPLC conditions for each herb should be specifically optimized, that is, to ensure a good separation. Second, the content of each pixel in the array should be examined before dotting on the chip, possibly by LC-MS analysis, which could speed up the identification process. Last but not least, an internal position marker, with known migration time in the HPLC analysis, should be included so that the result will not be affected by shifting in the chromatograms.

5. Conclusions

The bioactivities of quercetin and Ginkgo Folium on neural functions were illustrated. The potency of neurite development triggered by quercetin or extract from Ginkgo Folium may be due to the binding between NGF and quercetin. Moreover, the current result revealed the potential of transcending the applicability of HerboChips from a screening platform to become a tool for characterizing bioactive compounds on herbs.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary Materials

308 work flow of HerboChips screening and validation examination on biotinylated NGF probe are available as Supporting Information. Figure 1S: standard work flow of HerboChips screening. Figure 2S: validation on the efficiencies of biotinylation on nerve grow factor (NGF). Brain lysate and biotinylated NGF were loaded into gel and underwent SDS-PAGE. Images were captured after probing with Cy3 or Cy5 linked streptavidin by a fluorescence imager. *(Supplementary Materials)*

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Research Article

Effect of Zishen Jiangtang Pill, a Chinese Herbal Product, on Rats with Diabetic Osteoporosis

Huilin Li, Shufang Chu, Hengxia Zhao, Deliang Liu, Xuemei Liu, Xi Qu, Jianpin Chen, Zengyin Li, and Jinhua Li

Shenzhen Traditional Chinese Medicine Hospital, Guangzhou University of Chinese Medicine, Shenzhen 518033, China

Correspondence should be addressed to Huilin Li; sztcmlhl@163.com

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Diabetic osteoporosis (DO) is a complication of diabetes. Zishen Jiangtang Pill (ZJP) is a Chinese herbal product which has been used in clinic to maintain blood glucose level and bone density for decades. However, the evidence about its mechanism on diabetes and osteoporosis is still unknown. The aim of this study is to investigate therapeutic effect of ZJP on DO in streptozotocin- (STZ-) induced rats. Rats were randomly assigned to 4 groups: one control group (CON), one model group (MOD), and two ZJP treatment groups (1.5 and 3.0 g/kg/d). All rats were treated for 8 weeks. Results showed that ZJP decreased the blood glucose level during OGTT and prevented the changes of FBG and Fins. Similarly, ZJP inhibited the changes of BCa, P, TRACP-5b, CTX-1, BALP, and BGP and the reduction of BMD. In parallel, 1H-NMR metabolomic studies showed that ZJP significantly altered the metabolic fingerprints of blood and urine level. These findings suggest that ZJP can effectively improve glucose metabolism, abnormal bone metabolism, and metabolic disorders in DO rats, which may be a useful alternative medicine for DO therapy.

1. Introduction

Diabetes is a devastating and life-altering disease, which causes many complications and comorbidities. Peripheral vascular, renal, cardiovascular, and neurologic comorbidities are the four major chronic complications that affect total expenditures for diabetes management [1]. Type 2 diabetes (T2DM) constitutes about 90% of all diabetes cases worldwide [2]. Increasing evidences support that T2DM is due to impaired secondary signaling to the binding of insulin to its receptor. Insulin induced the activation of signaling proteins, such as insulin receptor substrate- (IRS-) 1 and IRS-2, and is further attenuated in many tissues, including liver, skeletal muscle, kidney, and bone [3–5]. Moreover, the endocrine and metabolic alterations of T2DM could cause disorders in different pathways related to forming and maintaining of bone [6]. Thus, T2DM can bring several serious consequences, in particular, osteoporosis [7, 8]. Although there are many factors that could increase the risk of osteoporosis including genetic, hormonal, and specific pharmacological therapies [9], diabetes became one of the common causes for osteoporosis.

Diabetic osteoporosis (DO) has been increasingly recognized as an important complication of diabetes [10, 11], the reason of which is resulting from reduced bone mineral content due to the abnormal levels of sugar, protein, fat, and microelements [12]. It has been reported that no difference was observed in the antifracture efficacy of bisphosphonates and raloxifene between patients with diabetes and nondiabetic controls or between patients with type 1 diabetes (T1DM) and T2DM [13], indicating that diabetic patients may receive treatment for osteoporosis in the same way as nondiabetic patients. Bisphosphonates are effective treatment methods for preventing fractures in glucocorticoid-induced osteoporosis. Currently, antiresorptive therapies was evaluated in diabetic patients. Strontium ranelate has also been reported to reduce bone resorption and decreases fracture risk [14, 15]. However, based on existing rodent models, the observation supported crosstalk between the skeleton and energy metabolism, suggesting that osteoporosis therapies may have effects on glucose metabolism, and risk of diabetes should be evaluated [16]. In particular, osteocalcin has beneficial effects on glucose metabolism in animal model [17].

Thus, it is important to look for drugs that are effective and have low side effects in the treatment of DO.

Recently, a number of patients also may choose alternative therapeutic approaches, such as traditional Chinese medicine (TCM). Zishen Jiangtang Pill (ZJP), a Chinese herbal medicine, was comprised of 14 herbs such as Astragalus, Radix Rehmanniae Recens, Radix Rehmanniae Praeparata, Schisandra, Herba Epimedii, Rhizoma Cibotii, and Plastrum Testudinis, which have been used for treatment of diabetic patients in clinic for many years. ZJP possessed the efficacies of tonifying qi and yin, nourishing kidney and bones. Pharmacological studies have reported that ZJP could regulate plasma glucose and lipid levels [18]. Moreover, we found that ZJP inhibited the adipogenic differentiation of mouse bone marrow mesenchymal stem cells (BMSCs) showing the potential role in antiosteoporosis [14, 15]. However, the molecular mechanism of ZJP in DO remains unclear. In the current study, we hypothesize that ZJP could improve DO by regulating glucose and bone metabolism by using a diabetes model of rat. We examined whether ZJP would regulate the glucose and bone metabolism and performed 1H-NMR based urinary and blood metabonomic studies in ZJP treated DO rats.

2. Materials and Methods

2.1. Animal. Male Sprague-Dawley rats (n = 105, 190–220 g, 7-8 weeks of age) were obtained from Experimental Animal Center of Guangdong Province. Animals were maintained in a specific pathogen-free laboratory with regular 12/12 hours light/dark cycles with the average temperature of 25°C and humidity conditions. All animal procedures were approved by the Animal Care and Use Committee at Experimental Animal Center of Guangdong Province and were conducted in accordance with the policies of the Ethics Committee for Animal Research.

2.2. Establishment of Rat Diabetes Model and Drug Administration. The animals were allowed to acclimatize for a week before beginning experiments. Rats were randomly assigned to 4 groups: (1) control group (CON) (injection with citrate buffer, intraperitoneally) (n = 10); (2) model group (MOD) (injection with streptozotocin (STZ), 55 mg/kg, intraperitoneally) (n = 10); (3) model group with 3.0 g/kg/d of ZJP orally (MOD + H-ZJP) (n = 10); (4) model group with 1.5 g/kg/d of ZJP orally (MOD + L-ZJP) (n = 10). ZJP was obtained from Shenzhen Traditional Chinese Medicine Hospital (Shenzhen, China). STZ was purchased from Sigma-Aldrich (St Louis, MO, USA).

Rats in diabetic and drug-treated diabetic groups were intraperitoneally injected with STZ on one week to induce diabetes. Rats in control group were fed with standard chow and water ad libitum and injected with citrate vehicle alone. After one week of STZ injection, rats were fasted for 12 hours; then venous blood was collected to examine the glucose levels. The glucose levels over 16.7 mmol/L were considered diabetic and selected for further studies. All rats were provided with a vehicle control or drug (ZJP) for 8 weeks after STZ injection. Rats were placed in metabolic cages to collect 24-hour urine after 8 weeks of oral gavage administration of drug. After the 24-hour urine was collected, oral glucose tolerance test (OGTT) was performed. Rats were fed with standard chow and water ad libitum for another week after OGTT; then all the animals were fasted for 12 hours and anesthetized using 2% (w/v) pentobarbital sodium (50 mg/kg, Solarbio Science & Technology, Beijing, China) via intraperitoneal injection. After abdominal aorta blood sampling, rats were killed. The serum was separated by centrifugation at 3000 rpm and stored at −80°C until analyzed. The bones were collected from each animal and dissected with care being taken to protect the periosteum. Each bone was individually wrapped in ddH2O-soaked gauze and stored at −80°C until analyzed.

2.3. Oral Glucose Tolerance Test (OGTT). After overnight fasting, all animals received glucose (3 g/kg) orally. Serum glucose was measured by using glucometer at 0, 0.5, 1.0, and 2.0 hours after glucose intake.

2.4. Examination of Glucose and Bone Metabolism. The levels of glycosylated hemoglobin (HbAlc), fasting insulin (Fins), tartrate-resistant acid phosphatase-5b (TRACP-5b), bone specific alkaline phosphatase (BALP), type I procollagen (PINP), and osteocalcin (BGP) were quantified using Quantikine ELISA kit (RD, USA). The experiment was carried out according to user's menu from the manufacturer. Plates were read using an ELISA reader (Hercules, CA, USA) at 450 nm. The concentrations of HbAlc, Fins, TRACP-5b, BALP, PINP, and BGP were calculated using standard calibration curve prepared by using serial dilutions of the standard provided with the kit. The levels of fasting blood glucose (FBG), blood calcium (BCa), phosphorus (P), and urinary calcium (UCa) were examined by using Hitachi fully automatic chemistry analyzer (Beijing Tailin Oriental Trading Company, Beijing, China).

2.5. Measurement of Bone Mineral Density (BMD). Total bone mineral density (T-BMD), spine bone mineral density (S-BMD), and left thigh bone mineral density (LT-BMD) were measured by using dual-energy X-ray absorptiometry (DXA) with Hologic DXA equipment (Hologic Discovery W 81507) using the software for small animals. Results were obtained as grams of mineral content per square centimeter of bone area (g/cm2). The scanner was calibrated daily by in-house certified technician.

2.6. Blood Sample Preparation and Pretreatment. Samples were vortexed for 30 seconds; aqueous layer was transferred to 0.5 mL 3 KDa ultrafiltration filter (Millipore, USA). Filtrate was collected by centrifuging the sample at 13000 rpm for 45 mins. 350 μL aqueous layer was transferred to a clean 2 mL centrifuge tube. 100 ul D2O and 50 μL DSS standard solution (Anachro Technologies Inc, Canada) was added. Samples were mixed well before transfer to 5 mm NMR tube (Norwell, USA). Sample spectra were collected using a 600 MHz Bruker NMR spectrometer. MetNOESY sequence was used for its superior solvent suppression result. 100 ms mixing time along with a 990 ms presaturation was employed to match the

acquisition parameters used in Chenomx Library. Spectra were collected at 25°C, with a total of 64 scans to obtain the required signal-to-noise ratio.

2.7. Urine Sample Preparation and Pretreatment. Samples were centrifuged at 13000 rpm for 2 mins; 540 μL aqueous layer was transferred to a centrifuge tube. 60 μL DSS standard solution (Anachro, Canada) was added. Samples were mixed for 10 seconds before being transferred to 5 mm NMR tube (Norwell, USA). Spectra were collected using the same protocol as described in Section 2.6. Because of lower concentration for most of the components, a total of 128 scans over a period of 15 min were used to acquire data for each sample.

2.8. Spectrogram Processing and Multivariate Pattern Recognition Analysis. The Free Induction Decay (FID) data was first processed using processor module in Chenomx NMR Suite 8.1. (Chenomx Inc., Edmonton, Canada). Briefly, data was automatically zero filled and underwent Fourier transform. The frequency domain data was then carefully phased and baseline corrected inside the same processor module. All spectra were referenced to DSS. Metabolites qualification and quantification were made by experienced analysts using Chenomx Compound Library. With the amount of 70 spectra, a total of 56 metabolites were identified and quantified. All metabolites' concentrations were used and normalized by Pareto scaling before multivariable analysis. R packages "pls" [19] and "ggplot2" [20] were used to perform PLS-DA analysis and plots, respectively.

2.9. Statistical Analysis. Data were expressed as means ± SD values. OGTT, ELISA, and fully automatic chemistry analyzer data analysis were performed by using GraphPad Prism 5 Software. One- or two-way ANOVA was performed to identify features with differential abundances across groups. Post hoc tests for the results were evaluated by Bonferroni test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. ZJP Partially Inhibits the Increase of Blood Glucose Concentration in Diabetic Rats. As shown in Figure 1, the blood glucose concentrations were significantly increased after receiving glucose orally in MOD, MOD + H-ZJP, and MOD + L-ZJP compared with the control group ($P < 0.001$), while the increases in MOD group were partially inhibited by ZJP treatment at the dosage of both 3.0 g/kg/d and 1.5 g/kg/d after 0.5 hours and 1.0 hour of glucose administration ($P < 0.001$). The blood glucose concentration reached similar levels in MOD and MOD + L-ZJP groups after 2 hours of glucose administration ($P < 0.001$), while the blood glucose concentration remains at lower levels in MOD + L-ZJP groups compared with the control group ($P < 0.001$).

3.2. ZJP Improves Abnormal Glucose Metabolism in Diabetic Rats. As shown in Figure 2(a), the concentration of FBG and the percent of HbAlc were significantly increased in

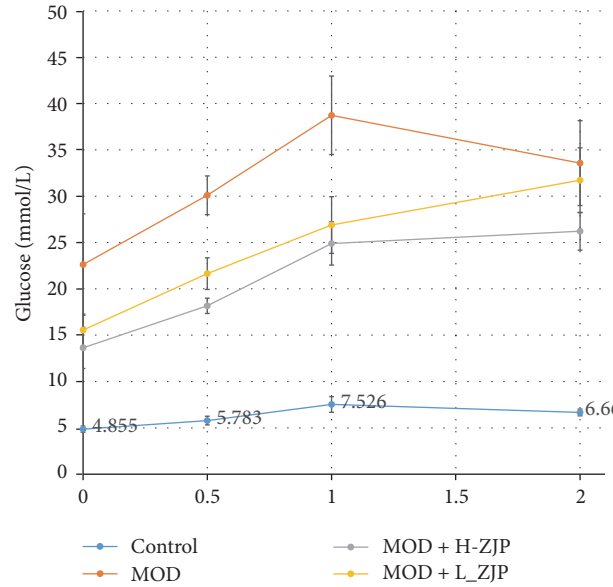

Figure 1: *Effect of ZJP on blood glucose concentration in diabetic rats.* The blood glucose concentrations were significantly increased after receiving glucose orally in MOD, MOD + H-ZJP, and MOD + L-ZJP compared with the control group; ZJP partially inhibits the increases of blood glucose concentration at the concentration of both 3.0 g/kg/d and 1.5 g/kg/d after 0.5 and 1.0 hours of glucose administration. ZJP partially inhibits the increases of blood glucose concentration at the concentration of both 3.0 g/kg/d after 2.0 hours of glucose administration.

MOD group, while the increase of FBG concentration was partially inhibited by ZJP at the dosage of both 3.0 g/kg/d and 1.5 g/kg/d ($P < 0.001$) and the percent of HbAlc was not changed with ZJP treatment ($P > 0.05$). As shown in Figure 2(b), STZ significantly reduced the concentration of Fins ($P < 0.001$), while ZJP completely inhibited the reduction induced by STZ ($P < 0.001$).

3.3. ZJP Partially Inhibits the Levels of BCa and P in Diabetic Rats. As shown in Figure 3, STZ significantly increased the levels of BCa, P, and UCa in MOD group ($P < 0.001$), while the increase of BCa level was partially inhibited by ZJP at the dosage of both 3.0 g/kg/d ($P < 0.01$) and 1.5 g/kg/d ($P < 0.05$) and the increase of P level was also partially inhibited by ZJP at the dosage of both 3.0 g/kg/d ($P < 0.001$) and 1.5 g/kg/d ($P < 0.001$). However, the increase of UCa in diabetic rats was not changed by ZJP administration ($P > 0.05$).

3.4. ZJP Improves Abnormal Bone Metabolism in Diabetic Rats. TRACP-5b and CTX are normally used to diagnose osteoporosis, malignant bone tumours, or other pathology and to monitor antiresorptive therapy, which indicate the changes of bone resorption most satisfactorily [21, 22]. As shown in Figures 4(a) and 4(b), STZ significantly increased the levels of TRACP-5b ($P < 0.01$) and CTX ($P < 0.05$) in MOD group, while the increase of TRACP-5b level was completely inhibited by ZJP at the concentration of both 3.0 g/kg/d ($P < 0.001$) and 1.5 g/kg/d ($P < 0.001$), and

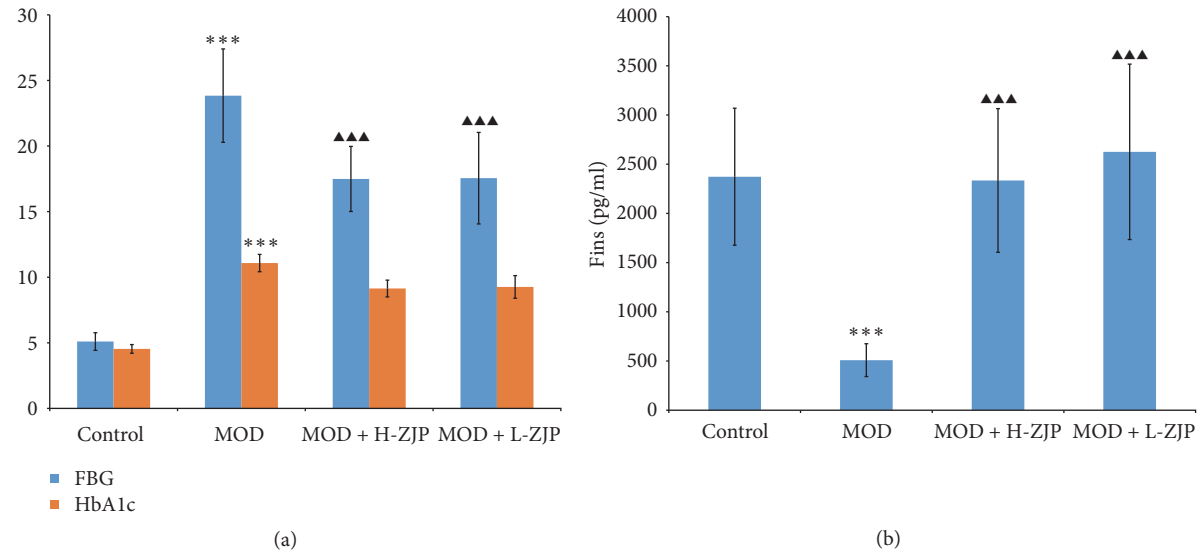

Figure 2: *Effect of ZJP on glucose metabolism in diabetic rats.* (a) The concentration of FBG and the percent of HbAlc were significantly increased in MOD group; ZJP partially inhibited the increases of FBG concentration but did not change the increased percent of HbAlc. (b) STZ significantly reduced the concentration of Fins; ZJP greatly inhibited the reduction of Fins concentration in diabetic rats. Asterisks indicate statistical significance ($^{**}P < 0.01$, $^{***}P < 0.001$, control versus MOD). Triangle indicates statistical significance ($^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$, MOD versus MOD + H-ZJP or MOD + L-ZJP).

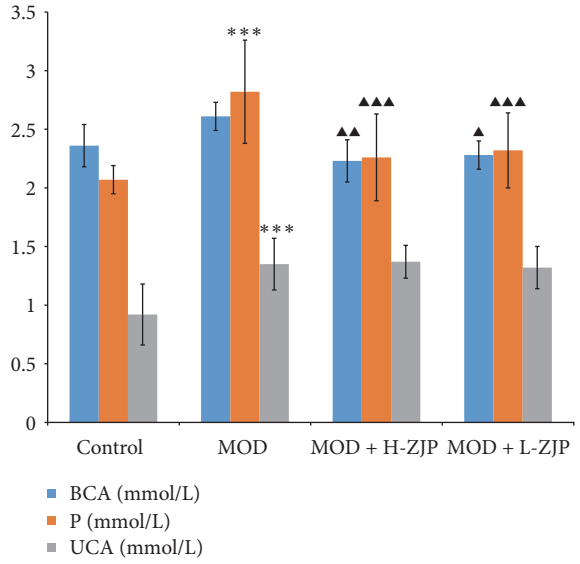

Figure 3: *Effect of ZJP on the levels of BCa, P, and UCa in diabetic rats.* STZ significantly increased the levels of BCa, P, and UCa in MOD group; ZJP partially inhibited the increases of BCa and P levels in diabetic rats but did not change the increase of UCa level. Asterisks indicate statistical significance ($^{***}P < 0.001$, control versus MOD). Triangle indicates statistical significance ($^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, and $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$, MOD versus MOD + H-ZJP or MOD + L-ZJP).

the increase of CTX-1 level was also completely inhibited by ZJP at the dosage of both 3.0 g/kg/d ($P < 0.001$) and 1.5 g/kg/d ($P < 0.001$). Serum BALP, PINP, and BGP have emerged as reliable markers of bone turnover in humans and is routinely used to monitor bone formation [23, 24]. As shown in Figure 4(c), the level of BALP was significantly decreased by STZ ($P < 0.001$), while ZJP attenuates the reduction at the concentration of 3.0 g/kg/d ($P < 0.01$). As shown in Figure 4(d), the level of PINP was not changed by STZ and ZJP administration ($P > 0.05$). As shown in Figure 4(e), the level of BGP was significantly decreased in diabetic rats ($P < 0.001$), while ZJP attenuates the reduction of BGP at the concentration of 3.0 g/kg/d ($P < 0.01$) and 1.5 g/kg/d ($P < 0.01$). As shown in Figure 4(f), the T-BMD, S-BMD, and LT-BMD were significantly decreased by STZ ($P < 0.001$), while the reduction of T-BMD was partially inhibited by STZ at the concentration of 3.0 g/kg/d ($P < 0.001$). Meanwhile, the reduction of S-BMD was partially inhibited by STZ at the concentration of both 3.0 g/kg/d ($P < 0.05$) and 1.5 g/kg/d ($P < 0.001$), and reduction of LT-BMD was also partially inhibited by STZ at the concentration of both 3.0 g/kg/d ($P < 0.01$) and 1.5 g/kg/d ($P < 0.01$).

