Identification of the Main Active Components and Mechanism of Wang Bi Tablet in Treating Rheumatoid Arthritis Based on Integrative Pharmacology

Yuanyuan Jiao1,2†, Jia Xu3†, Hong Chen1,4†, Qiuyan Guo1, Xiaofang Deng1, Tong Zhang1, Jingbo Zhang1, Chenjing Shi1 and Ping Wang1*

1Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China, 2College of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China, 3Affiliated Hospital of Guizhou Medical University, Guiyang, China, 4College of Traditional Chinese Medicine, Shenyang Pharmaceutical University, Shenyang, China

Wang Bi tablet (WBT) is used to treat rheumatoid arthritis (RA) in China. We employed integrative pharmacology, including rapid analysis of chemical composition, pharmacological experiment, and network pharmacology analysis, to elucidate the active components and mechanism underlying the effect of WBT against RA. The chemical fingerprint of WBT was revealed by UPLC-QTOF-MS/MS, and the chemical composition was identified. The anti-inflammatory effect of WBT was evaluated in TNF-α-stimulated RAW264.7 cells by ELISA and transcriptome sequencing. Network pharmacology analysis, functional enrichment analysis, and network visualization were performed. A total of 293 chemical constituents were preliminarily identified or tentatively characterized in WBT extract, and they effectively inhibited inflammatory response in TNF-α-stimulated RAW264.7 cells. Forty-eight key active constituents were identified based on high-frequency binding to hub targets and their corresponding targets number. Next, 135 corresponding hub genes, which may be the putative targets of WBT in treating RA, were selected. Functionally, the putative targets were significantly associated with the inflammatory immune response regulation module, energy metabolism regulation module, and cell function regulation module, corresponding to the traditional efficacy of WBT. In summary, this study revealed, for the first time using integrative pharmacology, that WBT may attenuate RA through the inflammation-immune regulation system.

Keywords: TCMIP, integrative pharmacology strategy, UPLC-QTOF-MS/MS, rheumatoid arthritis, Wang Bi Tablet

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial hyperplasia, inflammatory cell infiltration, pannus formation, and destruction of articular cartilage and bone matrix, and this disease develops symmetrically and destructively, eventually leading to articular deformity and loss of function (Smolen et al., 2016; Burmester and Pope, 2017; McInnes and Schett, 2017; Alehah and Smolen, 2018). RA has a worldwide prevalence of approximately 0.5–1%, affects women 2–3 times more often than men, and occurs in adolescents, adults, and the elderly, with a high...
incidence in people aged 40–60 years (Saraux et al., 2006; Myasoedova et al., 2010). Globally, the overall age-standardized prevalence and incidence rates of RA have been increasing since 1990 (Safiri et al., 2019). Between 2005–2014, the overall incidence of RA was stable compared with that in the previous decade, possibly owing to the changing prevalence of lifestyle factors, such as smoking and obesity (Myasoedova et al., 2020). As RA is a chronic, progressive disease, patients with RA have serious comorbid conditions, such as infection (Au et al., 2011), osteoporosis (Jin et al., 2018), cardiovascular disease (England et al., 2018), respiratory disease (Chatzidionisyou and Catrina, 2016), and cancer (Asking, 2007). Early diagnosis and treatment could slow down the progression of arthritic damage in 90% of RA patients, thereby preventing irreversible disability (Goekoop-Ruiterman et al., 2005). However, only a few patients can achieve long-term remission without long-term medical treatment (Matcham et al., 2014). At present, the western drugs for RA can be divided into five generations according to the development time and principles: nonsteroidal anti-inflammatory drugs, glucocorticoids, disease-modifying antirheumatic drugs, early biological agents mainly composed of TNF-α inhibitors, and new biological agents directly targeting T cells. In recent years, the development of effective biologics and small-molecule kinase inhibitors has markedly improved both the management and long-term prognosis of RA. However, these treatments are mainly used to relieve symptoms, and they cause adverse side effects including vomiting, rash, leukopenia, and liver and kidney damage. Therefore, it is urgent to find a safe and effective treatment strategy to improve both the management and long-term prognosis of RA.

Traditional Chinese medicine (TCM) is a medical and pharmaceutical system with unique theories and techniques that reflect the Chinese nations understanding of life, health, and disease. Wang Bi was first proposed by Mr. Shude Jiao in 1981 in China, mainly refers to RA and other diseases with joint deformation and bone damage, such as ankylosing spondylitis, tuberculous arthritis, and Kashin-Beck disease (Jiao and Wang, 2009). Owing to its minor side effects and reasonable treatment expenditure, TCM has become a significant strategy for treating chronic, complex, and geriatric diseases, such as RA, diabetes, emphysema, and chronic nephritis. Herbal TCMs, such as Dan-guine Sui decoction (Cheng et al., 2017), Guizhi Fuzi decoction (Feng et al., 2013), Huangqi Guizhi Wuwu decoction (Wang et al., 2010), and Wang Bi tablet (WBT) (Yang et al., 2009; Kang et al., 2011; Wu and Shen, 2011; Liu et al., 2012), have a long history of use in the treatment of RA. WBT is a Chinese patent medicine produced exclusively by Liaoning Haohushi Pharmaceutical (Group) Co., Ltd., and it has been recorded in the Pharmacopeia of the People’s Republic of China from the 2010 edition. WBT is composed of 17 herbal medicines, including *Rehmannia glutinosa* (Gaertn.) DC (Sheng Dihuang, SDH) 15.4g, processed *R. glutinosa* (Shu Dihuang, SDH) 15.4g, *Dipsacus asper* Wall. ex DC (Xu Duan, XD) 11.5g, *Aconitum carmichaelii Debeaux* (Fu Zi, FZ) 11.5g, *Angelica pubescens* Maxim (Du Huo, DH) 7.7g, *Drynaria fortunei* (Kunze ex Mett.) J. Sm (Gu Suibu, GSB) 11.5g, *Cinnamomum cassia* (L.).
WBT against RA is mediated via regulation of its candidate targets. The findings of this study would advance our understanding of the pharmacological mechanism of WBT against RA.

MATERIALS AND METHODS

Chemicals and Reagents
HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific Co. (Loughborough, United Kingdom). HPLC-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, United States of America). Distilled water was purchased from Watsons Water Co., Ltd. (Shenzhen, China). SDH, SD, XD, FZ, DH, GSB, GZ, YYH, FF, WLX, ZJC, BS, GJ, ZM, SJC, HH, and goat or sheep bones were purchased from Shanghai Traditional Chinese Medicine Co., Ltd. (Shanghai, China). The herbal medicines were identified by Mrs. Xirong He, a research assistant at the China Academy of Chinese Medical Sciences (Beijing), and the voucher specimens were deposited at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

Recombinant human TNF-α (catalog #300-01A) was purchased from PeproTech, Inc. (Rocky Hill, NJ, United States of America). An MTT assay kit (ab211091) as well as IL-1β (ab46052) and IL-6 (ab178013) ELISA kits were purchased from Abcam (Burlingame, CA, United States). Dulbecco's modified eagle medium (DMEM) (LOT26019006), 0.25% trypsin (LOT10519010), penicillin streptomycin solution (LOT30002341), and phosphate buffer saline (PBS) (LOT18919010) were purchased from Corning, Inc (NY, United States). Fetal bovine serum (LOT10099-141) was purchased from Gibco BRL Co. (Boise, Idaho, United States). Methotrexate was purchased from Sigma-Aldrich (St. Louis, MO, United States of America).