3.5. ^{1}H-NMR Based Metabolomics Study on Blood of Diabetic Rats. By using the targeted profiling method, the metabolites were quantified and qualified using Chenomx NMR Suite 8.0 by an experienced lab technician. All identified metabolites were used for multivariable analysis. As shown in Figure 5(a), PLS-DA was used to bring out the specific variation in the blood samples of MOD and CON groups. In the PLS-DA score plot, the metabolic state of MOD group was significantly different from the CON, indicating that diabetes changed the endogenous substances metabolism and significantly altered the metabolic fingerprints of rat blood. A Variable Importance in Projection (VIP) plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes from CON group is shown in Figure 5(b). As shown in Figure 5(c), in the

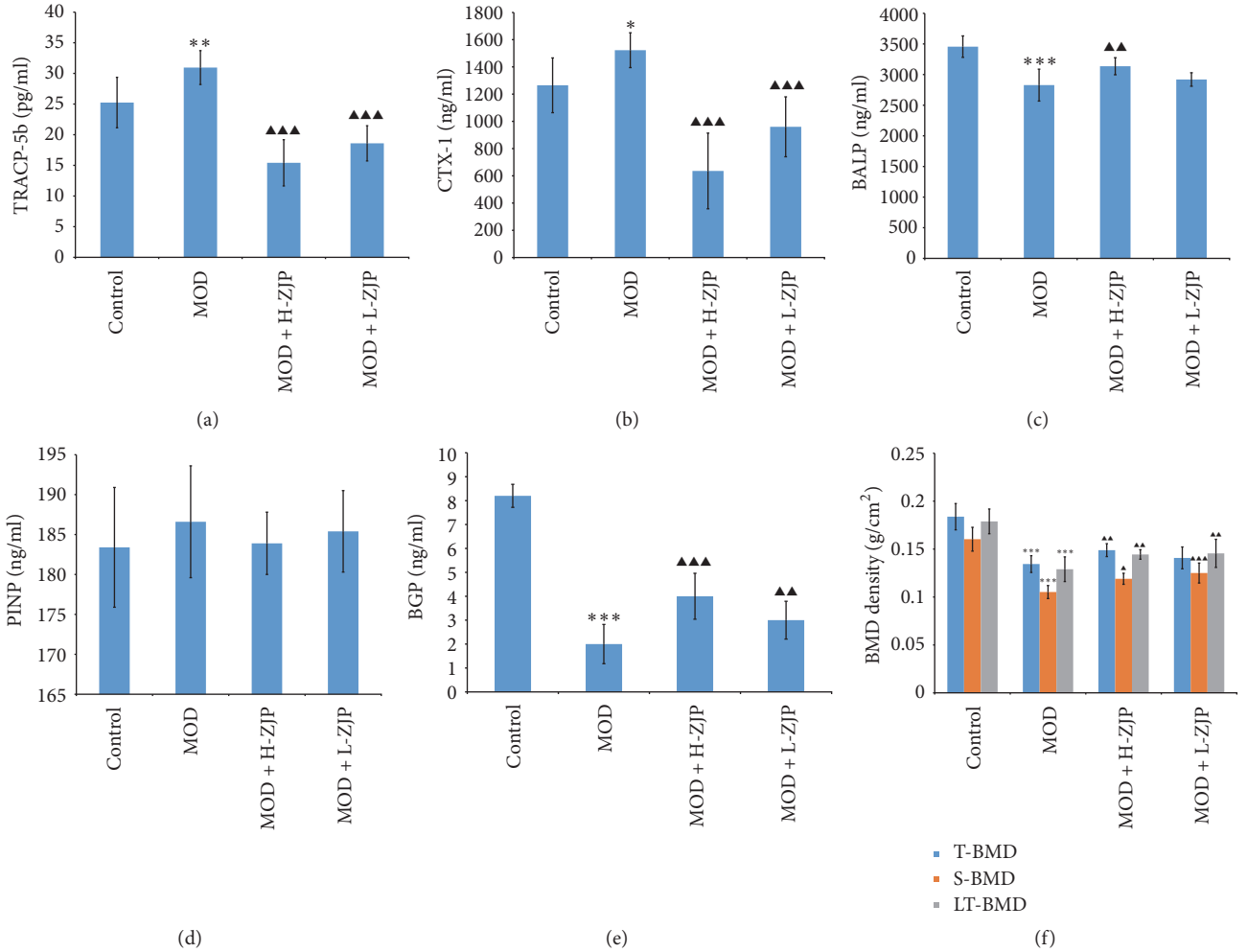

Figure 4: *Effect of ZJP on bone metabolism in diabetic rats.* (a) STZ significantly increased the level of TRACP-5b in MOD group; ZJP completely inhibited the increase of TRACP-5b level in diabetic rats. (b) STZ significantly increased the level of CTX in MOD group; ZJP completely inhibited the increase of CTX levels in diabetic rats. (c) STZ significantly decreased the level of BALP in MOD group; ZJP attenuates the reduction of BALP level at the concentration of 3.0 g/kg/d in diabetic rats. (d) The level of PINP was not changed by STZ and ZJP administration. (e) STZ significantly decreased the level of BGP in MOD group; ZJP attenuates the reduction of BGP level in diabetic rats. (f) STZ significantly decreased the T-BMD, S-BMD, and LT-BMD; ZJP partially inhibited the reduction of T-BMD, S-BMD, and LT-BMD in diabetic rats ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$, control versus MOD). Triangle indicates statistical significance ($^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, and $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$, MOD versus MOD + H-ZJP or MOD + L-ZJP).

PLS-DA score plot, the metabolic state of MOD group was significantly different from the MOD + H-ZJP and MOD + L -ZJP groups, indicating that different concentrations of ZJP can change metabolic fingerprints of rat blood, suggesting that ZJP played a therapeutic role in diabetic rats. A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes from ZJP treated groups is shown in Figure 5(d). As shown in Figure 5(e), in the PLS-DA score plot, the metabolic state of MOD group was significantly different from the MOD + H-ZJP groups, and the metabolic state of MOD + H-ZJP group was different from the MOD group, indicating that ZJP played a therapeutic role in diabetic rats. A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes from ZJP is shown in Figure 5(f).

3.6. ^{1}H-NMR Based Metabolomics Study on Urine of Diabetic Rats. PLS-DA was used to bring out the specific variation in the urine samples of MOD and CON groups. As shown in Figure 6(a), in the PLS-DA score plot, the metabolic state of MOD group was significantly different from the CON, indicating that diabetes changed the endogenous substances metabolism and significantly altered the metabolic fingerprints of rat urine. A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes from CON group is shown in Figure 6(b). As shown in Figure 6(c), in the PLS-DA score plot, the metabolic state of MOD group was significantly different from the MOD + H-ZJP and MOD + L-ZJP groups, indicating that different concentrations of ZJP can change metabolic fingerprints of rat urine. A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes

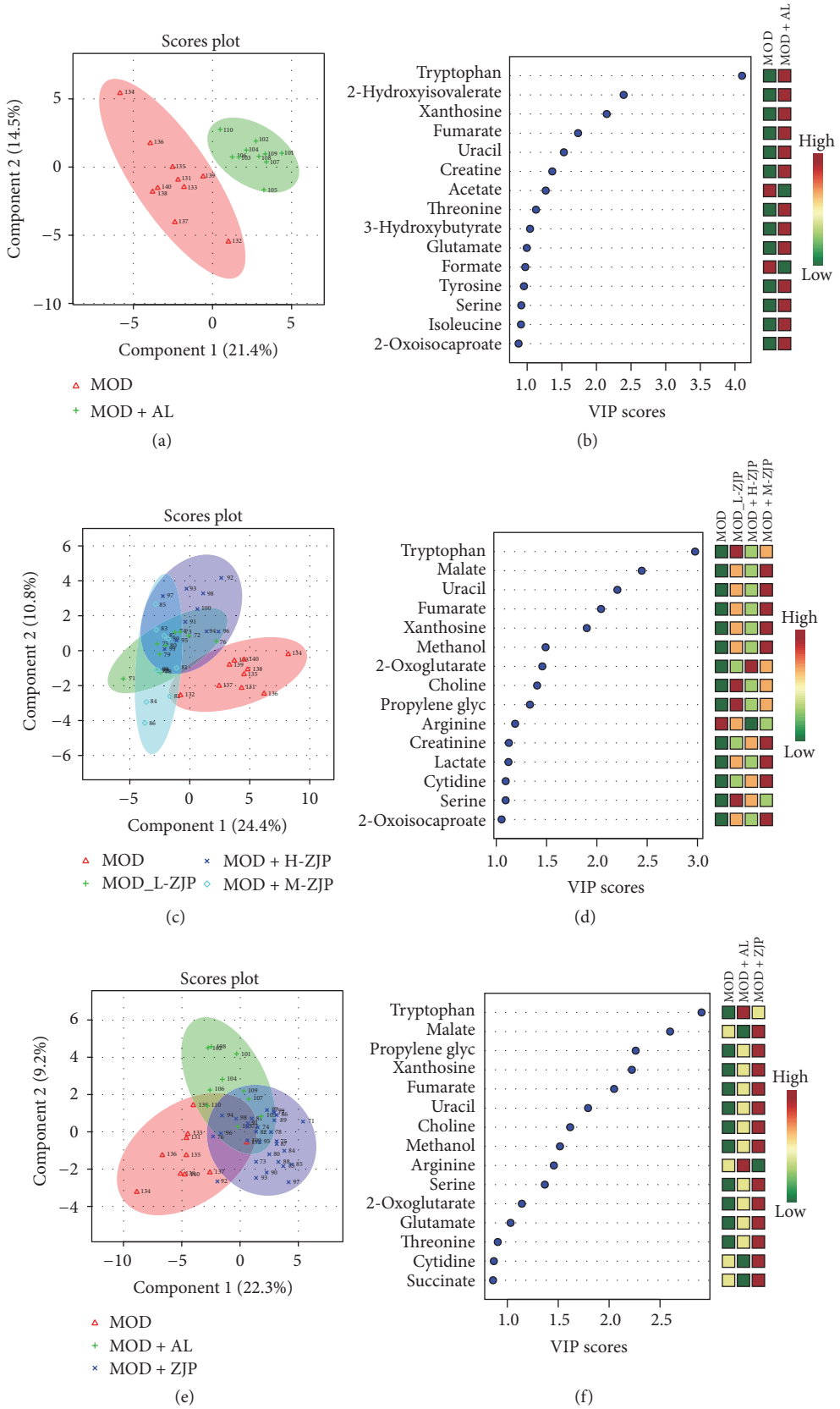

Figure 5: *Multivariate data analysis of blood metabolomics.* (a) The metabolic state of MOD and CON groups. (b) VIP scores of MOD and CON groups. (c) The metabolic state of MOD, MOD + H-ZJP, MOD + L-ZJP, and CON groups. (d) VIP scores of MOD, MOD + H-ZJP, MOD + L-ZJP, and CON groups. (e) The metabolic state of MOD and MOD + H-ZJP groups. (f) VIP scores of MOD and MOD + H-ZJP groups.

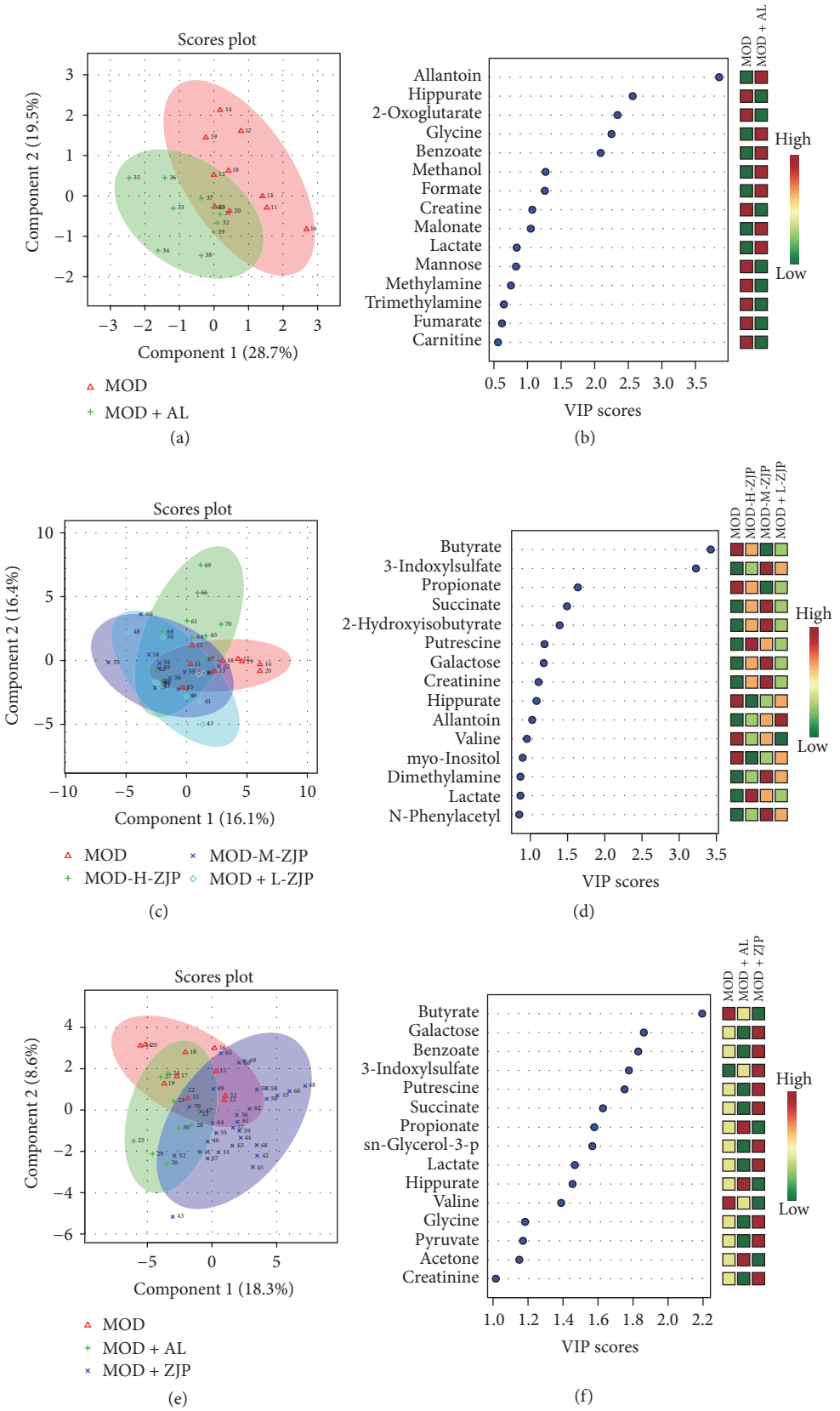

Figure 6: *Multivariate data analysis of urine metabolomics.* (a) The metabolic state of MOD and CON groups. (b) VIP scores of MOD and CON groups. (c) The metabolic state of MOD, MOD + H-ZJP, MOD + L-ZJP, and CON groups. (d) VIP scores of MOD, MOD + H-ZJP, MOD + L-ZJP, and CON groups. (e) The metabolic state of MOD and MOD + H-ZJP groups. (f) VIP scores of MOD and MOD + H-ZJP groups.

from ZJP treated groups is shown in Figure 6(d). As shown in Figure 5(e), in the PLS-DA score plot, the metabolic state of MOD group was significantly different from the MOD + H-ZJP and CON groups, and the metabolic state of MOD + H-ZJP group was different from the MOD group. A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of diabetes from ZJP groups is shown in Figure 6(f).

4. Discussion

Diabetic osteoporosis (DO), characterized by low bone mass, is a common complication of diabetes but asymptomatic in diabetic patients until the fracture [10]. It has been reported that fracture risk in T2DM patients with poor glycemic control increased by 47%–62% compared with nondiabetic patients and those with good glycemic control [25]. The basic mechanism underlying the development of osteoporosis is the bone-remodeling imbalance [26]. The characteristics of bone reconstruction associated with DO are thought to include a continuous increase in the number and activation level of osteoclasts with a stable proliferation and activity of osteoblasts [27, 28]. In previous epidemiological studies, diabetic patients show moderately increased risk for osteoporotic bone fractures compared to general population [29]. However, the underlying molecular mechanism causing osteoporosis in diabetes remains unclear. Numerous research studies are currently focusing on the effects of traditional Chinese medicine formulations on osteoporosis [30, 31].

In the present study, we investigate the effect of the traditional Chinese medicine ZJP on STZ-induced DO rat. We first examined the glucose tolerance in diabetic rats by using the OGTT; the result showed that ZJP significantly decreased the blood glucose level in diabetic rats compared with the MOD group (Figure 1), indicating that ZJP may exert a role in diabetes induced by STZ. In support of this, previous study has demonstrated that vildagliptin also elicited decrease in glucose during OGTT in patients with T2DM [32]. We further investigated the glucose metabolism in diabetic rats. We examined the levels of glucose metabolism markers including FBG, HbAlc, and Fins. Results showed that ZJP decreased the level of FBG and increased Fins level in diabetic rats but has no effect on HbAlc level (Figure 2). Generally, as the important glucose metabolism indexes, the levels of FBG and HbAlc were significantly higher, and the level of Fins was significantly lower in diabetic patients than that of nondiabetic patients. Studies showed that FBG and HbAlc levels were downregulated, and the level of Fins was upregulated in diabetic patients after drug treatment [33–35]. However, ZJP has no effect on HbAlc level in our study. This phenomenon is probably due to the fact that HbAlc is an indicator of long-term blood glucose control, whereas our experiment period is only 8 weeks. Moreover, once-daily treatment of ZJP cannot last 24 hours, which led blood glucose level to be fluctuated. Besides, we also examined the calcium-phosphorus metabolism in diabetic rats, and the results demonstrated that the levels of BCa, P, and UCa were also increased in diabetic rats, while the increase of BCa and P levels was partially inhibited by ZJP. However, the level of UCa was not changed in diabetic rats treated with ZJP (Figure 3). We next examined the bone metabolism to detect osteoporosis in diabetic rats. The bone resorption indexes TRACP-5b and CTX, as well as the bone formation indexes BALP, PINP, BGP, and BMD, were analyzed. Data showed that ZJP inhibited the increases of TRACP-5b and CTX levels (Figures 4(a) and 4(b)) and attenuated the reduction BALP and BGP levels (Figures 4(c) and 4(e)) in diabetic rats compared with the MOD group but did not change the level of PINP. Moreover, the T-BMD, S-BMD, and LT-BMD were also partially recovered by the treatment of ZJP (Figure 4(f)). These data supported that diabetes induced the formation of osteoporosis in rats, while treatment of ZJP markedly attenuated the development of osteoporosis.

Moreover, we further investigated the metabonomics of blood and urine in diabetic rats to determine the therapeutic role of ZJP on diabetic rats which has been identified with osteoporosis. Result showed that diabetes changed the endogenous substances metabolism and significantly altered the blood metabolic fingerprints of rats (Figure 5(a)). The levels of acetate, urea, acetone, and citrulline were significantly increased in the blood of DO rats (Figure 5(b)). Treatment with ZJP (H-ZJP, L-ZJP) significantly altered the endogenous substances metabolism and the metabolic fingerprints of rat blood (Figure 5(c)). The levels of acetate, urea, acetone, and citrulline are significantly decreased in the blood of DO rats after ZJP treatment (Figure 5(d)). These data suggested that ZJP might exert a therapeutic effect on DO rats.

Taken together, our results demonstrate that ZJP can effectively improve glucose metabolism through regulation of FBG and Fins, improving abnormal bone metabolism, such as inhibiting excessive bone absorption and promoting the reduced bone resorption through regulation of TRACP-5b, CTX, BALP, and BGP, enhancing BMD, and improving blood and urinary metabolism in DO rats. Our findings suggest that ZJP may be a potentially effective medicine for the treatment of DO.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Huilin Li and Shufang Chu contributed equally to this work.

Acknowledgments

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Research Article

Study of Pharmacodynamic and Pharmacokinetic Interaction of Bojungikki-Tang with Aspirin in Healthy Subjects and Ischemic Stroke Patients

Jung-Hwa Yoo,[1] Sung-Vin Yim,[2] and Byung-Cheol Lee [1]

[1]*Department of Internal Medicine, College of Korean Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun, Seoul 02447, Republic of Korea*
[2]*Department of Pharmacology, School of Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun, Seoul 02447, Republic of Korea*

Correspondence should be addressed to Byung-Cheol Lee; hydrolee@khu.ac.kr

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Background. Bojungikki-tang (BJIKT) is a widely used traditional herbal formula in China, Japan, and Korea. There have been reports that several herbs among BJIKT have interactions with antiplatelet drugs, such as aspirin. This study aimed to assess whether BJIKT interacts with aspirin in terms of pharmacokinetics (PK) and pharmacodynamics (PD) in healthy subjects and ischemic stroke patients. *Methods*. The phase I interaction trial was a randomized, open-label, crossover study of 10 healthy male subjects, and the phase III interaction trial was a randomized, placebo-controlled, parallel study of 43 ischemic stroke patients. Each participant randomly received aspirin + BJIKT or aspirin + placebo. For PK analysis, plasma acetyl salicylic acid (ASA) and salicylic acid (SA) were evaluated, and, for PD analysis, platelet aggregation and plasma thromboxane B_2 (TxB_2) were measured. *Results*. In the PK parameters, mean area under curve, maximum concertation, and peak concentration time of ASA and SA were not different between two groups in healthy subjects and ischemic stroke patients. In the PD profiles, TxB_2 concentrations and platelet aggregation were not affected by coadministration of BJIKT in healthy subjects and ischemic stroke patients. *Conclusions*. These results suggest that coadministration of BJIKT with aspirin may not result in herb-drug interaction.