Preparation of Herbal Extracts
WBT preparation was conducted in complete compliance with that recorded in the Chinese Pharmacopoeia (P985-986, 2015 edition). The procedure was as follows. Briefly, SDH 15.4 g, SD 15.4 g, GSB 11.5 g, GJ 11.5 g, and goat or sheep bones 15.4 g were decocted twice in eight and six volumes of water for 1.5 h. The decoctions were filtered and then combined to obtain extract S1. The remaining 12 herbal medicines were decocted as before to obtain extract S2. Next, S2 was evaporated under reduced pressure to obtain extract S3. S1 and S3 were combined and concentrated under reduced pressure to obtain a thick paste (S4) with a relative density of 1.27–1.30 (50°C). BS 46 g and ZM 57.5 g were ground into powder and then filtered through a 100-mesh sieve. The fine powder was mixed thoroughly with S4 before freeze drying, and the
lyophilized powder was screened through a 40-mesh sieve. Six batches of herbal medicines were extracted in parallel. The fine lyophilized powder of WBT was ultrasonically extracted using 20 volumes of 70% MeOH for 0.5 h before centrifugation at 12,000 × g for 10 min in an Eppendorf 5415D centrifuge (Eppendorf Co., Hamburger, Germany). After the supernatant was filtered through a 0.22 μm filter ( Pall Corporation, Beijing, China), 2 μl aliquots were transferred to the UPLC-QTOF-MS/MS system for analysis. The quality control (QC) samples of WBT were prepared by mixing the six batches of herbal medicines.

Instrumentation and UPLC-QTOF-MS/MS Conditions
The UPLC separation was performed using a Waters Acquity UPLC HSS T3 column (100 mm × 2.1 mm, i. d. 1.8 μm) on a Waters Acquity UPLC I-Class system (Waters Corp., Milford, United States of America). The column was maintained at 40°C. The mobile phases consisted of eluent A (0.1% formic acid in deionized water, v/v) and eluent B (0.1% formic acid in acetonitrile, v/v), and the linear gradient program was as follows: 0–1 min, 1% B; 1–10 min, 10% B; 10–20 min, 20–40% B; 20–25 min, 40–50% B; 25–28 min, 50–100% B; 28–33 min, 100% B; 33–33.1 min, 1% B; 33.1–35 min, 1% B. The flow rate was 0.5 ml/min, and the injection volume was 2 μl.

The MS experiment was performed on a Waters Xevo G2-S Q-TOF Mass System (Manchester, United Kingdom) equipped with electrospray ionization (ESI). The data acquisition modes were MS² centroid for all samples and the extra continuum mode for QC samples in both the ESI+ and ESI- ionization modes. The operating parameters were set as follows: mass range, 50–1,500 Da; source temperature, 100°C; desolvation temperature, 400°C; desolvation gas flow, 800 L/h; sampling cone, 40 V; capillary voltage, 2.5 KV for ESI-, 0.5 KV for ESI+. At low CE scan, the auto MS collision energy was 6 eV. At high CE scan, the collision energy was 15–45 eV ramp for ESI- and ESI+.

Leucine enkephalin was selected as the lock-mass at a concentration of 200pg/ml in acetonitrile (0.1% formic acid): H2O (0.1% formic acid) (50:50, v/v) for the positive ion mode ([M + H]+ = 556.2771) and negative ion mode ([M–H]− = 554.2615) via a lock spray interface.

Cell Culture
RAW264.7 murine macrophage-like cells were obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in a biochemical incubator (LRH-150, Shanghai, China) with humidified atmosphere containing 95% O2 and 5% CO2.

MTT Assay
The cytotoxicity of WBT was analyzed via MTT assay. RAW264.7 cells (1 × 10⁵ cells/well) were plated in 96-well plates (p = 6) and incubated for 24 h. After washing with PBS, WBT (12.8, 25.6, 128, 640, 3200, 1.6 × 10⁶, 8.0 × 10⁴, 4.0 × 10⁵, 2.0 × 10⁶, 1.0 × 10⁷ ng/ml) or PBS was added to the cells, which were then incubated for another 24 h. Cell viability was evaluated using an MTT assay kit (#ab211091; Abcam) according to the manufacturer’s protocol, and the absorbance was detected by a PerkinElmer EnVision multimode plate reader (2104, Wellesley, MA).

Drug Treatment
RAW264.7 cells were seeded on 96-well plates (p = 6) at a density of 1 × 10⁵ cells per well and incubated for 24 h. Next, the medium was discarded, and the cells were washed with PBS. The cells were subsequently incubated with PBS, TNF-α (20 ng/ml), TNF-α (20 ng/ml) + WBT (0.001, 0.01, 0.1, 1.0, or 10 µg/ml), or TNF-α (20 ng/ml) + methotrexate (MTX, 0.2 µg/ml) for another 24 h. Finally, the supernatant was collected for measurement of IL-1β and IL-6 using ELISA kits according to the manufacturer’s instructions.

RNA Extraction and Quality Control
Total RNA was isolated using a RNeasy mini kit (Qiagen, Germany) according to the manufacturer’s protocol. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States of America). The quantity of total RNA in the samples was measured using a Qubit®3.0 Fluorometer (Thermo Fisher Scientific, United States of America) and NanoDrop One (Wilmington, DE, United States of America).

mRNA-Seq
Paired-end libraries were synthesized using a TruSeq™ RNA Sample Preparation Kit (Illumina, United States of America) following the TruSeq™ RNA Sample Preparation Guide. Briefly, poly A-containing mRNA molecules were purified using poly T oligo-attached magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations at 94°C for 8 min. The cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. This was followed by second-strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then went through an end-repair process, the addition of a single A’ base, and then ligation of the adapters. The products were then purified and amplified via PCR to create the final cDNA library. Purified libraries were quantified using a Qubit® 2.0 Fluorometer (Life Technologies, United States of America) and validated using an Agilent 2100 Bioanalyzer (Agilent Technologies) to confirm the insert size and calculate the mole concentration.Clusters were generated by cBot with the library diluted to 10 pM and then sequenced on an Illumina NovaSeq 6000 (Illumina, United States of America). The sequencing work was performed by Beijing Zhimei Yinuo Biotechnology Co., Ltd. (Beijing, China). The sequencing data has been uploaded to GEO website with Series record GSE165272.

Bioinformatic Analysis
Bioinformatic analysis was also undertaken by Zhimei Yinuo Biotechnology Co., Ltd. The whole procedure is shown in Figure 2.
Prediction the Putative Targets of the Chemical Constituents of WBT

The TCM target prediction and function analysis module (TTFM) of TCMIP was employed to predict the putative targets of the compounds preliminarily identified in WBT. The prediction accuracy was set at 0.80 (moderate to high similarity) to select constituent-putative target pairs, and these genes were saved in the customer center. Simultaneously, the QED value was set at 0.49, and constituents with QED <0.49 will be filtered out.

Collection of RA-Related Genes From TCMIP

The disease name “rheumatoid arthritis” and symptom nouns, such as “joint swelling”, “morning stiffness”, and “arthralgia” were selected to search in TCMIP in order to obtain RA-related genes among those saved in the personal user center.

Network Visualization and Functional Enrichment Analysis

NaviGator v3.0 was employed to establish a network, and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov) was used to elucidate the biological functions of the core efficacy gene set.

Data Analysis

The UPLC-QTOF-MS/MS system was controlled by the Masslynx 4.1 software (Waters Corp.). The MS⁸ continuum data were processed by UNIFI 1.8 (Waters Corp.). The processes included data acquisition, data mining, library searching, and report generation. The chemical information of the 16 herbal medicines (except for goat or sheep bones) was collected in a list consisting of molecular name/formulae/weights and chemical structures (mol. format) from the literature and the Encyclopedia of Traditional Chinese Medicine (http://www.nrc.ac.cn:9090/ETCM/) (Xu et al., 2019). The list, as a customized library that could facilitate chemical identification, is shown in Supplementary Excel S1 [M+H]^+, [M+K]^+, [M+Na]^+, [2M+H]^+, and [M-e]^- were selected as additive ions in the positive ion mode. [M+COOH]^-, [M-H]^-, and [2M-H]^− were selected in the negative ion mode. The allowable maximum error range was 5 mDa/10 ppm, and the matched constituents were given the predicted fragments from the structure. The functional module of the mass fragment could facilitate the chemical identification of unmatched constituents based on the isotopic abundance, elemental composition, and i-FIT score.