1. Introduction

The use of combination of herbal supplements and modern drugs has become increasingly popular in recent years [1]. Although this combined therapy has been reported to be beneficial in strengthening therapeutic effects or reducing side-effects, numerous reports exist in which negative, undesired effects have been reported [1, 2].

A drug–herb interaction can be defined as pharmacologic or clinical response to the coadministration of modern pharmaceutical drugs and herbal products [3]. The high prevalence of both conventional pharmacological therapy and herbal medicines use draws attention to safety concerns. However, the prevalence of drug–herb interactions is unknown, signifying the negligence of the consumers in reporting adverse herb reaction or drug–herb interactions. [3, 4].

Bojungikki-tang (BJIKT; Bu-Zhong-Yi-Qi-Tang in Chinese or Hochu-ekki-to in Japanese) is a widely used traditional herbal formula in China, Japan, and Korea. BJIKT, originally meaning "Tonify the Middle and Augment the Qi Decoction," has been identified as an effective medication to improve conditions such as general fatigue and poor appetite and as an adjunct to treating stroke caused by qi deficiency and treating debilitating condition resulting from sequelae of cerebrovascular disease [5–8]. There have been reports that several medicinal herbs among BJIKT composed natural herbs have interactions with antiplatelet drugs, such as aspirin [9–14].

However, to date, there have been no randomized clinical trials focusing on herb-drug interaction. Accordingly, the objective of this study was to assess whether BJIKT interacts with an antiplatelet drug in terms of pharmacokinetics (PK)

Table 1: Baseline characteristics of healthy subjects and ischemic stroke patients.

	Healthy subjects (n = 10)	Ischemic stroke		
		Aspirin + placebo (n = 21)	Aspirin + BJIKT (n = 22)	P
Age	28.7 ± 4.2	60.0 ± 10.9	64.72 ± 7.1	NS
Male (n, %)	10 (100)	13 (61.9)	14 (63.6)	NS
BMI (kg/m^2)	23.7 ± 1.7	24.7 ± 3.1	24.1 ± 1.7	NS
Heart disease (n, %)	0	3 (14.2)	7 (31.8)	NS
HBP (n, %)	0	13 (61.9)	14 (63.6)	NS
Diabetes (n, %)	0	6 (28.5)	6 (27.2)	NS
Dyslipidemia (n, %)	0	10 (47.6)	9 (40.9)	NS
Smoking (n, %)	0	2 (9.5)	2 (9.1)	NS
Alcohol (n, %)	0	7 (33.3)	8 (36.3)	NS

BJIKT: Bojungikki-tang, BMI: body mass index, and HBP: high blood pressure.

and pharmacodynamics (PD) in healthy individuals as well as in ischemic stroke patients.

2. Materials and Methods

2.1. Subjects. The phase I study population consisted of 10 healthy adult male volunteers. The mean age was 25.4 ± 3.4 years (20–33 years). The mean body weight was 70.9 ± 10.2 kg and the mean height was 175.5 ± 4.9 cm. Significant exclusion criteria for study included a history of allergy to aspirin or herbal medication; history of renal, hepatic, cardiovascular, gastrointestinal, or neurologic diseases that might significantly alter the absorption, distribution, metabolism, and excretion of the study drug; known hypersensitivity to the study drugs; acute disease within the past 28 days from the administration of a drug; participation in another clinical study within the past 60 days; receiving medications that induce or inhibit drug-metabolizing enzymes, such as barbiturates, within past 30 days; history of excessive drinking; illiteracy; or inability to be protected by parental rights.

The phase III study population was selected from 322 ischemic stroke patients over 40 years old (range 41–77 years) who were living in Korea. Prospective participants were screened at Kyung Hee University Medical Center from March 2010 to April 2011. Of these, 43 subjects participated in this study. The inclusion criteria included individuals taking aspirin for over 3 months with a previous diagnosis of ischemic stroke, defined as an acute focal or global neurological deficit lasting more than 24 hours without an apparent cause other than vascular origin, consecutively confirmed by magnetic resonance imaging (MRI) within 72 hours of the onset of symptoms. Patients with cerebral hemorrhage, cerebral venous thrombosis, or a brain tumor and those who met any of the phase I exclusion criteria were excluded. Of the 43 ischemic stroke patients enrolled in phase III, 39 subjects completed the study and were included in PK and immunogenicity analyses (17, aspirin + BJIKT; 22, aspirin + placebo). Four subjects from the aspirin + placebo group and 0 subjects in the aspirin + BJIKT group withdrew consent after receiving the study drug. The baseline demographic characteristics of subjects in each group were well balanced within the groups (Table 1).

Both phase I and phase III studies were approved by the institutional review board of Kyung Hee University Medical Center (phase I: KMC IRB 0917-03-A2; phase III: KOMC IRB 2009-15) and were also approved by Korean Food Drug Administration (KFDA) (phase I: 2010-135; phase III: 2009-1080). Written informed consent was obtained from each participant, and studies were conducted in accordance with the principles of the International Conference of Harmonization for Good Clinical Practice (ICH-GCP) and the ethical standards for human experimentation established in the Declaration of Helsinki. The study was registered with Clinical Research Information Service (CRIS): KCT0002049.

2.2. Study Drugs. We purchased the BJIKT extract granules that contain a mixture of spray-dried hot water extracts of 10 medicinal plants from Hanpoong Pharmacy & Foods Company (Seoul, Korea). The 10 medical plants are Astragali radix (16.7%), Atractylodis lanceae rhizoma (16.7%), Ginseng radix (16.7%), Angelicae radix (12.5%), Bupleuri radix (8.3%), Zizyphi fructus (8.3%), Aurantii nobilis pericarpium (8.3%), Glycyrrhizae radix (6.3%), Cimicifugae rhizoma (4.2%), and Zingiberis rhizoma (2.0%). A voucher specimen (code number HX018) was deposited in herbarium in the department of herbal pharmacy, Kyung Hee Korean Medical Hospital.

Each herb in BJIKT was quality controlled from the places of origin to the final products. The active ingredients were also quality controlled by using high-performance liquid chromatography. According to the compilation of specification and test procedures of Hanpoong Pharmacy & Foods Company, BJIKT contains 52.0 mg of hesperidin ($C_{28}H_{34}O_{15}$ in Citri Unshii Pericarpium), 5.4 mg of ginsenoside Rb_1 ($C_{54}H_{92}O_{23}$ in Ginseng radix), 50.0 mg of decursin ($C_{19}H_{20}O_5$ in Angelicae Gigantis Radix), 6 mg of zingerol ($C_{17}H_{26}O_4$ in Zingiberis Rhizoma), and 67.5 mg of glycyrrhizic acid ($C_{42}H_{62}O_{16}$ in Glycyrrhizae Radix et Rhizoma) per pack.

And enteric-coated tablet aspirin (Aspirin Cardio™) 100 mg was donated from Bayer HealthCare Pharmaceuticals, Germany.

2.3. Protocol. The phase I interaction study was conducted in the Kyung Hee Clinical Research Institute, Kyung Hee Medical Center, Kyung Hee University (Seoul, Korea). It was a randomized, open-label, crossover study of 10 healthy male subjects. A day before the study, eligible subjects were hospitalized in the Kyung Hee Clinical Research Institute. After overnight fasting, each subject randomly received an oral administration of 3 packs of BJIKT or placebo (1 pack volume: 6.83 g) at 7 AM and additionally given the 2 capsules of Aspirin Cardio 100 mg (Bayer HealthCare Pharmaceuticals, Germany) at 8 AM with 240 ml tab water. For the pharmacokinetic analysis, blood acetyl salicylic acid and salicylic acid were measured 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 600 min after aspirin administration. For pharmacodynamic analysis, platelet aggregation was measured 0, 2, and 4 h, and plasma thromboxane B2 (TxB2) was measured 0, 5, 10, 20, 30, 45, and 60 min after aspirin administration. After 1 week wash-out period, administration of each group was exchanged, and all measurements were repeated in the same manner.

The phase III interaction study was conducted at Kyung Hee Korean Medical Hospital, Kyung Hee University (Seoul, Korea). It was a randomized, placebo-controlled, double-blinded, parallel study. Eligible ischemic stroke patients were randomly allocated to either BJIKT treatment or placebo, in addition to aspirin. After allocation, each subject randomly received oral administration of BJIKT or placebo 3 times a day (1 pack volume: 6.83 g) as well as 1 capsule of Aspirin Cardio 100 mg every morning for 2 weeks [15]. For PK analysis, blood ASA and SA were measured 2 hours after aspirin administration at 0, 1, and 2 weeks. For PD analysis, platelet aggregation and plasma TxB_2 were measured 1 hour after aspirin administration at 0, 1, and 2 weeks. At every visit, all of the laboratory tests done at screening as well as basic exams (weight, height) were repeated. Blood samples were collected for participant safety and outcome assessment. Participants were asked about adverse events, and BJIKT packs and aspirin capsules were counted to assess compliance (Table 5).

2.4. Measurement of Acetyl Salicylic Acid and Salicylic Acid. Plasma concentration of acetyl Salicylic acid (ASA) and salicylic acid (SA) which is the further metabolite of ASA was determined with liquid chromatography (UPLC, Waters Corp)/tandem mass spectrometry (Qtrap 5500, AB sciex). The compounds were separated using reverse column (ACQUITY UPLC C18 Column, 1.7 μm, 2.1 × 50 mm) with an isocratic mobile phase consisting of acetonitrile and water (70 : 30, v/v; with 0.1% formic acid) at flow rate 0.2 ml/min. Detection was performed using electrospray ionization (ESI) source in the negative ion mode at −4500 eV and 600°C. The operating conditions were optimized for each of the analytes and were determined as follows: nebulizing gas (Gas1), 60; heater gas (Gas2), 60; curtain gas, 30. Quantification was performed by multiple-reaction monitoring (MRM). The masses for ASA and SA were m/z 179 → 135 (with declustering potential −80, collision energy −16) and 136.8 → 93 (with declustering potential −140, collision energy −15.5). The internal standard of this study was simvastatin and the mass was m/z 434.6 → 367 (with declustering potential −60, collision energy −15). The lower limit of quantification (LLOQ) for ASA and SA was 5 ng/mL and 50 ng/mL, respectively.

Pharmacokinetic parameters for ASA and SA were determined including C_{max} (maximum plasma concentration), T_{max} (time point of maximum plasma concentration), and $AUC_{0-\infty}$ (area under the plasma concentration versus time curve from 0 h to infinity).

2.5. Measurement of Platelet Aggregation and Plasma Thromboxane B2 Level. Platelet aggregation experiments were performed using Chrono-Log model 700 two-channel whole Blood/Optical Lumi-Aggregometer (Chrono-Log Corporation). Platelet-rich plasma (PRP) was obtained from the citrated blood centrifugation (Beckman Allegra 6R) at 800 rpm for 10 min. Platelet-poor plasma (PPP) was obtained from PRP centrifugation (eppendorf 5415R) at 13000 rpm for 10 min. Collagen (Chrono-Log Corporation) was used to induce the platelet aggregation. The change in absorbance was recorded until the response reached a plateau or for 5 min.

Plasma thromboxane B_2 (TXB_2) was measured using an enzyme immunoassay kit (Thromboxane B_2 EIA Kit, Cayman Chemical, MI, USA). The standard curve was prepared as outlined in the manufacturer's instructions. The thawed test and control plasma samples were tested in duplicate.

2.6. Statistical Analysis. The study sample sizes were determined from variance estimates based on prior aspirin platelet aggregation PD data [15]. To evaluate clinically relevant interactions, we used the noninferiority approach with 90% test power and a two-sided alpha value of 0.05. Data were presented as the mean ± standard deviation. Baseline demographics and clinical variables in the phase III study were compared among treatment groups using a Student's t-test, chi-square test, or Fisher's exact test. The changes in PK or PD 1 and 2 weeks from baseline were compared between the BJIKT treatment group and the placebo group using a Student's t-test. All P values were two-tailed, and significance was set at $P < 0.05$. All statistical analyses were performed using the GraphPad Prism for Windows, Version 5.01 (GraphPad Software, Inc.).

3. Results

3.1. Pharmacokinetic Effects of Aspirin and Bojungikki-Tang Coadministration. In the phase I trial with healthy subject, the area under curve (AUC) of acetyl salicylic acid in placebo group was 60959.5 ± 14243.3 ng/ml, while in BJIKT group it was 53465.3 ± 6777.6 ng/ml. Mean ± SE peak plasma concentration (*C*max) value in the placebo was 440.8 ± 100.8 ng/ml, while in the BJIKT it was 418.2 ± 81.5 ng/ml. Time to peak concentration (*T*max) of salicylic acid in placebo was 312.0 ± 43.6 min, while in the BJIKT it was 312.0 ± 24.9 min. There were no significant differences between two groups in mean values of AUC, *C*max, and *T*max (Table 2, Figure 1). The plasma concentrations of salicylic acid at various time intervals have been plotted in Figure 1. When an Aspirin was administrated with BJIKT, the area under the curve (AUC) of salicylic acid in placebo

Table 2: Pharmacokinetic parameters among healthy subjects (phase I study).

	ASA			SA		
	Aspirin + placebo	Aspirin + BJIKT	P	Aspirin + placebo	Aspirin + BJIKT	P
AUC_{0-last} (ng m/ml)	60959.5 ± 14243.3	53465.3 ± 6777.6	N.S.	1704207.8 ± 276367.4	1986552.4 ± 252995.5	NS
C_{max} (ng/ml)	440.8 ± 100.8	418.2 ± 81.5	N.S.	6284.3 ± 1029.1	7550.8 ± 906.2	NS
T_{max} (min)	312.0 ± 43.6	312.0 ± 24.9	N.S.	414.0 ± 44.2	348.0 ± 21.5	NS
$t_{1/2}$ (min)	68.4 ± 21.6	50.1 ± 10.7	N.S.	319.2 ± 118.6	252.0 ± 57.4	NS

BJIKT: Bojungikki-tang, ASA: acetyl salicylic acid, SA: salicylic acid, AUC_{0-last}: area under the serum concentration-time curve from time 0 to 10 hours after aspirin administration, C_{max}: maximum plasma concentration, T_{max}: time point of maximum plasma concentration, and $t_{1/2}$: terminal elimination half-life.

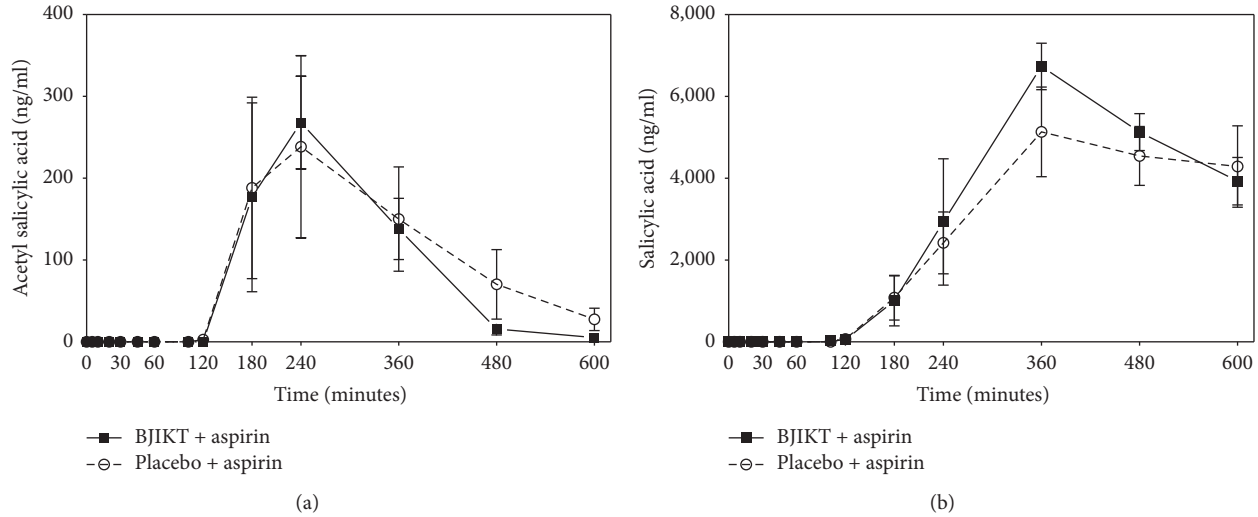

Figure 1: The pharmacokinetic profiles of healthy subjects (phase I study). Data are shown as mean and standard deviation of acetyl salicylic acid (a) and salicylic acid levels (b) in Bojungikki-tang (BJIKT) + aspirin and placebo + aspirin.

group was 1704207.8 ± 276367.4 ng/ml, while in BJIKT group it was 1986552.4 ± 252995.5 ng/ml. Mean ± SE peak plasma concentration (*C*max) value in the placebo was 6284.3 ± 1029.1 ng/ml, while in the BJIKT it was 7550.8 ± 906.2 ng/ml. Time to peak concentration (*T*max) of salicylic acid in placebo was 414.0±44.2 min, while in the BJIKT it was 348.0± 21.5 min. There were no significant differences between two groups in mean values of AUC, *C*max, and *T*max (Table 2, Figure 1).

In the phase III trial with ischemic stroke patients, the plasma concentration of ASA with 100 mg of aspirin in the placebo group was decreased to be −15.3 ± 14.5 ng/ml at 2 weeks, compared with 27.8 ± 17.4 ng/ml at baseline. The ASA in the BJIKT group was decreased to be −18.3±14.1 ng/ml at 2 weeks, compared with 19.1 ± 11.5 ng/ml at baseline (Table 4). However, there was no significant difference between the combination of aspirin with BJIKT and the placebo. When aspirin was administrated with BJIKT, the mean plasma SA concentration changed −374.1 ± 506.3 compared with baseline (2298.6 ± 598.2). In the placebo group, the mean plasma SA concentration decreased −244.1 ± 522.9 ng/ml at 2 weeks compared with baseline (2953.7 ± 571.2 ng/ml) (Table 4). A statistically significant change in SA was not detected in either the BJIKT or the placebo group.

3.2. Pharmacodynamic Effects of Aspirin and Bojungikki-Tang Coadministration. In the phase I trial with healthy subject, when aspirin was administrated with a placebo, a 60.5% rapid decrease in mean plasma TxB_2 concentrations compared with baseline was detected at 5 min ($P < 0.001$). In the BJIKT group, a 62.2% decrease in mean plasma TxB_2 was also detected at 5 min ($P < 0.001$) (Table 3, Figure 2).

Table 3: Pharmacodynamic parameters among healthy subjects (phase I study).

	Aspirin + placebo	Aspirin + BJIKT	P
TxB_2 (pg/ml)			
0 min	173.6 ± 41.2	163.4 ± 41.8	NS
5 min	68.5 ± 14.8	61.7 ± 21.5	NS
10 min	55.81 ± 10.9	47.6 ± 13.9	NS
20 min	58.8 ± 17.8	47.6 ± 16.8	NS
30 min	70.1 ± 36.7	59.9 ± 17.8	NS
45 min	70.9 ± 25.2	71.9 ± 15.7	NS
60 min	78.9 ± 18.7	75.4 ± 21.1	NS
PLT agg (%)			
0	77.4 ± 5.4	78.1 ± 5.4	NS
2 hours	76.1 ± 8.6	78.9 ± 5.5	NS
4 hours	66.7 ± 14.7	66.9 ± 14.9	NS

BJIKT: Bojungikki-tang, TxB_2: thromboxane B_2, PLT agg: platelet aggregation.

Table 4: Change in pharmacokinetic and pharmacodynamic profiles and blood tests among ischemic stroke patients (phase III study).

	Aspirin + placebo		Aspirin + BJIKT		P
	Baseline	Net change from baseline	Baseline	Net change from baseline	
PK					
ASA	27.8 ± 17.4	−15.3 ± 14.5	19.1 ± 11.5	−18.3 ± 14.1	NS
SA	2953.7 ± 571.2	−244.1 ± 522.9	2298.6 ± 598.2	−374.1 ± 506.3	NS
PD					
TxB_2 (pg/ml)	16.2 ± 3.4	−13.9 ± 2.5	21.1 ± 5.3	−13.6 ± 3.6	NS
PLT agg (%)	74.8 ± 3.6	−0.2 ± 5.1	73.6 ± 2.5	−1.1 ± 2.8	NS
Blood chemistry					
FBG (mg/dl)	117.9 ± 39.8	−0.4 ± 52.1	120.8 ± 48.7	−9.2 ± 59.9	NS
T-chol (mg/dl)	171.0 ± 41.7	30.8 ± 141.4	171.1 ± 38.5	−5.1 ± 22.9	NS
TG (mg/dl)	153.7 ± 82.3	−17.2 ± 68.5	161.7 ± 69.9	−5.3 ± 57.4	NS
Ca	9.2 ± 0.3	5.1 ± 21.2	9.1 ± 0.2	4.0 ± 18.1	NS
P	3.1 ± 0.6	0.01 ± 0.4	3.1 ± 0.5	0.08 ± 0.4	NS
Uric acid	5.5 ± 1.5	0.1 ± 0.6	5.4 ± 1.6	−0.3 ± 0.6	NS
Blood count					
WBC	6.5 ± 1.0	−0.1 ± 1.4	6.8 ± 1.1	−0.1 ± 0.5	NS
RBC	4.7 ± 0.5	0.4 ± 1.4	4.5 ± 0.5	0.0 ± 0.2	NS
Hgb	14.1 ± 1.6	0.0 ± 0.6	13.8 ± 1.5	0.1 ± 0.5	NS
Hct	41.4 ± 4.4	0.1 ± 1.7	40.6 ± 4.6	0.2 ± 1.9	NS
Platelet	277.1 ± 82.2	10.8 ± 24.1	263.7 ± 69.9	9.9 ± 37.6	NS

BJIKT: Bojungikki-tang, ASA: acetyl salicylic acid, SA: salicylic acid, TxB_2: thromboxane B_2, PLT agg: platelet aggregation, FBS: fasting blood glucose, T-chol: total cholesterol, TG: triglyceride, Ca: calcium, P: phosphorus, WBC: white blood cell, RBC: red blood cell, Hgb: hemoglobin, and Hct: hematocrit.

Table 5: Safety parameters among ischemic stroke patients (phase III study).

	Aspirin + placebo		Aspirin + BJIKT		P
	Baseline	Net change from baseline	Baseline	Net change from baseline	
Liver function					
AST	24.7 ± 4.9	1.8 ± 5.9	25.2 ± 5.4	2.0 ± 6.3	NS
ALT	23.5 ± 11.7	1.2 ± 8.6	21.4 ± 8.1	1.6 ± 6.2	NS
GGT	29.6 ± 12.5	0.0 ± 4.4	29.1 ± 16.2	0.1 ± 4.9	NS
T-bil	0.6 ± 0.2	−0.0 ± 0.1	0.6 ± 0.2	0.0 ± 0.1	NS
ALP	78.1 ± 16.9	0.7 ± 7.4	68.5 ± 15.8	0.8 ± 6.2	NS
Protein	7.6 ± 0.5	0.0 ± 0.4	7.4 ± 0.4	−0.0 ± 0.4	NS
Albumin	4.4 ± 0.1	−0.0 ± 0.2	4.3 ± 0.2	−0.0 ± 0.1	NS
Kidney function					
BUN	15.6 ± 2.5	−1.8 ± 4.1	16.6 ± 5.3	−2.3 ± 4.7	NS
Cr	0.7 ± 0.1	−0.0 ± 0.0	0.8 ± 0.4	−0.0 ± 0.0	NS
Adverse effects					
Indigestion		1		1	NS
Diarrhea		1		0	NS

BJIKT: Bojungikki-tang, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glutamyl transferase, T-bil: total bilirubin, ALP: alkaline phosphatase, and BUN: blood urea nitrogen.

A statistically significant decrease in plasma TxB_2 was not detected in either the BJIKT or placebo groups at baseline or various time intervals. The inhibition of platelet aggregation at various time intervals for the BJIKT or placebo groups combined with aspirin was analyzed. In the placebo group, platelet aggregation with 300 mg of aspirin was found to be 66.7 ± 14.7% at 4 h compared with 77.4 ± 5.4% at baseline ($P < 0.001$). In the BJIKT group, the platelet was decreased to be 66.9±14.9% at 4 h compared with 78.1±5.4% at baseline ($P < 0.001$). However, the combination of aspirin with either BJIKT or a placebo did not potentiate the inhibition of collagen-induced platelet aggregation (Table 3, Figure 2).

In the phase III trial with ischemic stroke patients, the effects of the combination of aspirin with either BJIKT or

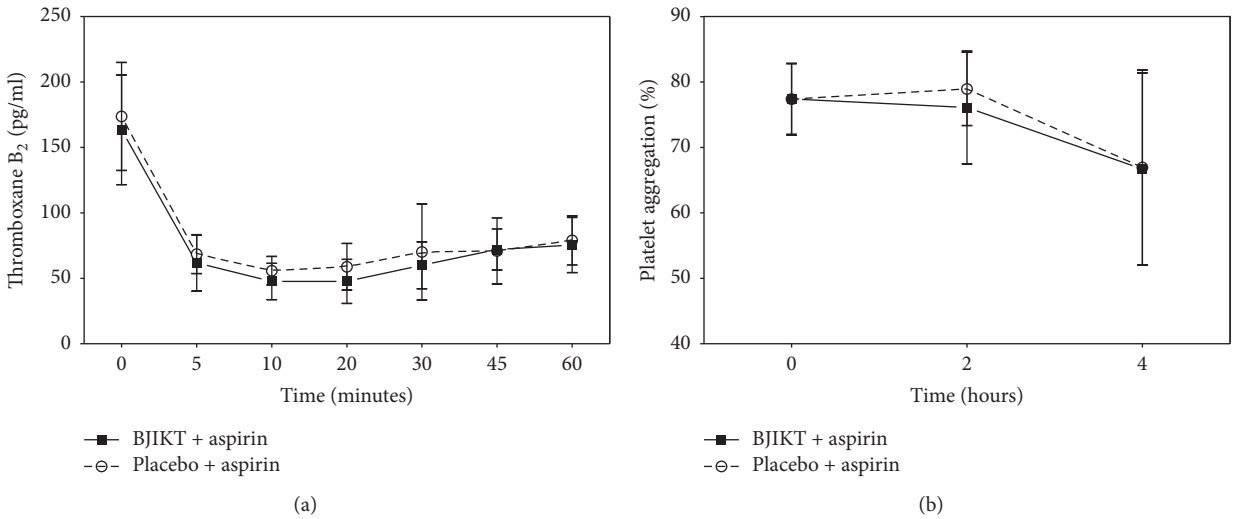

Figure 2: The pharmacodynamic profiles of healthy subjects (phase I study). Data are shown as mean and standard deviation of thromboxane B2 (a) and platelet aggregation (b) in Bojungikki-tang (BJIKT) + aspirin and placebo + aspirin.

placebo on plasma TxB_2 concentrations are shown in Table 4. When aspirin was administrated with a placebo, the mean plasma TxB_2 concentrations decreased -13.9 ± 2.5 pg/ml in 2 weeks compared with baseline (16.2 ± 3.4 pg/ml). In the BJIKT group, mean plasma TxB_2 concentration changed -13.6 ± 3.6 pg/ml in 2 weeks (Table 4). However, a statistically significant decrease in plasma TxB_2 was not detected in either the BJIKT or placebo groups. The inhibition of platelet aggregation at baseline and 1 and 2 weeks for the BJIKT and placebo groups combined with aspirin was accessed. In the placebo group, the platelet aggregation with 100 mg of aspirin was decreased to be $-0.2 \pm 5.1\%$ at 2 weeks compared with $74.8 \pm 3.6\%$ at baseline. In the BJIKT group, the platelet aggregation was found to be $-1.1 \pm 2.8\%$ at 2 weeks compared with $73.6 \pm 2.5\%$ at baseline. However, the combination of aspirin with either BJIKT or the placebo did not change the inhibition of collagen-induced platelet aggregation (Table 4).

4. Discussion

This is the first study to investigate the PK and PD profiles of the herb-drug interaction between BJIKT and aspirin among healthy subjects and patients with ischemic stroke. Overall, there were no apparent differences in the PK profiles of ASA and SA and PD profiles of TxB_2 and platelet aggregation between aspirin alone and coadministration with BJIKT among either healthy subjects or ischemic stroke patients, suggesting that BJIKT may not interact with aspirin.

The increased use of herbal medicines and supplements worldwide has substantially increased the number of potential drug interactions with modern drugs, and there has been the documented experimental and clinical evidence of interactions between herbal products and modern drugs [9]. Among these, herbs that may influence the effect of antiplatelet treatment are of particular interest, considering that treatment for coronary artery diseases and ischemic stroke is quite common, and aspirin is one of the drugs most frequently used to treat these diseases.

BJT may be prescribed for stroke caused by qi deficiency. It is also a typical prescription for deficiency of middle qi and overall symptoms of qi deficiency [16]. This prescription may tonify the spleen and raise and circulate pure qi, which is effective against fever, heart discomfort, sweating, fatigue, dizziness, numbness, and weakness [16]. BJIKT is composed of 10 natural herbs, among which Ginseng inhibited TxA_2 formation and thus platelet aggregation in *in vitro* studies [9, 17, 18] and impaired platelet aggregation in rats [19]. Astragalus could reduce platelet adhesion and aggregation, reduce plasma fibrinogen, and show antithrombus formation effect [10–12]. Glycyrrhizae can inhibit thrombin and platelet aggregation, therefore enhancing the risk of bleeding with antiplatelets and anticoagulants [4]. Hesperidin, which is major component of Citri Unshii Pericarpium, has been documented to inhibit TxB_2 formation and human platelet aggregation [14]. However, there are controversial reports that Ginseng inhibited CYP2D6, but the magnitude of the effect did not appear clinically relevant [20], and Citri Unshii demonstrated a relatively low frequency of drug interactions and had weak inhibitory effects on CYP2C9, which metabolizes NSAIDs [21, 22]. Furthermore, cocktail herbal medicine including Ginseng and Citrus, not single herb, indicated no significant effect on CYP1A2, CYP2D6, CYP2E1, and CYP3A4 activity in healthy volunteers [20]. Therefore, our results, along with previous reports, support the idea that BJIKT does not affect the antiplatelet effects of aspirin.

In the PK study, C_{max}, T_{max}, and AUC of plasma ASA and SA were not changed by BJIKT among healthy subjects and ischemic stroke patients. And PD profiles showed no apparent differences in the TxB_2 and platelet aggregation between aspirin alone and coadministration with BJIKT among healthy subjects and even among ischemic stroke patients.

Most herb-drug interaction studies have used *in vitro* testing of herbal constituents in microsomal system or conducted in healthy subjects, but most relevant results have

been obtained when conducted in the patients who have used the herb and drug together. Thus, the advantage of this study is conducted with patients as well healthy subjects. However, BJIKT consist of 10 medicinal plants and contain multiple compounds, which may not accurately represent all the effects of each plant and/or compound. Moreover, the sample size of this study is relatedly small and all participants are Korean, which may also have the limitation of applying study result to other races. Therefore, further study with large sample and various races should be required.

5. Conclusions

In conclusion, the PK and PD profiles of aspirin were not affected by combined treatment with BJIKT. No safety clinical concerns were raised among ischemic stroke patients. These results suggest that coadministration with BJIKT for the purpose of antiplatelet effects may not result in herb-drug interaction.

Disclosure

The authors are responsible for the writing and contents of the paper.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgments

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Review Article

Evidence-Based Study to Compare *Daodi* Traditional Chinese Medicinal Material and Non-*Daodi* Traditional Chinese Medicinal Material

Xingyue Yang[1,2] **Xin Tian**[3] **Yannan Zhou**[3] **Yali Liu**[4,5] **Xinlong Li**[4] **Tingting Lu**[5] **Changhe Yu**[6] **and Liyun He**[4]

[1]*Beijing University of Chinese Medicine, Beijing 100700, China*
[2]*State Key Laboratory Breeding Base of Dao-Di Herbs, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[3]*The First Clinical Medical College of Lanzhou University, Lanzhou 730000, China*
[4]*Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[5]*Evidence-Based Medicine Center, School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, China*
[6]*Department of Tuina and Pain Management, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100700, China*

Correspondence should be addressed to Liyun He; hely3699@163.com

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Background. *Daodi* medicinal material is widely used in Chinese herb medication. However, there is a lack of systematic methodology for identifying characteristics associated with good quality and reliable efficacy of *Daodi* med-material. *Purpose*. The purpose of this study is to provide some evidence to further substantiate the use of *Daodi* medicinal materials. *Methods*. Seven relevant databases were searched before July 2014. Two evaluators were responsible for screening and categorizing the results. The data was analyzed with Microsoft Excel 2007 and SPSS 21.0 statistical software. *Results*. Overall, 107 articles were systematically analyzed. Of these studies, 55.1% (59/107) focused on the methodology to assess *Daodi* med-material, and 38.3% (41/107) were interested in med-material ingredients, soil physical and chemical properties, and the geological background system (GBS). Only 6.5% (7/107) of studies were mainly conducted as clinical trials and animal experiments. *Conclusion*. Comparisons between *Daodi* and non-*Daodi* materials have been studied mainly in terms of the ingredients or composition of medical materials, soil physics and chemistry, and the GBS, and some identifying methodologies have been created to identify *Daodi* attributes. Until now, there is still no consensus of comparison criteria between *Daodi* and non-*Daodi* medicinal material. Only a few studies were conducted through animal experiments and clinical trials to determine *Daodi* superiority.

1. Introduction

The term *"Daodi"* medicinal material has often been used in Chinese ethnopharmacology, and it is usually defined as a material that has been screened after a long period of traditional medical practice, growing in a specific region, and associated with a unique production method. Thus, materials with this label are recognized as having high quality and being clinically effective, and they are a reputable hallmark compared to other medicinal materials that are non-*Daodi* and so forth [1].

The Chinese word *"Daodi"* accentuates some distinctive higher quality for the medicinal material that grows in a certain area. The pristine exploration can be retraced to the late Eastern Han Dynasty (25–220 CE) with the advent of the earliest Chinese medicine document *Divine Husbandman's Classic of Materia Medica* (Shennong Ben Cao Jing). In the Tang Dynasty (618–907 CE), the famous scholar Sun Simiao (the renowned Medicinal Material King) proposed in his classic work *Thousand Gold Pieces (Qian jin fang)* categorizing the original region for producing medicine in the contemporary administrative province and emphasized a concept

that medicinal material is essentially embedded in soil. Sun Simiao first used the term *"Dao,"* which was the rudimentary concept that later became known as *"Daodi."* The *"Daodi"* concept was initially defined in the Chinese medical classic *Essentials of Materia Medica Distinctions (Ben cao pin hui jing yao)* in the age of the Ming Dynasty (1368–1644 CE) [2]. The medicinal selection methods had in fact been gradually formulated during medical practice for thousands of years and were established as a unique way to identify materials. Being supported by profound Chinese medical theory, this method is still significant in modern times. Furthermore, the *Daodi* materials are the most thoroughly investigated materials, and they represent a large amount of the market with tremendous economic value. It is reported that there are 200 *Daodi* materials out of the 500 traditional medicinal materials, yet *Daodi* materials contribute approximately 80% to the overall usage [3].

Chinese herbal medicine has been used in China for over 2000 years [4]. In traditional Chinese medicine (TCM), *Daodi* materials are considered to have high medicinal efficacy [5]. Most of the studies comparing *Daodi* and *non-Daodi* materials were from China. The theory and practice have been developed over thousands of years. Considering territory distribution, *Daodi* medicinal material can be categorized into Chuan- (Sichuan) Guang- (Guangdong/Guangxi) Yun- (Yunnan) Gui (Guizhou), Nan (Southern China) Bei- (Northern China) Zhe (Zhejiang) Huai (Henan), and Shan- (Shanxi) Gan- (Gansu) Qing- (Qinghai) Ning (Ningxia). Each medicinal material is rooted in locations with optimal breeding conditions. Because it is the delicate complexity in a natural land that endows a *Daodi* material with its perplexing mechanism, it has been conventionalized, probably for an expedient solution, through the use of empiricism to differentiate *Daodi* and *non-Daodi* materials. As a result, although *Daodi* medicinal materials are highly valued and renowned nationwide, until now, there has been a lack of sufficient evidence to corroborate traditional practices during *Daodi* material selection and to identify the superiority, in terms of either quality or clinical efficacy [6].

The purpose of this study is to provide some evidence to further substantiate the perspective on *Daodi* medicinal materials. We searched the relevant modern literature to analyze and assess comparative studies of *Daodi* and non-*Daodi* materials.

2. Methods

2.1. Inclusion/Exclusion Criteria. Studies that compared *Daodi* Chinese traditional medicinal material and *non-Daodi* traditional medicine were retrieved and included. Review articles were excluded. Article abstracts that could not be traced to their source data and full-text were also ineligible.

2.2. Search and Retrieval Strategy. Seven databases were searched, including PubMed, EMBASE, Web of Science, the Chinese Biomedical Literature Database (CBM), the Chinese Journal Full-Text Database (CJFD), the Chinese Scientific Journal Full-Text Database (CSJD), and the Wanfang database. The key words included "famous-region drug", "authentic medicinal Medicine", "genuine medicinal material", "genuine crude drugs", *"Daodi"* (道地), and "Didao" (地道).

2.2.1. Screening Procedure. Two evaluators (Xin Tian and Yannan Zhou) screened the articles independently by reviewing the title and the abstract. If they reached an agreement that the article met the literature identification standard, the full-text version was sought. Any disagreement was settled by a third evaluator, Yali Liu.

2.2.2. Data Retrieval and Analysis. The data of general characteristics and the overall study/processing report were collected. Two evaluators screened data from each article independently, and any discordance from the search results was discussed or another evaluator consulted to reach a resolution (Yali Liu).

A retrieval form was designed according to the study strategy, which mainly included (1) basic information, such as publication journal and time, academic institution, and study funding and (2) data to compare the *Daodi* and *non-Daodi* medicinal materials for possible differences: ① clinical trials and animal experiments that included basic medicinal material information, methodology, results, and conclusions; ② medicinal material ingredients, the geological background system (GBS), and different chemical and physical soil properties; and ③ a study methodology for identifying a *Daodi* material, including general med-material information, methodological contrast, and other aspects. The producing regions of the *Daodi* and *non-Daodi* described in the basic information and the sample size in either a clinical trial or an animal experiment were recorded as numbers. The following information was described in the form for data collection: growth mode and material sourcing in the basic information section; the reported dose formation and the dosing methods, as well as ingredients, during clinical trials and animal experiments; and other information needed for morphological comparisons between the materials, for the GBS and for a description of the physical and chemical properties and their final comparison conclusion. The other items or contents were recorded as 1 (described in the article) or 0 (not described in the article).

The data were summarized and analyzed by Microsoft Excel 2007 and SPSS 21.0 statistical software, and all the results were described statistically (frequency and percentage).

3. Results

3.1. Search and Retrieval Outcomes. Overall, 849 articles were originally acquired, including 324 written in Chinese and 551 articles in English. After deleting repetitive or redundant information, screening was implemented after perusing the abstract section, 120 articles were consequently confirmed through the literature identification criteria, and the 120 full-text articles were then evaluated. After scrutiny of the full text, another 13 articles were excluded from the study. The

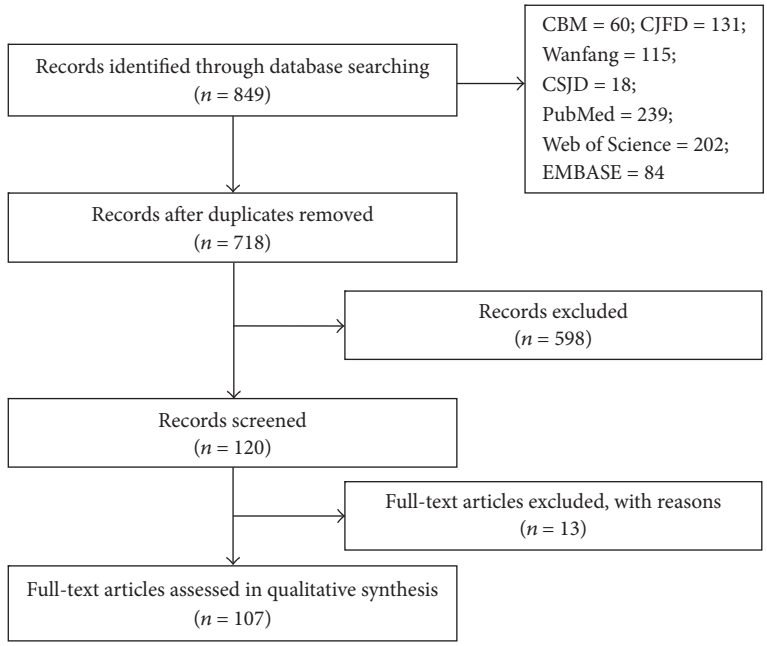

Figure 1: Flow chart of study inclusion.

remaining 107 articles were included, including 4 studies in English (Figure 1).

3.2. Basic Characteristics of the Included Articles

3.2.1. Basic Information of Included Studies. This study included 67 (67/107) journal theses, 32 (32/107) academic degree dissertations (30 Master's degree dissertations and 2 Ph.D. dissertations), 5 (5/107) conference papers, and 3 published conference recordings. Ten (10/107) authors, constituting the maximal proportion, were scholars from the China Academy of Chinese Medical Sciences. Of the studies, 58.9% (63/107) had a funding source, the other 41.1% (44/107) had no funding support, and 72.7% (32/44) were academic degree dissertations. The four English studies reported that there were no conflicts of interest, and not any claims of interest conflict were written in the Chinese articles. Of the 67 journals, the Chinese Journal of Chinese Materia Medica was the most proportional one at 23.9% (16/67) (Table 1).

The first publication about a *Daodi* medicinal materials and non-*Daodi* comparison study was issued as *Zhong Yao Cai* in 1990. However, from 1990 to 1999, only 3 studies were published, including two studies about traceable mineral elements; the other was a clinical trial. After 2000, there was a continuous increase in the number of relevant studies. The first genetic comparison of the two types of material was published in 2001, and also in this year an animal experiment to investigate the difference in the two types of material was performed. Studies pertaining to the physical and chemical properties of the soil began in 2002. A comparison of med-material ingredients, soil physical and chemical properties, and the GBS was initiated.

3.2.2. Study Categorization. Six different categories were summarized in Table 2, including 1 clinical trial, 6 animal experiments, 2 articles pertinent to the GBS and physical or chemical properties of the soil, 24 articles that exclusively investigated medicinal ingredients, 15 articles involving the medicinal composition, the GBS, and the chemical or physical properties of the soil, and 59 articles seeking a methodology to assess non-*Daodi* materials.