RESULTS

Characterization and Identification of Chemical Constituents in WBT

A total of 293 compounds were identified or tentatively characterized in both the positive (165) and negative (229) ion modes, including flavonoids, alkaloids, glycosides, coumarins, saponins, phenolic acid, and iridoids. The base peak intensity (BPI) chromatograms of WBT in two ion modes are depicted in Figures 3A,B. Among the 293 constituents characterized, 45 were identified in SDH or SD, 28 in XD, 23 in FZ, 23 in DH, 15 in GB, 14 in GZ, 36 in YYH, 22 in FF, 7 in WLX, 8 in ZIC, 28 in BS, 8 in GJ, 26 in ZM, 16 in SJC, and 31 in HH. Detailed information of the 293 compounds is listed in Supplementary Excels S2, S3, including RT, M/Z, error, response, adducts, formula, name, fragments, category, and origin.

The BPI chromatograms of the water extract of WBT corresponding to the positive and negative ion modes are shown in Figures 3A,B. The mass spectra of two compounds are shown below. The ion at RT = 15.37 and [M + H]^+ = 823.022 was primarily identified as baohuoside VI (C₃₉H₅₀O₁₉) in ESI+ after matching with data from the customized database (Supplementary Excel S4) of UNIFI. The main fragments were m/z 677.2438 [M + H-rha]^+, 531.1860 [M + H-2rha]^+, 369.1328 [M + H-Glc-2rha]^+, and 313.0703 [M + H-Glc-2rha-C₄H₇]^+, which were consistent with those in literature (Yu et al., 2016). In the same way, the ion at RT = 17.27 and [M-H]^− = 927.4931 was primarily identified as akebiasaponin D (C₄₇H₇₆O₁₈) in ESI−. The main fragments were
m/z 603.3904 [M-H-2Glc]⁻, 323.0984 [M-H-Glc-C₄H₈O₄]⁻, and 179.0563 [M-H-Glc-C₃H₆O₃]⁻, which were consistent with those in literature (Yang et al., 2016). The mass spectra and structures of the two compounds are displayed in Figures 3C,D.

WBT Reduces the Release of IL-1β and IL-6 in TNF-α Stimulated RAW264.7 Cells

The MTT assay showed that WBT exerted no significant cytotoxicity to RAW264.7 cells even at a concentration as high as 1.6 × 10⁴ ng/ml (Figure 4A). Therefore, WBT concentrations ranging from 0.001 to 10 μg/ml were selected for the examination of the anti-inflammatory activity of WBT in TNF-α-stimulated RAW264.7 cells. In RAW264.7 cells stimulated with TNF-α (20 ng/ml) for 24 h, IL-1β and IL-6 levels in the culture medium were significantly increased (p < 0.01) compared with that in the control group. MTX, as a positive drug, exhibited an excellent effect against inflammatory responses, suggesting a good pharmacodynamic evaluation system. In RAW264.7 cells co-cultured with TNF-α and WBT at 0.1, 1, and 10 μg/ml, IL-1β and IL-6 levels were significantly reduced (p < 0.01) in a dose-dependent manner. WBT at a low dose (0.001, 0.01 μg/ml) did not exhibit any anti-inflammatory effects (Figures 4B, C).

RNA-Seq Transcriptional Comparison Between Different Groups and Functional Enrichment Analysis of Core Genes