A total of 24 med-materials were included in this investigation and published in the 107 articles. The investigated med-materials were listed below: Pheretima Aspergillum (the only material with a zoological origin), Morindae Officinalis Radix, Atractylodis Rhizoma, Citri Reticulatae Pericarpium, Carthami Flos, Chuanxiong Rhizoma, Rhei Radix et Rhizoma, Salviae Miltiorrhizae Radix et Rhizoma, Moutan Cortex, Angelicae Sinensis Radix, Codonopsis Radix, Rehmanniae Radix, Poria, Aconiti Lateralis Radix Praeparata, Polygoni Multiflori Radix, Magnoliae Officinalis Cortex, Coptidis Rhizoma, Scutellariae Radix, Astragali Radix, Lonicerae Japonicae Flos, Ophiopogonis Radix, Cyathulae Radix, Ginseng Radix et Rhizoma, Notoginseng Radix et Rhizoma, Dioscoreae Rhizoma, Paeoniae Radix Alba, Himalaica Mirabilis, Asari Radix et Rhizoma, Scrophulariae Radix, Polygalae Radix, Alismatis Rhizoma, Anemarrhenae Rhizoma, Aurantii Fructus, and one from a mineral (Gypsum Fibrosum).

3.3. Comparison of Daodi Medicinal Materials

3.3.1. General Information of the Origins and Identification of Daodi Medicinal Materials. Because the production regions were divided according to rather inconsistent definitions in these studies, we described producing regions as more than one and only one. More than half of the studies did

Table 1: Basic information of the included studies.

Category	Characteristic	Number (%) of studies, $n = 107$
Year	1990–1999	3 (2.8%)
	2000–2009	56 (52.3%)
	2010–2014.7	48 (44.9%)
Ref type	Journal thesis	67 (62.6%)
	Academic degree dissertation*	32 (29.9%)
	Conference paper	5 (4.7%)
	Conference recording	3 (2.8%)
Title	Comparison of *Daodi* and non-*Daodi* Medicinal Materials	21 (19.6%)
	Comparison of Different Medicinal Materials	11 (10.3%)
	Others	75 (70.1%)
Author address	China Academy of Chinese Medical Sciences	10 (9.3%)
	China Pharmaceutical University	8 (7.5%)
	Henan University of Chinese Medicine	8 (7.5%)
	Beijing University of Chinese Medicine	7 (6.5%)
	Chengdu University of TCM	7 (6.5%)
	Hubei University of Traditional Chinese Medicine	6 (5.6%)
	Peking University	5 (4.7%)
	Chinese Academy of Medical Sciences	4 (3.7%)
	Tsinghua University	4 (3.7%)
	Others	48 (44.9%)
Funding source	Natural Science Foundation of China	20 (18.7%)
	National Basic Research Program of China	7 (6.5%)
	National Administration of Traditional Chinese Medicine Fund Projects	5 (4.7%)
	Support fund not indicated	44 (41.1%)
	Others	31 (29.0%)
Competing interests	Not mentioned	103 (96.3%)
Journal (n = 67)	China Journal of Chinese Materia Medica	16 (23.9%)
	Chinese Pharmaceutical Journal	4 (6.0%)
	Journal of Chinese Medicinal Materials	4 (6.0%)
	Lishizhen Medicine and Materia Medica Research	3 (4.5%)
	Others	40 (59.7%)

*Some authors were affiliated with different research institutions, and the first affiliated institution was used; academic degree dissertations were categorized according to the university or college.

not describe the growth mode. The majority of the studies (78.5%, 84/107) provided information about the process of acquiring med-materials. For example, procurement from a certain Chinese traditional medicine market or going to the original region for purchase was described by some professors or labs. The med-materials were identified by some experts in only 35.5% (38/107) of the articles. No study specified identification methods in detail. Some studies only mentioned that the med-material was identified; however, there was often no record of any professional assigner, and in some cases, the material extraction process was erroneously considered as the identification process. Of the studies, 52.3% (56/107) described specific timing about natural med-material reaping (at least roughly for a specified lunar-month). Most of the studies described the basic properties, pharmacological activity, and/or documented efficacy of the Chinese medicine (Table 3).

3.3.2. Data from Clinical Trials and Animal Experiments. Only 6 studies were designed with animal experiments to compare *Daodi* and *non-Daodi* materials. One clinical trial investigated differences in the effectiveness between the two material types. The first clinical study was published in 1990, and it compared the effectiveness and safety between rhubarbs. *Daodi* or *non-Daodi* materials were used to treat upper gastrointestinal hemorrhage, and the results demonstrated that the *Daodi* rhubarb had a more effective cure rate and reduced adverse events. Most of the animal experiments in both mice and rats (83.3%) were published after 2010, with 16.7% (1/6) describing *in vitro* experiments and 83.3% (5/6) describing *in vivo* experiments. The sample size varied from 42 to 140 (mean 96). All the *in vivo* experiments included randomization into groups, and an aqueous extract was administered most often. All these studies reported pharmacodynamics results, and there were no adverse events.

Table 2: Study content categorization of the included studies.

Category	Characteristic	Number (%) of studies, $n = 107$
Contents of article	Clinical trial	1 (0.9%)
	Animal experiment	6 (5.6%)
	GBS and soil physical/chemical properties	2 (1.9%)
	Medicinal composition	24 (22.4%)
	Medicinal composition, GBS, and soil physical-chemical property	15 (14.0%)
	Methodology to ascertain *Daodi*	59 (55.1%)
Medicinal species	Atractylodis Rhizoma, Lonicerae Japonicae Flos	13 (12.1%)
	Angelicae Sinensis Radix	9 (8.4%)
	Dioscoreae Rhizoma	8 (7.5%)
	Scutellariae Radix, Achyranthis Bidentatae Radix	7 (6.5%)
	Salviae Miltiorrhizae Radix et Rhizoma	6 (5.6%)
	Chuanxiong Rhizoma	5 (4.7%)
	Rehmanniae Radix, Paeonia Lactiflora	4 (3.7%)
	Aconiti Lateralis Radix Praeparata, Alismatis Rhizoma	3 (2.8%)
	Ginseng Radix et Rhizoma, Scrophulariae Radix, Polygalae Radix	2 (1.9%)
	Others	1 (0.9%)

Table 3: General information on Chinese traditional medicinal materials.

Category	Characteristic	Number (%) of studies, $n = 107$
Number of the *Daodi* medicinal material original areas	1	44 (41.1%)
	>1	56 (52.3%)
	Not mentioned	7 (6.5%)
Number of the non-*Daodi* medicinal material origin areas	1	10 (9.3%)
	>1	90 (84.1%)
	Not mentioned	7 (6.5%)
Growth mode of the *Daodi* medicinal material	Artificial feeding	41 (38.3%)
	Feral	19 (17.8%)
	Not mentioned	55 (51.4%)
Growth mode of the non-*Daodi* medicinal material[#]	Artificial feeding	49 (45.8 %)
	Feral	19 (17.8%)
	Not mentioned	58 (54.2%)
Medicine acquirement	With origin, without source	26 (24.3%)
	With origin and source	84 (78.5%)
Whether it has been identified	Yes	38 (35.5%)
	No	69 (64.5%)
Specific time for reaping	Described	56 (52.3%)
	Not described	51 (47.7%)
Basic property	Described	91 (85.0%)
	Not described	16 (15.0%)
Pharmacological activity and documented efficacy record	Described	89 (83.2%)
	Not described	18 (16.8%)

One study included two med-materials; either the *Daodi* or the *non-Daodi* was produced in more than one region, which resulted in >100%. [#]Of the basic information, some of the med-materials had been recorded with more than one item about growth mode and material origin, which also produced greater than 100%.

The majority of these studies (83.3%) reported that the *Daodi* materials were more effective than the non-*Daodi* materials, and 25% reported that the two med-materials had similar effective results.

3.3.3. Medicinal Material Ingredients, Soil Properties, and the GBS. Forty-one studies investigated the med-material ingredient, soil properties, and GBS. Two studies compared the GB and soil properties, 24 studies were exclusively

about the material ingredients, and 15 articles included all the subjects mentioned above. Three articles examined the different arable soils (different region) that were selected to raise the same herbs, and the possible effect of changes in some inorganic or mineral elements could be analyzed. In this review, we put this article in the category of covering all subjects. For expediency, we added associated study subjects, which included endophyte microbes and metabolites, morphological contrast, and germplasm resource and genetic analysis, Table 4.

Med-material ingredient comparisons were performed mostly to examine mineral and organic compositions. Of these, 63.4% (26/41) compared the active ingredients of an organic composition, and one study investigated pesticide residue, heavy metal substances, and the ineffective ingredients (phenylformic acid) of two med-materials. Overall, 58.5% (24/41) analyzed mineral element discrepancies, and 4.9% (2/41) reported the probable influence on pharmacodynamics from these minerals. For organic ingredient comparison, many of the studies were focused on the active component of med-materials. The difference of volatile oil between Atractylodis Rhizoma and Chuanxiong Rhizoma was the most frequently analyzed, accounting for approximately 38.5% of studies (10/26). Some studies examined extracts, polysaccharides, and total ash proportions as the main discrepant indicators between the *Daodi* and *non-Daodi* materials. Morphological contrasts were presented in 26.8% (11/41) of the studies, including physiological anatomy or functional observation. Two studies were conducted to contrast the microanatomy, and one observed some ultramicrostructures.

Some scholars observed the constituents, soil features, and GBS to try to find possible differences in certain med-materials. Mineral quantities were analyzed in 36.6% (15/41) of the studies, and 22.0% (9/41) examined the pH of soil samples. Most GBS studies were conducted to determine geographic characteristics of growing locations, such as altitude and latitude. There were also considerable studies about climatological parameters (i.e., type of climate or conditions, annual sunshine duration, rainfall, and temperature) and the geological background (topography and landforms). A few studies (2.4%, 1/41) addressed concerns about wind velocity and the humidity of different breeding places (Table 4).

3.3.4. Methods for Identifying Daodi Med-Material. Most of the methods used to define a *Daodi* med-material have depended on physics, chemistry, and biological techniques, as well as statistics, to distinguish apparently similar natural materials, or different production, processing, or concoction procedures have been analyzed to further investigate effective components that might have been affected by processing (Table 5). Fifty-five studies described a methodology to identify a *Daodi* material, and most of these involved a fingerprint technique. These techniques included chromatography, spectrometry, MRI (magnetic resonance imaging), and DNA fingerprint. Thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) were used. Diode array detector (DAD), Fourier Transform Infrared (FT-IR), and Near Infrared (NIR) were used for spectrometry. MS was the only MRI analysis. HPLC has been the most frequently used approach, with 50.8% (30/59), and the other widely used techniques included GC-MS and HPLC-DAD. Fingerprint DNA was reported in 11 studies. The profile of fingerprint DNA in *Daodi* med-materials was established in 37.3% (22/59) of the included studies. Cluster analysis was the most frequent method for statistical analysis (52.5%,31/59). Another 8.5% (5/59) included principal component analysis (PCA) processing. Five (8.5%) studies investigated a process to prepare, produce, and concoct a medicine, which might also contribute to identifying the quality of *Daodi*.

3.3.5. Results and Clinical Practices. Several studies were investigated in this review. Of these studies, 64.5% (69/107) claimed that *Daodi* med-materials were superior to *non-Daodi* med-materials. Only a small number, 1.9% (2/107), suggested that statistical significance could not be found. Additionally, 2.8% (3/107) of these studies analyzed the active ingredients of the materials and discovered that the *Daodi* med-materials had fewer active ingredients. Another 30.8% (33/107) did not make any comparison of superiority or inferiority between the two types of material (Table 6).

4. Discussion

4.1. Complicated Environments of Daodi Breeding and Incomplete Studies according to the Identification of a Daodi Med-Material. Previous studies about *Daodi* med-materials mostly involved medical material tests [7–9], the foundation for formation and development [7, 10–12], quality evaluation and identification/verdicts [13–16], and GAP development [17–22]. In addition, these approaches had mainly been conducted through review and experiment studies. The first study about comparisons was published approximately 20 years ago, and pertinent studies have been increasing gradually. To the best of the author's knowledge, this study is the first review that presents a comparison between *Daodi* and *non-Daodi* med-materials.

The pertinent studies on the comparison of *Daodi* and *non-Daodi* materials were first published in the 1990s. Beginning in 2000, well-rounded development began again, except for contrasting the ingredients or compositions and pharmacodynamics; other aspects, such as soil characteristics, the GBS, the evaluation methods, and animal experiments, were also inclusively conducted. In 2001, the first exploratory study for some *Daodi* and non-*Daodi* genetic comparisons produced a study that has substantiated our molecular insight into these plants. From 2000 to 2010, the published studies were mostly performed through animal experiments, soil analysis, and GBS comparison. Since 2010, the study trends included animal experiments and the introduction of contemporary techniques to identify *Daodi* and non-*Daodi* Chinese traditional medicinal material.

When studying med-material ingredients and composition or soil properties, most often, scholars will compare either mineral or organic ingredients. For example, mineral

Table 4: Ingredients of the studied medicine/soil physical and chemical properties/GBS.

Comparison of medicinal composition, GBS, and soil properties	Number (%) of studies, $n = 41$
Medicinal composition	
Inorganic elements	
Element differences	24 (58.5%)
Detection method	22 (53.7%)
Correlation analysis	12 (29.3%)
Accumulation ability	11 (26.8%)
Character index	7 (17.1%)
Others	7 (17.1%)
Organic component	
Active substance	26 (63.4%)
Extracts	5 (12.2%)
Total ash proportion	4 (9.8%)
Polysaccharide	4 (9.8%)
Others	6 (14.6%)
Morphologic	
Physiological anatomy or functional observation	11 (26.8%)
Microanatomy	2 (4.9%)
Ultrastructure	1 (2.4%)
Germplasm resource	7 (17.1%)
Genetic contrast	4 (9.8%)
Endophyte microbes and metabolites	3 (7.3%)
Others	6 (14.6%)
GBS and soil physical-chemical properties	
Soil properties	
Inorganic elements	15 (36.6%)
pH	9 (22.0%)
Soil characteristics and type	8 (19.5%)
Available nutrients	7 (17.1%)
Organic material	6 (14.6%)
Physical clay	4 (9.8%)
Soil structure	3 (7.3%)
BS	3 (7.3%)
CEC	3 (7.3%)
Total nutrients	2 (4.9%)
Soil color	2 (4.9%)
Soil moisture	2 (4.9%)
Others	2 (4.9%)
GBS	
Altitude	8 (19.5%)
Latitude	7 (17.1%)
Type of climate or conditions	7 (17.1%)
Annual sunshine duration, rainfall, and temperature	7 (17.1%)
Soil parent material	5 (12.2%)
Topography	4 (9.8%)
Climatic regionalization	4 (9.8%)
Landforms	3 (7.3%)
Vegetation regionalization	3 (7.3%)
Hydrological regionalization	3 (7.3%)
Clay mineral composition	2 (4.9%)
Others	3 (7.3%)

Table 5: Methods for assessing *Daodi* medicinal materials.

Methods for assessing *Daodi* medicinal materials	Number (%) of studies, $n = 59$
Fingerprint	
Chromatography	
Thin-layer chromatography/TLC	4 (6.8%)
High-performance liquid chromatography (HPLC)	30 (50.8%)
Gas chromatography (GC)	10 (16.9%)
Spectrometry	
Diode array detector (DAD)	8 (13.6%)
Fourier Transform Infrared (FT-IR)	7 (11.9%)
Near Infrared (NIR)	4 (6.8%)
MRI (magnetic resonance imaging)	
Mass spectra (MS)	12 (20.3%)
DNA fingerprint	
Randomly amplified polymorphic DNA (RAPD)	5 (8.5%)
Amplified fragment length polymorphism (AFLP)	1 (1.7%)
Intersimple sequence repeat (ISSR)	1 (1.7%)
Expressed sequence tags-simple sequence repeat (EST-SSR)	1 (1.7%)
Polymerase chain reaction (PCR)	5 (8.5%)
Characteristic fingerprint	21 (35.6%)
Statistical analysis	
Cluster analysis	31 (52.5%)
Principal component analysis (PCA)	5 (8.5%)
Processing methods	5 (8.5%)
Others	7 (11.9%)

Table 6: Results and clinical practice.

Characteristic	Number (%) of studies, $n = 107$
Results	
Daodi medicinal materials were better than non-*Daodi* medicinal materials	69 (64.5%)
No difference between *Daodi* medicinal material and non-*Daodi* medicinal material	2 (1.9%)
Non-Daodi medicinal materials were better than *Daodi* medicinal materials	3 (2.8%)
No clear comparison	33 (30.8%)
Use in clinical practice	
Having a significant use for clinical practice after finding that the genuine material is distinguishable from the nongenuine material and confirming the quality for the GAP (good agricultural practice) system	98 (91.6%)
Not mentioned	9 (8.4%)

differences in the two categories of med-materials were usually investigated. However, only a few of these studies established certain mineral characteristic as a significant indicator for claiming authenticity.

The GB is defined as the specific synthesis of attributes in a geologic body and geologic agents that are highly related to the med-materials, including quaternary sediment, the mineral distribution or rock mass, tectonics, crustal movement, geographic and geomorphic factors, topography and landforms, geochemistry, hydrogeology, and other multifarious considerations. In fact, the GBS is defined as a GB 2-dimensional integrality (GB, climate, biology, etc.), which is part of modern system theory about the foundation of natural nonequilibrium open system consistence law [9]. Most of the studies in this review are about the original production regions in terms of their geographic features, climate data, GB, and so on. Regarding differences in pivotal ingredients, the pharmacodynamics of the two types of med-material have high relationships with the geological milieu. Considering the complications in geographic environments, there are remarkable discrepancies in various soil and water conditions, climates, sunlight durations, and distributions of biological beings. All these factors may affect the quality of the med-materials, and as they may play a role in the differences in medical efficacy, these issues require further clinical trials to provide substantial evidence of these effects.

A majority of the studies to identify *Daodi* Chinese traditional medicinal material used a fingerprint method, and HPLC was the second most common approach. Many

studies identified the med-materials via only one method for fingerprint examination, and a combination of two methods for fingerprint study was also frequently applied, especially GC-MS and HPLC-DAD [19]. Few studies were facilitated by DNA fingerprint graphing. However, there are great variations in applying specific methods. In addition, NMR was also used in the analysis of plant extracts [20].

4.2. The Current Challenges, Issues, and Prospective Solutions. Even among the wide range of data sources sought in this review study to compare the overall investigational results between *Daodi* and *non-Daodi* materials, only 34 med-materials were covered, which contrasts with the more than 200 *Daodi* materials that have been recorded. This outcome suggests that other materials have not been sufficiently investigated. Ingredient differences were unanimously considered to be one of the standards for claiming superiority. However, for many studies in this review, a view of the growth mode was not taken for either *Daodi* or non-*Daodi* med-materials; further investigation on specific provenances, sourcing status, and reaping time was not considered; there was no report about whether an experimental identification was conducted between the two types of material; and there was a similar deficiency for articulating the processes and methods to claim a difference. In fact, this information has great significance in regard to comparing the functional ingredients of *Daodi* and non-*Daodi* materials as well as clinical effectiveness. Therefore, it is highly recommended that producing region information be considered for clarification. In cases regarding the necessary identification of the attributes of some med-materials, experts and professionals should be invited to consult. If so, the study's accuracy would be enhanced with reliable and intelligible scientific evidence, which would benefit the development of future translational medical studies.

Suitable geographical conditions are exterior factors for producing *Daodi* ingredients [1]. Admittedly, the Chinese geological environment has evolved on a large scale over time and under natural conditions. As a result, it may play a role in the transference of the *Daodi* med-material producing region. However, the fundamental principle for trustworthiness of *Daodi* med-material has always been stated as "Good Quality and High Efficacy" for its label and "Foundation on the Primacy" [23] for its essence. Two studies [24, 25] included in this review have distinguished the original region using the "ancient" tract and "current" tract, and both are attributed to the *Daodi* category, which disregards a possible effect on the medicine quality when considering the changes in the methods applied for producing region division or categorization.

Until now, clinical discrepancies were derived from empiricism rather than from more substantial trial results. In addition, modern studies still take material ingredients, soil properties, and the GBS as the investigational cynosure. Studies have seldom been performed through animal experiments and clinical practical tests. Furthermore, both of these types of study have only been sparsely and fragmentally reported. In most of the real cases and dosing in Chinese traditional medicine, a combination recipe with different materials was prescribed, and it was seldom administered as just a single medicinal ingredient/dose. Because there are interactions between different medicine ingredients and agents, with different mechanisms that could complicate the results, some scientists consider that even clinical trials, which are a widely accepted study method, cannot be taken as a valuable way to interpret whether any single medicine from different producing areas can be differentiated or confirmed to have an exclusive effectiveness. There might be only some slight or subtle difference between the *Daodi* and non-*Daodi* materials that is not as remarkable as the contrast between a medicinal agent and a placebo in established Western clinical pharmacology. As a result, the study has to include many more subjects to present a difference, which therefore poses a great challenge to obtaining clinical testimony. None of these included studies has investigated toxicity and adverse events, and there is evident incompleteness of data for toxic comparison between the medicinal materials. If the effectiveness of *Daodi* and non-*Daodi* materials in animal models is to be compared, it has been reasonably suggested that some parallel studies with a well-controlled design, as well as with wholeness and precision, be preferably established to investigate the relevant toxicity to further dig deeply into the discovery of the pharmacodynamics and safety profile.

One certain medicinal material may be produced from different regions, and its active ingredients and quantities can vary. Active ingredients or components are a uniquely important attribute for any *Daodi* med-material [23]. However, it is necessary to note here that a quality verdict might not be proportionally related to certain chemical compositions. In fact, certain levels of some ingredients cannot substantially indicate a remarkable or significant difference between one *Daodi* and another non-*Daodi* material [26]. Endophytes are some fungi or bacteria that have been living in healthy plant tissues or organs throughout the plants' whole life or some special growth stage; in addition, endophytes can beneficially play a role in some formation or accumulation of active ingredients [27]. Derived metabolites are usually regarded as the main ingredients or components in Chinese traditional medicine. The benefits of these derived metabolites have been commonly agreed on, such as disease-resistance, antipest, and counter-environmental adversary effects [28]. Only three studies were conducted on endophytes and compared derived metabolites in Chuanxiong Rhizoma and Lonicerae Japonicae Flos to investigate their *Daodi* attributes [29–31].

The superior germplasm resource is an internal factor of the formation of a *Daodi* medicinal material [1], and for this reason, many modern studies compare soil properties, such as mineral differences in producing soil that may breed a *Daodi* or *non-Daodi* material. However, there is still a lack of evidence that necessitates some further studies to investigate the relationships between soil minerals and the medicine toxicity, and the compound minerals that may be formed into any quality, as well as med-material yields, especially indispensable minerals that could improve the quality of traditional medicine, are still an open issue. We suggest that these aspects require further study to clarify the association between soil minerals and the chemical composition of the med-materials as well as to determine

if pragmatic significance was involved in the process of cultivation and quality assurance, along with fertilization for time and quantity considerations [32]. All the comparisons of the soil properties and various environmental factors in this study to ascertain significant differences between *Daodi* and non-*Daodi* med-materials are designed to discover natural conditions that formulate and compose *Daodi* supreme qualities. However, there is a lack of objective and clear methods as well as standards to produce reliable contrasts. Therefore, these pertinent studies have unfortunately been bogged down in a perplexing impasse, "purposed for a study, but without any credible measurement" and "wheeling into repetitive inanity." As a consequence, it is very difficult to disclose any ecological factor to identify a *Daodi* material. From this review, we recognize that assessment methods and standards must be established if the relationship between *Daodi* attributes and the ecological environment is to be studied [26].

Morphological comparison between *Daodi* and *non-Daodi* med-materials is one of the key aspects; however, it often highlights physiological states directly from observations, appearances, or phenomena, which usually come from individual empirical knowledge and are probably prejudiced by different viewpoints subjected to a provincial judgment. Attributed to these incomplete and nonquantified standards, these methods can be applied and developed with limitations.

Recently, new technologies and methods have been developed rapidly to be used for *Daodi* med-material identification, and these approaches will hopefully overcome some difficulties of traditional methods for identifying differences. Shilin Chen suggested that DNA barcoding [33] can be used for med-material sample identification when there is little background information. This method is advantageous for methodological generalization and digitalization as well as developmental feasibility [34]. He also proposed the Herb Genome Project (HerbGP) (Chen et al., 2010) and established a DNA barcoding [35] system, which has great potential. Xiaohe Xiao suggested establishing a "synergic *Daodi* med-material standard" (*Daodi* Indicator, DDI), and this method might be more systematic and objective for a control assessment as well as for confirming high quality med-materials [36] (Xiao et al., 2012). Xueyong Wang proposed some more systematic and precise methods for Chinese traditional medicine study to produce reliable evaluations [37]. In 2002, a complicated systematic theory about the Chinese traditional medicinal material GAP tool was suggested by Luqi Huang [17]. The author applied modern physics, chemistry, biology, and statistical methods to identify ingredients and confirm the quality of two med-materials, even though no agreement was reached.

4.3. Limitations of This Study. Above all, only seven Chinese or English databases were included as data sources, and the final studies included were entirely written in Chinese. No English articles were retrieved. Second, this review is only based on modern studies, and no associated provenance from ancient studies has been retraced. The third limitation is that it is possible to miss some studies because the designated keywords have not been included anywhere in an article's title, abstract, and keyword list, especially in cases where there was some specific description with different vocabularies, although this study began with a systematic retrieval method and plan.

5. Conclusion

Comparisons between *Daodi* and non-*Daodi* materials have been studied mainly in terms of med-material ingredients or composition, soil properties, the GBS, and some identifying methodologies to assess *Daodi* attributes. These factors are closely related to med-material production and effective ingredient identification, yet these factors are incapable of providing direct evidence to demonstrate safety and effectiveness. Some studies applied modern biomedicine or biostatistics methods for quantity analysis to compare the two types of med-material, even though there is still no consensus of comparison criteria between *Daodi* and non-*Daodi* medicinal material. Until now, only a few studies were conducted through animal experiments and clinical trials to discern the superiority of *Daodi*. These results highly suggested that clinical trials and fundamental studies are needed to explore the effectiveness and safety profiles as well as to further translate the benefits of clinical Chinese medicine into practice.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Xingyue Yang, Yali Liu, and Liyun He conceived and designed the study. Yali Liu, Xin Tian, and Yannan Zhou searched for data. Xin Tian, Yannan Zhou, Xingyue Yang, Xinlong Li, Tingting Lu, and Changhe Yu extracted the data and assessed the reporting quality of the animal experiments. Xin Tian and Yali Liu analyzed the data. Yali Liu and Liyun He interpreted the data. Xingyue Yang, Xin Tian, and Yali Liu participated in writing the manuscript. All authors read and approved the final manuscript.

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Research Article

Study on Quality Standard of Processed *Curcuma Longa Radix*

Zhimin Chen,[1,2] Yongfeng Zhao,[1] Liang Quan,[1] Haiting Zhou,[1] Dong Cao,[2] Changjiang Hu,[1,3] Wenbing Li,[3,4] and Zhuo Yang[1]

[1]*Chengdu University of TCM, Chengdu 611137, China*
[2]*Chengdu Institution of Chinese Herbal Medicine, Chengdu 610016, China*
[3]*Key Laboratory of Chinese Medicine Formulations Particle Mass and Clinical Evaluation, Chengdu 611900, China*
[4]*Neo-Green Pharmaceutical Co., Ltd., Chengdu 611900, China*

Correspondence should be addressed to Changjiang Hu; hhccjj@hotmail.com

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To control the quality of *Curcuma Longa Radix* by establishing quality standards, this paper increased the contents of extract and volatile oil determination. Meanwhile, the curcumin was selected as the internal marker, and the relative correlation factors (RCFs) of demethoxycurcumin and bisdemethoxycurcumin were established by high performance liquid chromatography (HPLC). The contents of multicomponents were calculated based on their RCFs. The rationality and feasibility of the methods were evaluated by comparison of the quantitative results between external standard method (ESM) and quantitative analysis of multicomponents by single-marker (QAMS). Ethanol extracts ranged from 9.749 to 15.644% and the mean value was 13.473%. The volatile oil ranged from 0.45 to 0.90 mL/100 g and the mean value was 0.66 mL/100 g. This method was accurate and feasible and could provide a reference for further comprehensive and effective control of the quality standard of *Curcuma Longa Radix* and its processed products.

1. Introduction

Curcuma Radix (Yujin) is derived from the dried root of *Curcuma wenyujin, Curcuma longa*, *Curcuma kwangsiensis,* or *Curcuma phaeocaulis*, which first appeared in the Theory of Drug Properties *(Yao Xinglun)* and has the effects of invigorating the circulation of blood, relieving pain, dispersing stagnated qi for relieving qi stagnation, clearing heat of heart and cooling blood, and curing jaundice [1–3]. *Curcuma Longa Radix* (Huangsiyujin, HSYJ), as one of the main varieties of *Curcuma Radix*, is the famous genuine medicinal herbs produced in Sichuan. The *Tu Jing Ben Cao* which was written by Su Song recorded that "Guangnan and Jiangxi also have, but not as good as Sichuan" [4].

Modern pharmacological studies have shown that *Curcuma Longa Radix* has many functions, such as anti-inflammatory [5–7], easing pain [8], antithrombosis and platelet aggregation [9], antioxidation [10–12], antidepression, and cholagogic [13]. But the quality standard of *Curcuma Radix* is insufficient. In the Chinese Pharmacopoeia 2015 edition, only moisture and total ash are used as indicators for quality control of *Curcuma Radix*. It is difficult to fully reflect and control the quality of *Curcuma Longa Radix* due to the lack of quality control indicators. To date, HPLC has applied in determination of curcuminoids in *Curcuma Longa Radix* simultaneously [14]. However, due to high experimental cost, the application of this method was limited. Therefore, there is a clear need for the development of a quality control method. In this paper, in order to explore a comprehensive quality control criterion for *Curcuma Longa Radix* and guarantee the effective and safe use of clinical practice. We not only added the contents of extract and volatile oil determination but also developed and validated a quantitative analysis of multicomponents by single-marker (QAMS) for the simultaneous determination of polar active components in *Curcuma Longa Radix*, including curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

2. Materials and Methods

2.1. Chemicals and Reagents. The reference standards of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (purity ≥ 98%) were purchased from the National Institutes

Table 1: Information of *Curcuma Longa Radix* samples.

Number	Place of purchase	Time	Place of origin
(S1)	Co.A	20170104	Sichuan
(S2)	Co.B	20160824	Sichuan
(S3)	Co.B	20160728	Sichuan
(S4)	Co.B	20160310	Sichuan
(S5)	Zhoudu Village of Shuangliu	201602	Shuangliu (Sichuan)
(S6)	Zhoudu Village of Shuangliu	201510	Shuangliu (Sichuan)
(S7)	Zhoudu Village of Shuangliu	201701	Shuangliu (Sichuan)
(S8)	Sichuan Hehuachi medicine market	20170518	Sichuan
(S9)	Qianwei	20170705	Qianwei (Sichuan)
(S10)	Co.C	20161025	Sichuan
(S11)	Co.C	2017519	Sichuan
(S12)	Co.C	20170206	Sichuan

Table 2: Results of ethanol extracts and volatile oil ($n = 3$).

Number	Extracts (%)	Volatile oil (mL/100 g)
(S1)	14.602	0.55
(S2)	14.382	0.45
(S3)	13.509	0.75
(S4)	14.749	0.45
(S5)	15.644	0.70
(S6)	9.749	0.80
(S7)	10.109	0.50
(S8)	12.334	0.80
(S9)	14.987	0.90
(S10)	13.940	0.60
(S11)	13.671	0.75
(S12)	14.001	0.70

for Food and Drug Control (Beijing, China). Methanol and acetonitrile (Fisher, USA) were of HPLC grade. Other reagents were of analytical purity. Water was glass-distilled and filtered through a Milli-Q water purification system (Millipore, Bedford, MA) prior to use. *Curcuma Longa Radix* was gathered from geoauthentic habitats, such as Qianwei, Shuangliu, and Chongzhou in Sichuan province and processed in our own laboratory. We also purchased *Curcuma Longa Radix* from different TCM enterprises. These samples were authenticated by Professor Xianming Lu and Professor Guihua Jiang (Chengdu University of Traditional Chinese Medicine, Chengdu, China). The information of samples is shown in Table 1.

2.2. Determination of Ethanol Extracts. The ethanol extracts were determined according to the 2201 of Chinese Pharmacopoeia 2015 edition Volume IV. Homeopathic alcohol was used as the extraction solvent. The results were shown in Table 2.

2.3. Determination of Volatile Oil. The volatile oil was determined according to the 2204 of Chinese Pharmacopoeia 2015 edition Volume IV. The results were shown in Table 2.

2.4. Instrumentation and Separation Conditions. HPLC determinations were performed using an Agilent HPLC 1200 instrument (Agilent Technologies, Palo Alto, CA), equipped with a diode array detector (DAD) detector, an auto sampler, a column heater, and a Welch Ultimate® XB-C18 (250 mm × 4.6 mm, 5 μm) column. The mobile phase consisted of A (acetonitrile) and B (4% glacial acetic acid aqueous) (V/V). Optimum separation was 48% A. The flow rate was 1.0 mL·min^{-1} and injection volume was 10 μL. The column temperature was set at 30°C and the wavelengths were monitored at 425 nm.

2.5. Sample Preparation. Powder of *Curcuma Longa Radix* was precisely weighed (1 g) and immersed in 25 mL of methanol. Additional methanol was added to make up the loss after ultrasonic extraction for 30 min. For HPLC analysis, the filtrate was filtered through a filter (0.45 μm pore size) prior to injection. And the negative control groups were prepared in the same manner.

2.6. Preparation of Standard Solution. A mixed stock solution containing reference standards was prepared by dissolving weighed accurately samples of each compound in methanol, which obtained curcumin 138.51 μg·mL^{-1}, demethoxycurcumin 28.20 μg·mL^{-1}, and bisdemethoxycurcumin 19.58 μg·mL^{-1}. The calibration curves were established by further dilution with methanol gives at least six different concentrations, and the six different concentrations of mixed standard solutions were injected (10 μL) and calculated. The series of working solutions are within the ranges of 3.3242 to 13.851 μg·mL^{-1} for curcumin, 0.6768 to 2.82 μg·mL^{-1} for demethoxycurcumin, and 0.4699 to 1.958 μg·mL^{-1} for bisdemethoxycurcumin.

2.7. Method Validation. The HPLC method was employed and methodology was examined for linearity, recovery, precision, repeatability, and stability. The validation was implemented based on the relative peak areas, the linear regression analysis was used to prepare calibration curves, and relative standard deviation (RSD) was used to evaluate precision, repeatability, stability, and recovery.

Table 3: Linear ranges survey of 3 curcuminoids in *Curcuma Longa Radix*.

Standard substance	Regression equation	Linear range (μg/mL)	Correlation coefficient (r^2)
Curcumin	$y = 9096.4x - 20.331$	3.3242~13.851	0.9997
Desmethoxycurumin	$y = 9194.2x - 5.6895$	0.6768~2.82	0.9997
Bisdesmethoxycurum	$y = 8601.1x + 2.2162$	0.4699~1.958	0.9993

Table 4: Precision, repeatability, stability, and recovery of 3 curcuminoids.

Compound	Precision	Repeatability	Stability	Recovery	
	RSD (%)	RSD (%)	RSD (%)	Mean	RSD (%)
Curcumin	0.38%	1.50%	0.74%	96.26%	2.58%
Desmethoxycurumin	0.40%	1.12%	0.83%	94.43%	2.47%
Bisdesmethoxycurum	0.39%	1.24%	0.95%	95.86%	3.97%

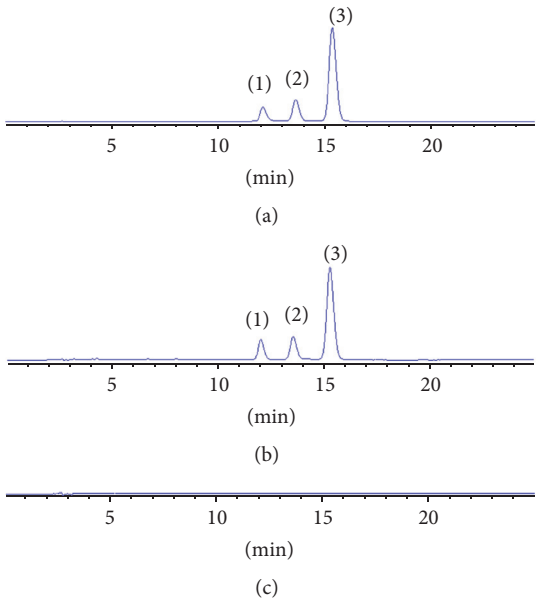

Figure 1: HPLC of reference substance (a), *Curcuma Longa Radix* sample (b), and negative control sample (c). (1) Bisdemethoxycurcumin, (2) demethoxycurcumin, and (3) curcumin.

3. Results

3.1. Chromatographic Conditions. Acetonitrile (A) and 4% glacial acetic acid aqueous (B) (V/V) were chosen as the composition of mobile phases for all the analyses. The chromatogram of mixed standard compounds, sample, and negative control sample were shown in Figure 1. In Figure 1, the three peaks marked with (1), (2), and (3) were bisdemethoxycurcumin, demethoxycurcumin, and curcumin. Mixed standard compounds and samples had the same retention time. The degrees of separation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were all greater than 1.5 and theoretical plate number was greater than 9000. The negative control sample had no peaks at the corresponding positions of *Curcuma Longa Radix* sample, which illustrated the other medicine herbs did not interfere with determination. The method gave good specificity.

3.2. Calibration Curves. Using the above chromatographic conditions, the calibration curves of 3 compounds exhibited good linear regressions. Table 3 gave the linear ranges survey of contents of three curcuminoids.

3.3. Precision, Repeatability, Stability, and Recovery. The precision was obtained by six copies of determinations individually of the standard solution and their RSD were calculated. The repeatability was performed by six-time determinations continuously for a sample (S9). The stability was tested with the sample solution that was stored at room temperature at several time points (0, 2, 4, 8, 12, and 24 h after preparation), and the 3 compounds were found to be rather stable within 24 h (RSD < 3%). In the recovery test, 6 samples were prepared by spiking known quantity of each of the 3 standards into the *Curcuma Longa Radix* sample that had been measured and then extracted according to sample preparation and analyzed. All of these data were shown in Table 4.

3.4. Relative Correlation Factors (RCFs, f). The mixed standard solution was injected 1 μL, 2 μL, 4 μL, 6 μL, 8 μL, 10 μL, and 20 μL. Curcumin was used as the internal marker to calculate RCFs of demethoxycurcumin (A) and bisdemethoxycurcumin (B). The results were shown in Table 5.

A_S and C_S were, respectively, the peak area and concentration of internal marker, and A_X and C_X were, respectively, the peak area and concentration of analyte [15].

3.5. Effects of Various Factors on RCFs(f). In this study, the effects of different instruments (Shimadzu LC-20A and Agilent 1200), chromatographic column (Welch Ultimate XB, SPOLAR, and Diamonsil), column temperature (25°C, 30°C, and 35°C), wavelength (423 nm, 425 nm, and 427 nm), and flow rate (0.90 mL/min, 0.95 mL/min, 1.00 mL/min, 1.05 mL/min, and 1.10 mL/min) on RCFs were investigated. All of these data were shown in Table 6.

3.6. Location of Chromatographic Peaks to Be Measured. The relative retention value ($r_{X/S}$, which was the retention time ratio of analyte and internal marker) of *Curcuma Longa Radix* of each component under test was used for the location of the chromatographic peak. Curcumin was used as the

Table 5: $f_{S/X}{}^{*}$ determination results with curcumin as internal content.

Injection volume/μL	1	2	4	6	8	10	20	Mean	RSD%
$f_{S/A}$	0.988	0.995	0.994	0.991	0.990	0.991	0.988	0.991	0.25
$f_{S/B}$	1.080	1.068	1.060	1.054	1.050	1.090	1.045	1.064	1.54

$^{*}f_{S/X} = f_S/f_X = (A_S \times C_X)/(A_X \times C_S)$.

Table 6: Effects of various factors on RCFs(f).

Effect	$f_{S/A}$		$f_{S/B}$	
	Mean	RSD (%)	Mean	RSD (%)
Instruments				
Shimadzu LC-20A	0.9788	1.02	1.0580	1.31
Agilent 1200	0.9931		1.0778	
HPLC column				
Welch Ultimate XB	0.9752		1.0398	
SPOLAR	0.9993	1.22	1.0563	0.8
Diamonsil	0.9886		1.0457	
Column temperature				
25°C	0.9903		1.0472	
30°C	0.9899	0.06	1.0469	0.02
35°C	0.9908		1.0467	
Wavelength				
423 nm	0.9798		1.0239	
425 nm	0.9894	0.96	1.0445	1.95
427 nm	0.9989		1.0645	
Flow Rate				
0.90 mL/min	0.9895		1.0460	
0.95 mL/min	0.9892		1.0460	
1.00 mL/min	0.9889	0.02	1.0448	0.06
1.05 mL/min	0.9889		1.0447	
1.10 mL/min	0.9892		1.0452	

internal marker to calculate $r_{X/S}$ of demethoxycurcumin and bisdemethoxycurcumin. $r_{X/S}$ of demethoxycurcumin and bisdemethoxycurcumin were 0.888 and 0.788 and RSD were 0.02% and 0.02%, respectively.

$$r_{X/S} = \frac{t_{R(X)}}{t_{R(S)}} \quad (1)$$

(X was analyte and S was the internal marker.)

(see [16]).

3.7. Comparison between QAMS and External Standard Method (ESM). Samples were prepared according to the preparation method of the sample solution, and the contents of curcumin, demethoxycurcumin, and bisdemethoxycurcumin in the samples were determined by HPLC. The contents of each component were calculated by using RCFs. Comparing results of QAMS and ESM, we find that the RSD were within 3%. So the QAMS used in the study of multicomponents quality evaluation of *Curcuma Longa Radix* was feasible. The results were shown in Table 7.

4. Discussion

In this paper, based on Chinese Pharmacopoeia 2015 edition Volume IV, preliminary experiments, and literature, we not only determined the homeopathic alcohol as the solvent and the extract of *Curcuma Longa Radix* were determined by hot dipping method but also increased the content of volatile oil of *Curcuma Longa Radix* on quality control.

The TCM theory believes that the efficacy of TCM is due to the multicompounds which consist of many different kinds of chemical constituents [17–21]. So it is difficult to accurately reflect the quality of traditional Chinese medicine by a single component as quality control indicators. In order to control the quality of traditional Chinese medicine, it is necessary to select a number of effective components or main components as indicator, especially the chemical components related to efficacy.

Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are the major active components of *Curcuma Longa Radix*. Three components have clear chemical structure, obvious pharmaceutical properties, and convenient detection, so they should be considered for study first. QAMS, which has many advantages such as low cost and high efficiency, has

Table 7: Results of sample determination by ESM and QAMS.