To elucidate the mechanism by which WBT altered the transcriptomic profile of RAW264.7 cells stimulated by TNF-α,
RNA-seq experiments were performed. The control, TNF-α, and TNF-α + WBT (1.0 μg/ml) groups were selected as the biological samples according to the level of inflammatory factors. To ensure the accuracy of the analysis, we set three biological replicates for RNA-seq, a fold change of more than 2, and a p value of less than 0.05. Principal component analysis showed good separation between different groups and good consistency in the same group (Figure 5A). After treatment, 593 and 177 genes were differentially regulated between TNF-α vs. Con and between TNF-α+WBT vs. TNF-α, respectively (Supplementary Excel S5, S6). Between TNF-α vs. Con, 376 genes were upregulated, and 217 genes were downregulated; these genes were regarded as “Inflammatory Immune Dysregulation Genes.” Between TNF-α+WBT vs. TNF-α, 92 genes were upregulated, and 85 genes were downregulated; these genes were regarded as “Anti-inflammatory Effect Genes.” Heat maps were generated to visualize the gene expression patterns in the three groups (Figures 5B,C). Interestingly, 45 genes that were differentially expressed between TNF-α vs. Con were significantly counter-regulated by WBT; thus, these genes may be the potential gene set responsible for the efficacy of WBT in reducing inflammatory responses. The 593 and 177 different genes were imported into FIGURE 5 | RNA-seq transcriptional comparison between different groups, and functional enrichment analysis based on network pharmacology. Principal component analysis of transcriptome sequencing results (A). Differential gene expression profiles of TNF-α vs. Con (B) and TNF-α+WBT vs. TNF-α (C) were visualized in heat maps (fold change ≥2, p < 0.05). Pathway enrichment analysis of the hub genes responsible for the anti-inflammatory effect of WBT. The purple box indicated the pathways closely related to the occurrence and development of RA (D).
the STRING database for construction of a PPI network including 264 nodes and 599 edges (Supplementary Excel S7). Thirty-three core nodes of the network were obtained by calculating the topological feature values, including degree, betweenness, and closeness. Generally, the node with a degree value of two-fold the median as well as betweenness and closeness values of one-fold the median is selected as the hub node. The 33 hub nodes included ABL1, ACTG2, ALDH1A7, B2M, C3, CCL2, CCL5, CCL9, CES2C, CSF1, CXCL1, CXCL10, CXCR3, EPHA3, EPHA5, H2-K1, HVCA1, IRF7, JUN, MIF2, MMP3, MMP9, NMU1, OLFM4, PLXNB3, RAB27A, RHBDL2, SEMA3G, THY1, TLR2, TRF, and VCAM1. Based on functional enrichment analysis, these 33 genes were mainly involved in the inflammation-immune regulation module, and the purple box indicates that the pathway information was closely related to the occurrence and development of RA (Figure 5D), such as cytokine-cytokine receptor interaction, chemokine signaling pathway, TNF signaling pathway, NOD-like receptor signaling pathway, and Toll-like receptor signaling pathway.

Underlying Mechanism and Key Active Components of WBT in Treating RA

By using the TTFM of TCMIP, we predicted a total of 1,210 putative targets based on the chemical structures of the 293 identified compounds (Supplementary Excel S8). Moreover, a total of 1681 RA-related genes were collected from the Disease-related Gene Database of TCMIP, as shown in Supplementary Excel S9. To illustrate the underlying mechanisms of WBT against RA, an interaction network of “Anti-inflammatory Hub Genes-Putative Targets-Disease Genes” was constructed using the TCM Association Network Mining Module of TCMIP. The interaction network included 2,251 nodes and 53,254 edges, with a network density of 0.011. A total of 566 core nodes of the network were obtained by calculating the topological feature values. In addition, a PPI network of the 566 core nodes was
constructed, which included 566 nodes and 23,558 edges and had a network density of 0.074, which was significantly higher than that of the initial network (density 0.011) and in accordance with network centrality of hub nodes. Finally, 135 hub nodes were obtained by calculating the topological feature values, of which 81 were putative targets of WBT corresponding to 225 chemical constituents.

To explore the biological function of these 135 hub genes, DAVID v6.8 was employed for KEGG analysis. As shown in Figure 6, the pathway information could be divided into five function modules: 1) the synovial inflammation-immune imbalance regulation module. It was the most significant functional module that included NOD-like receptor signaling pathway, B cell receptor signaling pathway, T cell receptor signaling pathway, Fc epsilon RI signaling pathway, Toll-like receptor signaling pathway, primary immunodeficiency, leukocyte transendothelial migration, chemokine signaling pathway, complement and coagulation cascades, natural killer cell-mediated cytotoxicity, regulation of actin cytoskeleton, Fc gamma R-mediated phagocytosis, and cytokine-cytokine receptor interaction; 2) energy metabolism regulation module, including citrate cycle (TCA cycle), oxidative phosphorylation, adipocytokine signaling pathway, neurotrophin signaling pathway, insulin signaling pathway, valine, leucine and isoleucine biosynthesis, glycolysis/gluconeogenesis, and pyrimidine metabolism; 3) cell function regulation module, including focal adhesion, mTOR signaling pathway, ECM-receptor interaction, apoptosis, TGF-beta signaling pathway, and MAPK signaling pathway; 4) synovial pannus formation module, including VEGF signaling pathway and vascular smooth muscle contraction; 5) bone destruction regulation module, including osteoclast differentiation.