Number	Curcumin mg/g	Demethoxycurcumin mg/g		Bisdemethoxycurcumin mg/g	
		ESM	QAMS	ESM	QAMS
(S1)	0.919	0.153	0.152	0.078	0.080
(S2)	0.948	0.166	0.165	0.090	0.091
(S3)	1.040	0.167	0.167	0.088	0.089
(S4)	0.735	0.127	0.126	0.079	0.080
(S5)	1.369	0.241	0.239	0.144	0.145
(S6)	0.696	0.121	0.120	0.041	0.042
(S7)	0.104	0.029	0.030	0.037	0.040
(S8)	0.750	0.124	0.123	0.072	0.074
(S9)	0.766	0.160	0.160	0.137	0.139
(S10)	0.823	0.131	0.130	0.068	0.069
(S11)	1.087	0.160	0.159	0.069	0.070
(S12)	1.217	0.221	0.221	0.107	0.109

been widely used in the determination of traditional Chinese medicine and multi-index components in recent years [17, 22–26]. In this experiment, the 3 components to be tested were curcumin derivatives and curcumin was a representative component of these compounds and is easy to obtain. So we established QAMS of *Curcuma Longa Radix* by using curcumin as the internal reference. The effects of different instruments, chromatographic column, column temperature, wavelength, and flow rate on RCFs were investigated. At the same time, 12 batches of *Curcuma Longa Radix* for verification were selected. The results showed that RCFs has good reproducibility under the experimental conditions and has no significant difference with ESM. Compared with ESM, QAMS overcomes the shortage of standard goods and saves testing fees, which makes it possible to apply to production practice.

5. Conclusions

TCM is the important part of Chinese culture. And how to establish an effective and reasonable method to monitor the quality of Chinese medicine is necessary. This study provides a new HPLC method for quality control of *Curcuma Longa Radix*. The results showed that the QAMS method for determination of curcumin derivatives of *Curcuma Longa Radix* was fast, accurate, and stable. The method could be suitable for quality control of *Curcuma Longa Radix*.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Zhimin Chen and Yongfeng Zhao contributed equally to this manuscript.

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Review Article

Clinical Effects and Safety of Zhi Sou San for Cough: A Meta-Analysis of Randomized Trials

Ningchang Cheng,[1] Jia Zhu,[2] and Pinpin Ding[1]

[1]*Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210023, China*
[2]*Department of Respiratory Medicine, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210029, China*

Correspondence should be addressed to Jia Zhu; jsnjzj@163.com

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Introduction. Zhi Sou San (ZSS), a traditional Chinese prescription, has been widely applied in treating cough. The purpose of this meta-analysis was to evaluate the effectiveness and safety of ZSS for cough. *Methods*. We searched relevant articles up to 5 March 2017 in seven electronic databases: the Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE, PubMed, Chinese National Knowledge Infrastructure (CNKI), Cqvip Database (VIP), China Biology Medicine disc (CBM), and Wanfang Data. Randomized controlled trials (RCTs) were eligible, regardless of blinding. The primary outcome was the total effective rate. *Results*. Forty-six RCTs with a total of 4007 participants were identified. Compared with western medicine, ZSS significantly improved the total effective rate (OR: 4.45; 95% CI: 3.62–5.47) and the pulmonary function in terms of FEV1 (OR: 0.35; 95% CI: 0.24–0.46) and decreased the adverse reactions (OR: 0.05; 95% CI: 0.02–0.01) and the recurrence rate (OR: 0.30; 95% CI: 0.16–0.57). However, there was no significant improvement in the cough symptom score comparing ZSS with western medicine. *Conclusions*. This meta-analysis shows that ZSS has significant additional benefits and relative safety in treating cough. However, more rigorously designed investigations and studies, with large sample sizes, are needed because of the methodological flaws and low quality of the included trials in this meta-analysis.

1. Introduction

Cough is the most common symptom among individuals seeking medical care. According to the duration, cough is divided into three types: acute, subacute, and chronic [1]. Acute cough (less than 3 weeks in duration) is the most predominant symptom of common cold or acute viral upper respiratory tract infection (URTI) [2, 3]. Acute cough from the common cold is usually transient and minor, but it may be life-threatening when it is caused by a serious illness [2]. Dyspnea, tachypnea, thoracic pain, hemoptysis, a severely worsened general state, and changes in vital signs are the major danger signs of acute cough [4]. Cough lasting from 3 to 8 weeks is categorized as subacute cough [5]. Postinfectious cough (PIC) is the most common cause of subacute cough [5]. Chronic cough is described as a cough that persisted for more than 8 weeks in adults [6] and more than 4 weeks in children (age < 15 years) [7]. Gastroesophageal reflux disease (GERD), asthma syndromes, smoker's cough, nonasthmatic eosinophilic bronchitis, and upper airway cough syndrome (UACS) associated with postnasal drip (rhinitis or rhinosinusitis) are the most common conditions associated with chronic cough in adults who are nonsmokers and are not receiving therapy with angiotensin converting enzyme (ACE) inhibitor [6, 8–10]. Unexplained chronic cough should be diagnosed as chronic cough with no etiology identified after evaluation and supervised therapeutic trial(s) that follow published best-practice guidelines [11].

Recent guidelines have attempted to provide directions in the treatment and management of cough. Patients with acute cough associated with the common cold can be treated with first-generation antihistamine/decongestant (A/D) preparation, expectorants, or mucolytics [3, 8]. Acute cough accompanying a cold or acute bronchitis/sinusitis usually resolves

without any specific medicinal treatment. Any antibiotic treatment of uncomplicated upper respiratory tract infections should be avoided according to the guideline [4]. Although the inhaled ipratropium or inhaled corticosteroids (ICSs) may be useful for PIC, the optimal treatment is not known [8, 57]. Cough variant asthma (CVA) should be initially treated with inhaled bronchodilators and ICSs [8]. Dietary and lifestyle modification, acid suppression therapy, and prokinetic therapy are recommended in patients with chronic cough due to GERD [8]. Current recommendations on managing UACS include A/D or intranasal corticosteroids [6]. Multimodality speech pathology therapy and therapeutic trial of gabapentin are recommended for adults with unexplained chronic cough [11].

ZSS, a formula originating from Qing Dynasty, is commonly used in treating cough nowadays. Tan et al. [58], Zhang [59], and Meng et al. [60], respectively, reported that ZSS is the most frequently used formula in the treatment of PIC and CVA. ZSS is composed of seven herbs: *Platycodon grandiflorum*, Fine Leaf Schizonepeta herb, Tatarian Aster root, Sessile Stemona root/Japanese Stemona root/Tuber Stemona root, Willowleaf Swallowwort Rhizome, tangerine peel, and liquorice root. According to the theory of TCM, ZSS could loose the evil Qi and calm the lung Qi. Compared to its counterparts, ZSS is peaceful and gentle, not too cold or hot.

Modified Zhi Sou San (MZSS) could improve the symptoms of chronic obstructive pulmonary disease (COPD) in rats of northwest China with cold dryness syndrome and delay the velocity of decreased lung function [61]. An experiment showed that the antiasthmatic mechanisms of MZSS were related to its significant reduction in contents of endothelin-1 and nitric oxide, eosinophilia, and the damage of lung tissue [62].

Several systematic reviews and meta-analyses showed that ZSS might be effective in treating diseases-induced cough (including PIC, CVA, and laryngeal cough) [63–65]. Jing et al. reported that MZSS or MZSS combined with western medicine had better safety and efficacy than western medicine alone in treating PIC [63]. ZSS or ZSS combined with western medicine had superior effect and lower recurrence rate than western medicine alone in treating CVA [64]. Wang et al. reported that, compared with western medicine, ZSS had good efficacy and less adverse reactions in treating laryngeal cough [65]. Although the aforementioned reviews and meta-analyses elaborated that ZSS was more effective and safe than western medicine in treating diseases-induced cough, whether ZSS is the alternative medicine in treating cough is not confirmed. More trials and evidences are needed. So this meta-analysis was aimed at summarizing and evaluating the evidence from RCTs and determining whether ZSS is more effective and safer than western medicine in the treatment of cough.

2. Methods

2.1. Research Protocol. This meta-analysis is reported in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

2.2. Databases and Search Strategies. We searched relevant articles up to 5 March 2017 in seven electronic databases: CENTRAL, MEDLINE, and PubMed in English and CNKI, VIP, CBM, and Wanfang Data in Chinese. The search terms used for databases were as follows: (cough or cough*) for cough AND (zhisousan or zhisou san or zhisou powder) for ZSS AND randomized or controlled or clinical research.

2.3. Eligibility Criteria. Studies included had to meet the following criteria: (a) types of studies: any RCTs with ZSS or MZSS administrated orally in patients with cough were eligible, regardless of blinding; (b) types of participants: any patients diagnosed with cough regardless of sex, age, country, or underlying disease were included; (c) types of interventions: any variants of ZSS regardless of the herbs in the ZSS archetype replaced, added, or removed were included; the control group was taking the western medicine; (d) types of outcomes: the primary outcome was the total effective rate (clinical cure rate plus obvious cure rate plus showing effective rate). The clinical efficacy classified as clinical cure, obvious cure, and showing effective and not effective rate was based on the guiding principle of clinical research on new drugs of TCM and/or the diagnostic criteria of TCM syndrome. The secondary outcomes were the score of TCM symptom, cough symptom score (such as cough diary or visual analog scale), the adverse reactions, the pulmonary function test results, and the recurrence rate.

2.4. Study Selection and Data Extraction. Two independent investigators (Ningchang Cheng and Jia Zhu) screened the titles and abstracts of the searched articles. The trials obviously not meeting the inclusion criteria were excluded. We emailed the corresponding authors of the trials which possibly meet the inclusion criteria to ensure that the included trials were RCTs. Any disagreements were dissolved by consensus and discussions. All articles included were judged by the third reviewer (Pinpin Ding). Data extracted included the authors, year of publication, country, sample size, participants (mean age, cough duration), cough inducing disease, details of ZSS interventions, details of control inventions, treatment duration, outcome measurements, and adverse events [66].

2.5. Assessment of Risk of Bias. According to the Cochrane Handbook for Systematic Reviews of Interventions (version 5.0.2), the risk of bias was assessed in seven domains, such as random sequence generation and allocation concealment for selection bias, blinding of participants and personnel for performance bias, blinding of outcome assessment for detection bias, incomplete outcome data for attrition bias, selective outcome reporting for reporting bias, and other sources of bias.

2.6. Data Analysis. Review Manager Software (Version 5.3, Copenhagen, the Nordic Cochrane Centre, the Cochrane Collaboration, 2014) was used for data analysis. Heterogeneity between similar studies is evaluated by chi-square test and I^2 statistic. There is moderate heterogeneity between studies, if $P < 0.05$ and $I^2 > 50\%$, and sensitivity

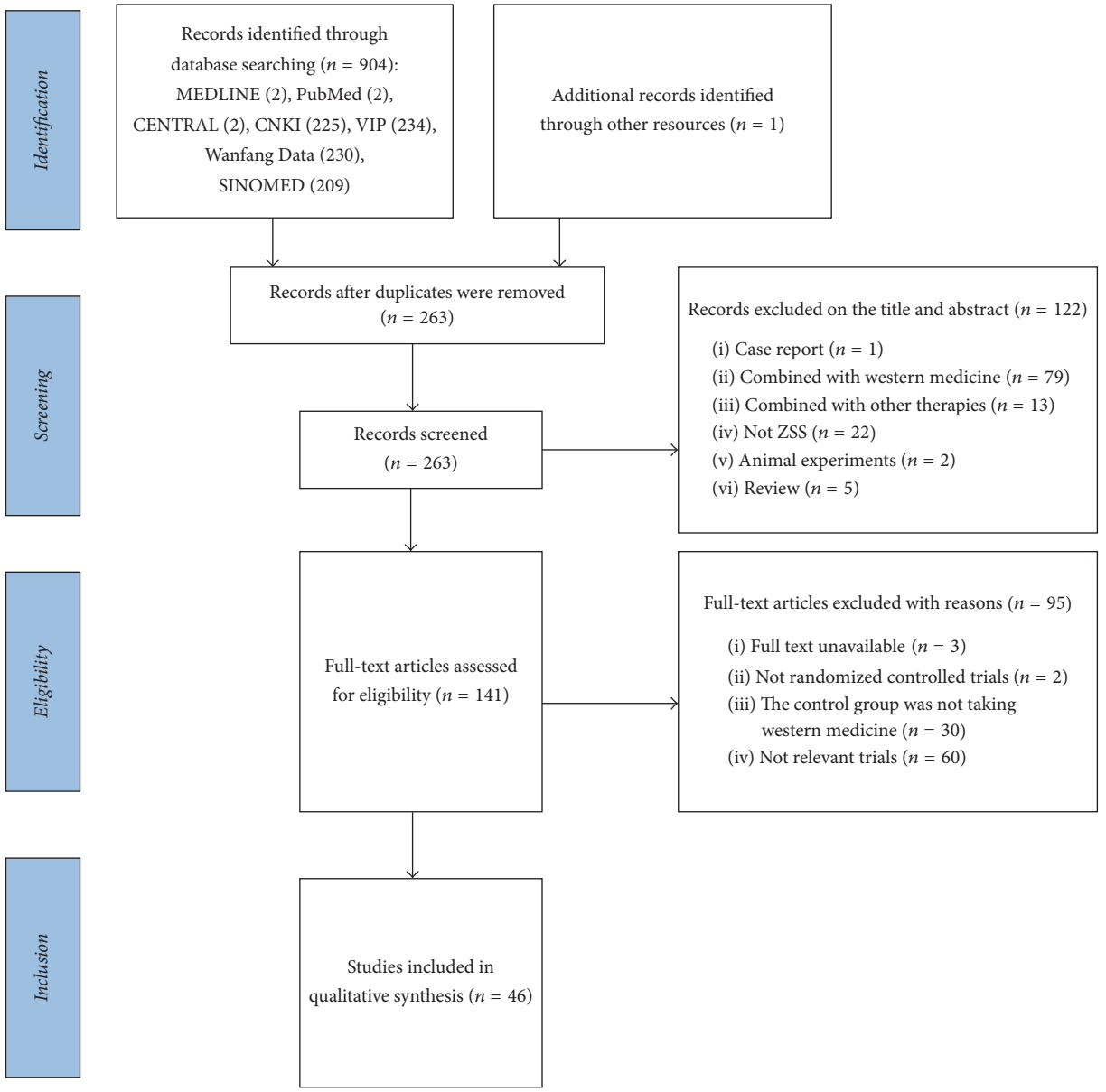

Figure 1: Flowchart of literature searching.

analysis is needed. The enumeration data is evaluated as dichotomous data and expressed as odds ratio (OR) with 95% confidence interval (CI). The measurement data is evaluated as continuous data and expressed as mean difference (MD) with 95% CI. Statistical significant difference was considered as $P < 0.05$.

3. Results

3.1. Characteristics of the Included Studies. We identified 905 articles through electronic searching. After duplicates were removed, 263 records were screened. The full texts of 141 studies were assessed for eligibility after screening the titles and abstracts. 95 studies were excluded with reasons of full text being unavailable, not being RCTs, the controlled group not taking western medicine, and not relevant trials. Finally, 46 studies [12–56, 67] were included in this meta-analysis. A flowchart in the form of PRISMA is presented in Figure 1.

All the included trials originated from China and were published from 2004 to 2016. The total number of participants analyzed in the meta-analysis was 4007, of which 2077 received ZSS or MZSS, while 1930 received western medicine alone. The baseline characteristics of the included trials were shown in Table 1.

In the final selected studies, postsurgical cough accounted for one study [12], cough accounted for one study [43], allergic cough accounted for two studies [53, 54], acute cough accounted for one study [56], laryngeal cough accounted for

Table 1: Baseline characteristics of the included trials.

Author	Year	Country	Cough inducing disease	Sample size (I/C)	Intervention group	Control group	Age (years) (I/C)	Cough duration (I/C)	Intervention duration	Outcome measurements
Dong [12]	2014	China	Postsurgical cough	52/53	ZSS	Gentamicin + α-chymotrypsin	32–65	NR	3 d	①
Cai [13]	2013	China	PIC	36/36	MZSS	Ketotifen or ambroxol	6–15/7–19	NR	5 d	①
Cai [14]	2011	China	PIC	30/30	MZSS	Meptin + ketotifen + azithromycin	18–60/21–55	21–55 d/22–54 d	7 d	①
Cao [15]	2011	China	PIC	30/27	ZSS	Cephalexin + compound guaiacol potassium oral solution	3–8/4–10	15 d–1 y/10 d–10 m	7 d	①
Jin [16]	2016	China	PIC	40/40	MZSS	Pentoxyverine + loratadine	50.08 ± 7.82/49.85 ± 7.85	24.53 ± 8.40 d/23.88 ± 7.90 d	10 d	①
Ju [17]	2010	China	PIC	56/56	MZSS	Chlorphenamine maleate	37.5l ± 9.47/40.18 ± 10.56	40.5 ± 11.5 d/41.2 ± 12.7 d	15 d	① + ②
Kan [18]	2010	China	PIC	30/30	MZSS	Dextromethorphan + chlorphenamine maleate	45.47 ± 8.36/44.59 ± 8.72	4.8 ± 1.2 wk/4.6 ± 1.5 wk	10 d	①
Kan [19]	2011	China	PIC	33/32	MZSS	Roxithromycin id	2–10/2–10	4 wk–3 m/4 wk–3 m	14 d	①
Li [20]	2013	China	PIC	30/30	MZSS	Asmeton	32.19 ± 5.86/33.35 ± 5.72	36.6 ± 6.9 d/33.2 ± 7.5 d	7 d	① + ③
Liang [21]	2014	China	PIC	84/84	MZSS	Pentoxyverine	32.1 ± 1.7/31.9 ± 2.4	14.3 ± 2.0 d/14.0 ± 1.9 d	NR	① + ③
Liu and Qiu [22]	2012	China	PIC	38/40	MZSS	Pentoxyverine + chlorphenamine maleate	19–53/18–55	3–8 wk	NR	① + ③
Liu [23]	2014	China	PIC	68/64	MZSS	Ketotifen + carbetapentane citrate	36.0 ± 6.8/38.0 ± 7.5	33.0 ± 7.5 d/35.0 ± 8.5 d	10 d	①
Lu [24]	2012	China	PIC	43/43	MZSS	Ketotifen + meptin + azithromycin	34.3 ± 1.4	38 ± 2.1 d	7 d	①
Qiu [25]	2012	China	PIC	28/28	MZSS	Chlorphenamine maleate + cartussin	22–55/20–60	19–49 d/23–46 d	15 d	①

Table 1: Continued.

Author	Year	Country	Cough inducing disease	Sample size (*I*/*C*)	Intervention group	Control group	Age (years) (*I*/*C*)	Cough duration (*I*/*C*)	Intervention duration	Outcome measurements
Qiu [26]	2011	China	PIC	82/78	MZSS	Dextromethorphan + loratadine	33.5 ± 8.6/34.3 ± 8.8	18 ± 3.0 d/17.6 ± 2.0 d	7 d	① + ③
Qiu [27]	2012	China	PIC	35/30	MZSS	Compound pholcodine syrup + asmeton + azithromycin	14–65/15–65	NR	3 d	①
Sun [28]	2011	China	PIC	58/52	MZSS	Chlorpheniramine maleate + methyl bromide	19–57/20–58	14–40 d/16–41 d	7 d	①
Tao [29]	2011	China	PIC	30/30	MZSS	Pseudoephedrine hydrochloride sustained release capsules	40.2 ± 8.7/39.5 ± 7.5	40.5 ± 13 d/42.8 ± 10.0 d	7 d	① + ②
Zhu [30]	2010	China	PIC	62/30	MZSS	Azithromycin	2 m–13/5 m–13	2–4 d/1–5 d	3 wk	①
Chen [31]	2004	China	Laryngeal cough	39/39	MZSS	Roxithromycin id + Zhikelu	5–87/7–82	5 d–2 y/3 d–1.5 y	5 d	①
Fang [32]	2014	China	Laryngeal cough	43/43	MZSS	Setastine + carbetapentane citrate + new bromide	40/41	3–12 wk/3–12 wk	14 d	①
Hu [33]	2013	China	Laryngeal cough	59/54	MZSS	Budesonide	34–63/32–64	3–8 wk/3–7.3 wk	7 d	① + ⑤
Huang [34]	2013	China	Laryngeal cough	60/60	MZSS	Cefuroxime axetil + phenergan syrup	16–64/17–65	7 d–3 m/7 d–3 m	10 d	① + ③ + ⑤
Liu [35]	2004	China	Laryngeal cough	72/38	MZSS	Amoxicillin or cefradine + phenergan cough syrup	31.3 ± 2.1/30.1 ± 2.3	42 ± 2.3 d/43.3 ± 6.7 d	4 d	①
Gong [36]	2007	China	CVA	40/40	MZSS	Ketotifen + doxofylline + salbutamol aerosol	41.3 ± 10.3/38.5 ± 11.8	2–36 m/2–30 m	28 d	① + ④
Lu [37]	2007	China	CVA	35/34	MZSS	Terbutaline + cetirizine	9.6/9.2	1.3 m/1.4 m	14 d	①
Liu [35]	2012	China	CVA	28/25	MZSS	Shah Mette Lo fluticasone propionate	43.79 ± 12.58/42.16 ± 10.77	3.42 ± 2.17 m/3.55 ± 2.29 m	14 d	① + ②
Qu [38]	2006	China	CVA	30/30	MZSS	Terbutaline	38.2 ± 12.5/40.3 ± 13.2	7.5 ± 8.5 m/8.0 ± 7.0 m	30 d	① + ④
Tao [39]	2014	China	CVA	55/55	MZSS	Montelukast sodium + budesonide aerosol	2–11/2.5–12	3–33 m/4–36 m	4 wk	①
Wang [40]	2011	China	CVA	60/60	MZSS	Conventional treatment for cough	2–9/2.5–10	NR	10 d	①
Yu et al. [41]	2016	China	CVA	52/52	MZSS	Shah Mette Lo fluticasone powder	40.9 ± 6.8/41.5 ± 7.2	8.5 ± 2.2 m/8.2 ± 2.4 m	30 d	① + ② + ④

Table 1: Continued.