The constituents that showed high-frequency binding to hub targets and had high content were selected as the key constituents (the target frequency and content were higher than the corresponding medians). A total of 48 key active constituents were selected, which corresponded to 13 herbal medicines. The detailed information is shown in Supplementary Excel S10. A multidimensional association network of “Herbal Medicines of WBT-Key Active Components-Core Modules-Traditional efficacy” was visualized using NaviGator v3.0, as shown in Figure 7. Among the hub targets, 13 red
triangles and 13 blue triangles represented the genes upregulated or downregulated, respectively, by WBP in the transcriptome data. These hub targets involved in different pathways were divided into four functional models corresponding to different traditional efficacies. The insulin signaling pathway; valine, leucine, and isoleucine biosynthesis; and glycolysis/gluconeogenesis were related to the tonifying of the liver and kidney as well as warming of the kidney to invigorate yang. The focal adhesion mTORC signaling pathway, ECM-receptor interaction apoptosis, TGF-beta signaling pathway, and MAPK signaling pathway were consistent with the elimination of malpractice, relief of pain, and strengthening of tendons and bones. The TCA cycle, oxidative phosphorylation, and adipocytokine signaling pathway were related to tonifying of the liver and kidney as well as warming and transforming of phlegm-damp. The other pathways were consistent with the boosting of blood essence, strengthening of tendons and bones, clearing and activation of the channels and collaterals, and promotion of blood circulation to remove stasis.

**DISCUSSION**

In the present study, we analyzed the chemical compositions of WBT and explore its molecular mechanisms against RA using integrative pharmacology. This is the first study to explore the active constituents and underlying mechanism of WBT in treating RA using integrative pharmacology, including rapid analysis of chemical composition, transcriptome sequencing, and network pharmacology analysis. In this study, the BPI chromatogram of WBT extracts was obtained by UPLC-QTOF-MS/MS, and 293 chemical compounds were preliminarily identified or tentatively characterized by matching the MS² raw data to in-house library data using the UNIFI 1.8 software. WBT at 0.1, 1.0, and 10 μg/ml significantly reduced IL-1β and IL-6 levels in RAW264.7 cells stimulated by TNF-α. Moreover, 593 and 177 differential genes were screened between TNF-α vs. Con and between TNF-α+WBT vs. TNF-α based on transcriptome sequencing analysis. An interaction network of “Inflammatory Immune Dysregulation Genes” and “Anti-inflammatory Effect Genes” was constructed, and the hub nodes, as calculated by the topological feature values, were mainly involved in the inflammation-immune regulation module, which is closely related to the occurrence and development of RA. To explore the underlying mechanism of WBT against RA and identify its key pharmacologically active substances, a multidimensional association network of Real-World Medicines of WBT-Key Active Components-Core Targets-Functional Modules-RA Pathology” was visualized using NaviGator v3.0. A total of 48 key active constituents were obtained based on their high-frequency binding to hub targets and contents in WBT, and 135 hub corresponding genes were selected, which may be the putative targets of WBT in treating RA. Functionally, the 135 putative targets were significantly associated with the inflammatory immune response regulation module, energy metabolism regulation module, and cell function regulation module, corresponding to their traditional efficacy.