Author	Year	Country	Cough inducing disease	Sample size (*I*/*C*)	Intervention group	Control group	Age (years) (*I*/*C*)	Cough duration (*I*/*C*)	Intervention duration	Outcome measurements
Zhang [42]	2009	China	CVA	30/30	MZSS	Ceftazidime + dexamethasone	2–66	NR	14 d	① + ②
Shao [43]	2004	China	Cough	60/48	MZSS	Cefradine	14–66/12–62	NR	14 d	①
Cai [44]	2011	China	Chronic cough	24/24	MZSS	Conventional treatment for cough	26–70/20–65	8–36 wk/10–42 wk	14 d	①
Dai et al. [45]	2015	China	Chronic cough	26/26	MZSS	Desloratadine + ambroxol	37.83 ± 10.43/36.40 ± 11.89	28.55 ± 26.71 wk/34.73 ± 24.17 wk	14 d	①
Huang [46]	2014	China	Chronic cough	45/45	MZSS	Chlorphenamine + aminophylline + ambroxol	24–75	8 wk–2 y	14 d	① + ③
Qiao [47]	2016	China	Chronic cough	40/40	MZSS	Cefuroxime axetil + ambroxol + dextromethorphan	54.6 ± 7.9	3.3 ± 1.6 y	1 m	① + ③
Tao [48]	2013	China	Chronic cough	40/40	MZSS	Azithromycin + ambroxol	20–76	NR	7 d	①
Wang and Guo [49]	2016	China	Chronic cough	50/50	MZSS	Chlorpheniramine maleate + salbutamol + methyl bromide	4.65 ± 1.94/5.46 ± 2.53	NR	7 d	①
Yang [50]	2015	China	Chronic cough	47/47	MZSS	Asmeton	41.6 ± 5.7	10.4 ± 1.9	7 d	① + ③
Ye [51]	2009	China	Chronic cough	30/30	MZSS	Asmeton	19–46	NR	7 d	①
Zhang [52]	2016	China	Chronic cough	44/44	MZSS	Ambroxol	50 ± 2.96/51 ± 2.63	NR	14 d	① + ⑤
Dang and Yang [53]	2008	China	Allergic cough	38/38	MZSS	Ketotifen + aminophylline	2–12/2–12	1–6.6 m/1–5.9 m	7 d	①
Zhang [54]	2014	China	Allergic cough	25/25	MZSS	Terbutaline	6.25 ± 3.05/6.05 ± 3.12	8.09 ± 15.45 m/7.86 ± 15.36 m	4 wk	① + ③
Hu [55]	2015	China	Acute cough	60/60	ZSS	Asmeton	37 ± 11/36 ± 10	18 ± 6 d/16 ± 7 d	14 d	①
Huang [56]	2011	China	Acute cough	80/40	MZSS	Amoxicillin	19–35/20–34	0.5–7 d/0.5–6.5 d	3 d	①

y, year; m, month; wk, week; d, day; NR: not reported. *Note.* ① The total effective rate, ② cough symptom score, ③ the adverse reactions, ④ the pulmonary function test results, and ⑤ the recurrence rate.

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95% CI
Dai et al., 2015	25	26	22	26	0.8%	4.55 [0.47, 43.78]
Zhang, 2016	41	44	26	44	2.4%	9.46 [2.53, 35.32]
Shao, 2004	56	60	27	48	3.1%	10.89 [3.40, 34.86]
Liu and Qiu, 2012	36	38	37	40	1.2%	1.46 [0.23, 9.25]
Qiu, 2012	26	28	21	28	1.5%	4.33 [0.81, 23.10]
Zhang, 2009	27	30	24	30	1.9%	2.25 [0.51, 9.99]
Gong, 2007	38	40	33	40	1.6%	4.03 [0.78, 20.76]
Cai, 2013	33	36	24	36	2.3%	5.50 [1.40, 21.65]
Yu et al., 2016	47	52	38	52	3.5%	3.46 [1.14, 10.48]
Huang, 2014	44	45	39	45	0.9%	6.77 [0.78, 58.72]
Sun, 2011	54	58	37	52	3.0%	5.47 [1.68, 17.80]
Tao, 2014	50	55	43	55	3.4%	2.79 [0.91, 8.55]
Zhu, 2010	58	62	25	30	2.2%	2.90 [0.72, 11.71]
Cai, 2011	22	24	18	24	1.4%	3.67 [0.66, 20.42]
Dong, 2014	49	52	41	53	2.4%	4.78 [1.26, 18.10]
Fang, 2014	40	43	35	43	2.2%	3.05 [0.75, 12.39]
Kan, 2010	28	30	24	30	1.5%	3.50 [0.65, 18.98]
Tao, 2011	24	30	15	30	3.2%	4.00 [1.27, 12.58]
Lu, 2007	31	35	22	34	2.7%	4.23 [1.20, 14.85]
Yang, 2015	45	47	38	47	1.7%	5.33 [1.08, 26.18]
Jin, 2016	37	40	28	40	2.3%	5.29 [1.36, 20.53]
Huang, 2013	55	60	45	60	3.6%	3.67 [1.24, 10.86]
Qiao, 2016	36	40	31	40	2.6%	2.61 [0.73, 9.32]
Qu, 2006	28	30	24	30	1.5%	3.50 [0.65, 18.98]
Miao et al., 2012	26	28	18	25	1.5%	5.06 [0.94, 27.19]
Cai, 2011	28	30	25	30	1.4%	2.80 [0.50, 15.73]
Cao, 2011	28	30	21	27	1.5%	4.00 [0.73, 21.84]
Huang, 2011	78	80	17	40	1.8%	52.76 [11.34, 245.44]
Qiu, 2011	32	35	26	30	1.7%	1.64 [0.34, 8.00]
Qiu, 2012	77	82	74	78	2.3%	0.83 [0.22, 3.22]
Zhang, 2014	24	25	21	25	0.8%	4.57 [0.47, 44.17]
Wang and Guo, 2016	47	50	39	50	2.3%	4.42 [1.15, 16.97]
Hu, 2015	58	60	44	60	1.8%	10.55 [2.30, 48.28]
Hu, 2013	52	59	35	54	4.5%	4.03 [1.53, 10.60]
Liu, 2004	69	72	31	38	2.1%	5.19 [1.26, 21.43]
Chen, 2004	38	39	32	39	0.9%	8.31 [0.97, 71.18]
Dang and Yang, 2008	36	38	29	38	1.6%	5.59 [1.12, 27.90]
Kan, 2011	30	33	21	32	2.2%	5.24 [1.30, 21.10]
Li, 2013	25	30	16	30	2.9%	4.38 [1.32, 14.50]
Ye, 2009	29	30	21	30	0.9%	12.43 [1.46, 105.74]
Tao, 2013	39	40	30	40	0.9%	13.00 [1.58, 107.23]
Lu, 2012	40	43	35	43	2.2%	3.05 [0.75, 12.39]
Ju, 2010	53	56	42	56	2.5%	5.89 [1.59, 21.85]
Liang, 2014	76	84	60	84	5.6%	3.80 [1.59, 9.06]
Wang, 2011	56	60	43	60	3.1%	5.53 [1.74, 17.65]
Liu, 2014	65	68	49	64	2.5%	6.63 [1.82, 24.19]
Total (95% CI)		*2077*		*1930*	*100.0%*	*4.45 [3.62, 5.47]*
Total events	1936		1446			

Heterogeneity: $\text{Tau}^2 = 0.00$; $\text{Chi}^2 = 31.38$; df = 45 ($P = 0.94$); $I^2 = 0\%$
Test for overall effect: $Z = 14.23$ ($P < 0.00001$)

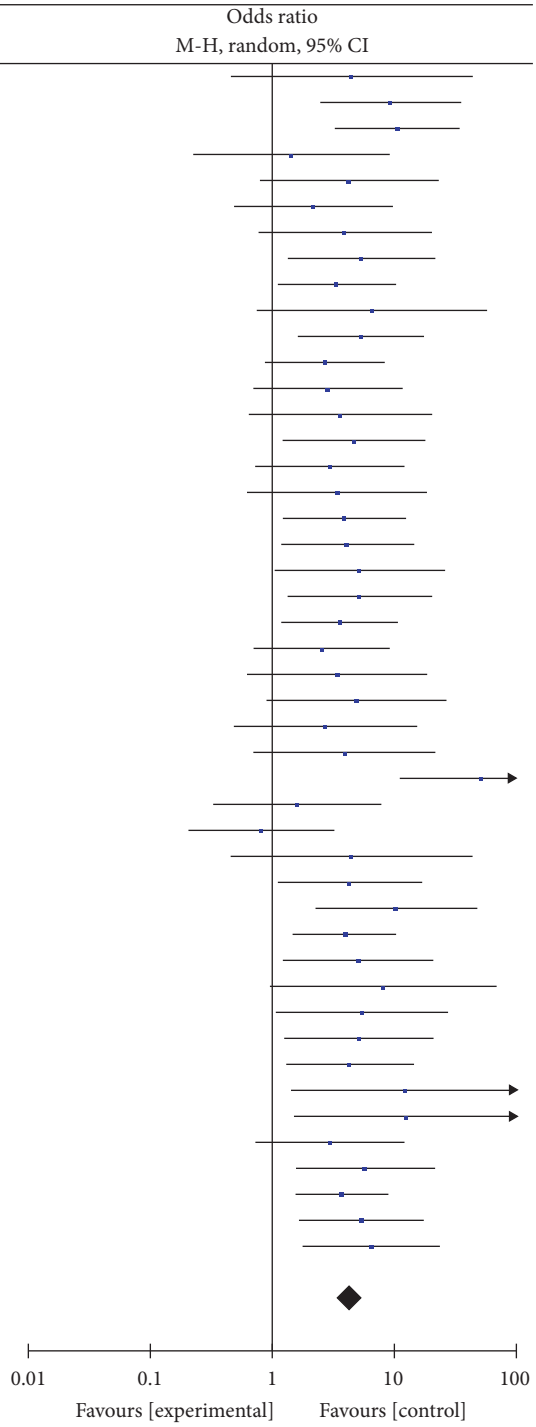

Figure 2: The effective rate comparing ZSS to western medicine alone.

six studies [31–35, 55], CVA accounted for eight studies [36–42, 67], chronic cough accounted for nine studies [44–52], and PIC accounted for eighteen studies [13–30].

3.2. The Total Effective Rate. All trials finally selected reported data on total clinical response rate [12–56, 67]. As for the fact that there was no significant heterogeneity ($I^2 = 0\%$; $P = 0.94$), a random-effects model was applied (Figure 2). The meta-analysis showed that a significant improvement in the total effective rate (OR: 4.45; 95% CI: 3.62–5.47) was observed when comparing ZSS to western medicine alone.

3.3. Cough Symptom Score. Five trials reported data on cough symptom score [17, 29, 41, 42, 67]. After sensitivity analysis,

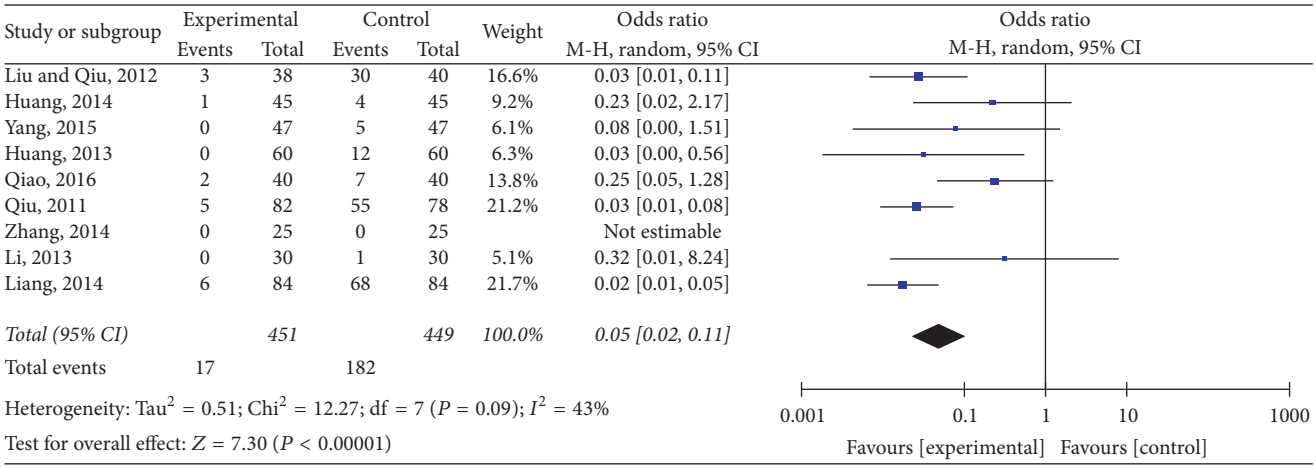

Figure 3: The adverse reactions comparing ZSS to western medicine alone.

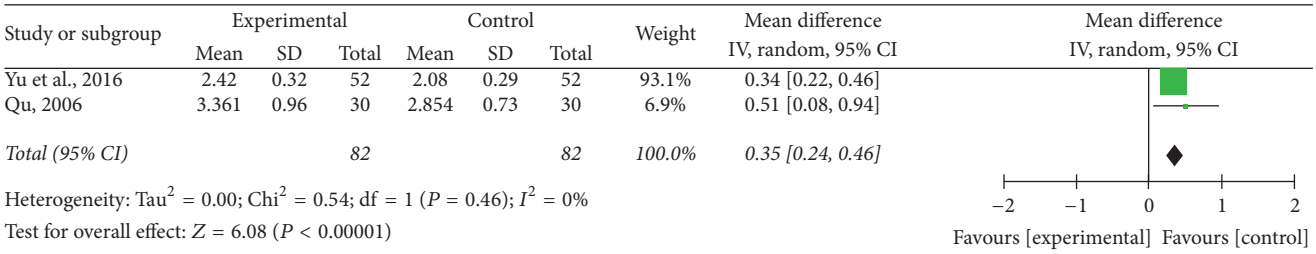

Figure 4: The pulmonary function test results comparing ZSS to western medicine alone.

a trial [17] was excluded. As for the fact that there was no significant heterogeneity (I^2 = 0%; P = 0.53), the meta-analysis showed that there was no significant improvement in the cough symptom score (OR: −0.72; 95% CI: −0.79–−0.65).

3.4. The Adverse Reactions. Nine trials mentioned the adverse reactions as the secondary outcome [20–22, 26, 34, 46, 47, 50, 54]. As is shown in Figure 3, a random-effects model was applied; there was no significant heterogeneity (I^2 = 43%; P = 0.09). The meta-analysis showed that a significant decrease in the adverse reactions (OR: 0.05; 95% CI: 0.02–0.01) was observed when comparing ZSS to western medicine alone.

3.5. The Pulmonary Function Test Results. Three trials provided data on the pulmonary function test results [36, 38, 41]. Two trials reported FEV1 (L) [38, 41]. The meta-analysis with random-effects model showed that ZSS significantly improved the pulmonary function in terms of FEV1 (I^2 = 0%; P = 0.46; OR: 0.35; 95% CI: 0.24–0.46) in comparison to western medicine alone (Figure 4).

3.6. The Recurrence Rate. There were three studies that talked about the recurrence rate [33, 34, 52]. Results (Figure 5) indicated that ZSS obviously decreased the recurrence rate compared with western medicine (I^2 = 0%; P = 0.55; OR: 0.30; 95% CI: 0.16–0.57).

3.7. Assessing the Risk of Bias of the Included Studies. The risk of bias of the finally included trials was not low. The selection bias was high due to the fact that wrong methods were applied in random sequence generation. Because multiple studies failed in blinding of participant and outcome assessment, the performance and detection biases were high (Figures 6 and 7) (+ indicates low risk of bias, − indicates high risk of bias, and ? indicates unclear risk of bias).

4. Discussion

A wide variety of pharmacological agents have been used in treating cough, such as antibiotic, ketotifen, asmeton, dextromethorphan, and ICSs [68]. No matter what caused cough, those mentioned agents were broadly used in remedying cough. "Zhisou," by its Chinese definition, means relieving cough. So ZSS is one formula that has the behavior of relieving cough and it is widely applied in China, Korea, and Japan. It is necessary and attractive to compare ZSS and western medicine commonly used in curing cough. This meta-analysis was aimed at evaluating the effect and safety between ZSS and aforementioned western medicines. However, we could not find any studies originating from Japan or Korea in those databases that we have searched. It

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95% CI
Zhang, 2016	2	44	8	44	15.3%	0.21 [0.04, 1.07]
Huang, 2013	5	60	18	60	34.8%	0.21 [0.07, 0.62]
Hu, 2013	15	52	17	35	49.8%	0.43 [0.18, 1.05]
Total (95% CI)		*156*		*139*	*100.0%*	*0.30 [0.16, 0.57]*
Total events	22		43			

Heterogeneity: Tau^2 = 0.00; Chi^2 = 1.19; df = 2 (P = 0.55); I^2 = 0%

Test for overall effect: Z = 3.72 (P = 0.0002)

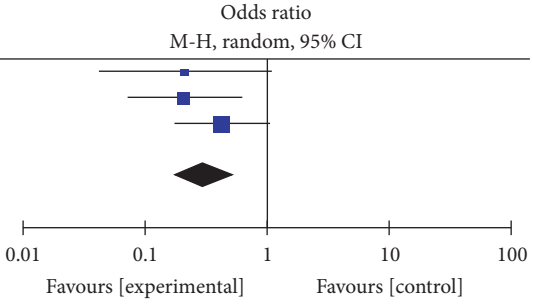

Figure 5: The recurrence rate comparing ZSS to western medicine alone.

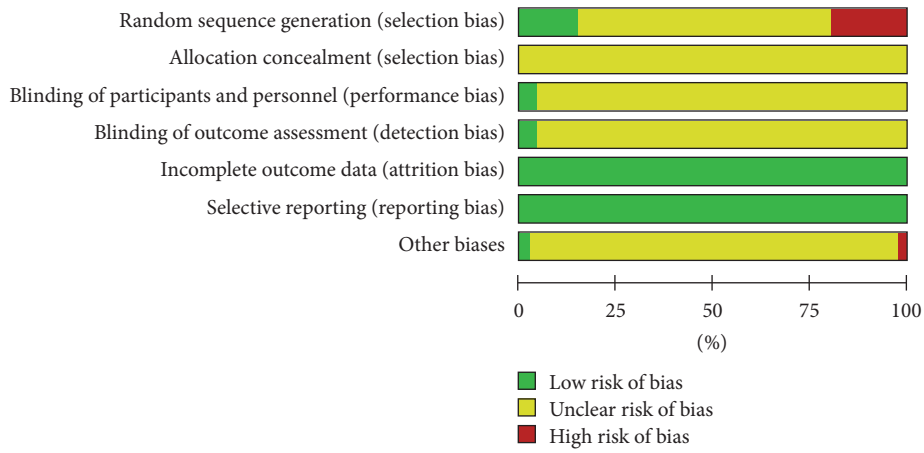

Figure 6: Risk of bias graph of the included trials.

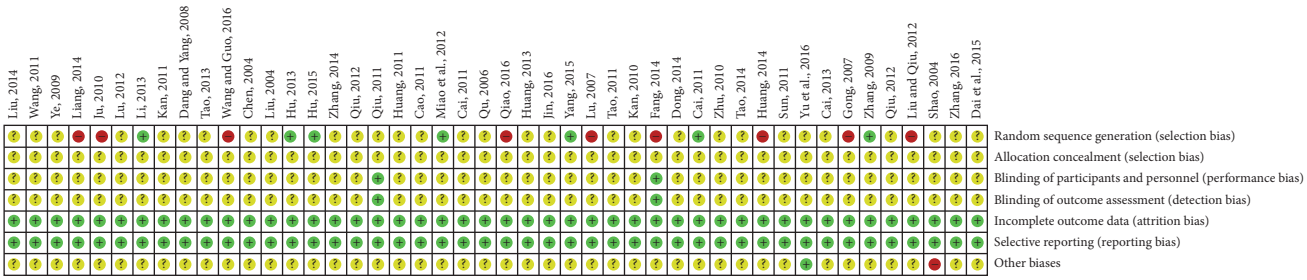

Figure 7: Risk of bias summary of the included trials.

may be due to the fact that the authors of this meta-analysis all come from China; they do not master Japanese language and Korean language.

As we know, this meta-analysis is the first one about using ZSS in treating cough. This meta-analysis included studies of a wide range of conditions, such as acute cough, chronic cough, PIC, and CVA. The main findings of this meta-analysis are as follows: ZSS significantly improved the total effective rate and the pulmonary function in terms of FEV1, ZSS decreased the adverse reactions and the recurrence rate compared with western medicine, and ZSS appeared to be safe, well-tolerant, and more effective in treating cough.

However, we should admit that several limitations exist concerning this study. Firstly and foremost, the sample sizes of RCTs were small and limited. So, it was difficult to find out the influence of contingency factors. Secondly, insufficient reporting of random sequence generation and allocation concealment were the major methodological flaws in most of the included trials, which could result in selection bias and decrease the reliability of the evidence. Thirdly, the overall methodological quality of included trials was low due to the lack of blinding of participants and personnel and outcome assessment. On the whole, the finally included studies failed to follow CONSORT guidelines for RCTs; the risk of bias assessment was assessed as high risk or unclear risk in a majority of the RCTs. Therefore, RCTs with high quality and large sample are required to be done in the future.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Ningchang Cheng and Jia Zhu designed this study, interpreted the results, made the literature research, extracted data, performed the statistical analysis, and revised the manuscript. Pinpin Ding evaluated the quality of the included studies and drafted the manuscript.

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