WBT is composed of 17 herbal medicines that fit the compatibility principle of Monarch, Minister, Assistant, and Guide. We herein discuss the mechanism of WBT in treating RA according to this principle, as the 48 key active constituents were mainly involved in 13 herbal medicines: SDH and SD are Monarch drugs; YYH, XD, GJ, FZ, DH, FF, WLX, ZC, HH, and BS are Minister drugs; and ZM and BS are Assistant and Guide drugs. The other three herbal medicines and one animal drug may exert therapeutic effects through other pathways or systems than the inflammation-immune regulation system. 21 of the 48 key active constituents belong to flavonoids, 7 alkaloid, 7 steroidal saponin, 3 monoterpenoid, 3 iridoid, 2 coumarins, 1 ribonucleoside, 1 sterol, and 1 tannin. According to our experience, flavonoids may have greater potential against RA. RAW 264.7 cell which we all know is one of the media for RA testing, Inflammation is an important pathological process of RA, so we selected TNF-α as inflammatory inducer.

Interestingly, the enrichment pathways are consistent with the traditional efficacy of the corresponding herbal medicines (Figure 7). For example, 20 hub genes corresponding to 5 chemical constituents of SDH and DH, are correlated with the regulation of glucose and lipid metabolism and blood circulation, which are consistent with the traditional effect of SDH and SD in “tonifying the liver and kidney, benefiting essence and blood”. 32 hub genes corresponding to 9 chemical constituents of FZ, FF and WLX, are mainly involved in signal transduction and inflammatory response of the nervous system, which are consistent with the traditional effect of the three herbs in “clearing and activating the channels and collaterals”. 24 hub genes corresponding to 8 chemical constituents of YYH, XD, GSB and GJ, are correlated with various regulation pathways and energy metabolism in the body, such as the citrate cycle; oxidative phosphorylation; adipocytokine signaling pathway; neurotrophin signaling pathway; insulin signaling pathway, which consistent with the “warming the kidney and strengthening yang” efficacy of Minister drugs. 44 hub genes corresponding to 19 chemical constituents of ZC and BS, are mainly involved in inflammatory immune regulation pathways, signal transduction, and neuroinflammatory response, which are related to the effects of warm channels and freeing of vessels, invigorating blood circulation and dispersing stasis. 48 hub genes corresponding to 14 chemical constituents of ZM and BS, were mainly involved in the regulatory pathways of nutrients and energy metabolism, blood circulation regulation pathway, which were related to the overall regulation of physical fitness and the enhancement of the effects of other drugs.

In the transcriptome sequencing experiment, we found that IL-13Ra2, Tnfaip3, Tnfrsf14, and Tnfrsf9 were upregulated in TNF-α-stimulated RAW264.7 cells, which was related with the development of inflammation (Wilson et al., 2011). The expression of Il1rn was upregulated in WBT + TNF-α-stimulated RAW264.7 cells, suggesting WBT could ameliorate inflammatory conditions stimulated by TNF-α.
CONCLUSION

In summary, the 48 key active constituents contained in WBT may attenuate the major pathological changes in RA through their 135 candidate targets, which were involved in the inflammation-immune regulation system, energy metabolism regulation module, and cell function regulation module. TCMIP v2.0 undoubtedly accelerates the process of chemical composition identification and network analysis, and transcriptome sequencing increases the accuracy of network prediction. This research strategy provides an efficient way to analyze chemical constituents and explore the pharmacological mechanism of TCM.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165272

AUTHOR CONTRIBUTIONS

PW performed the experimental design and drafted the manuscript. YJ and HC performed the analysis of chemical constituents. YJ and HC performed the analysis of network prediction. This research strategy provides an efficient way to analyze chemical constituents and explore the pharmacological mechanism of TCM.

ACKNOWLEDGMENTS

Thank Liaoning Haohushi Pharmaceutical (Group) Co., Ltd. for providing WBT samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.669551/full#supplementary-material
Wang, H., Guan, Y., Wu, R., Lv, X., Shen, X., and Ye, G. (2019). UPLC-Q-TOF/MS Characterization of Efficacy Substances on Osteoblasts Differentiation and Function in Rat Serum after Administration of Wang-Bi Tablet. Biomed. Chromatogr. 33 (10), e4628. doi:10.1002/bmc.4628

Wang, J., Luo, J., Xu, Y., Yan, Z., Qu, X., Chen, M., et al. (2020). Wang-Bi Tablet, a Patented Chinese Medicine, Maintains the Balance of Th1/Th2 in Mice with Collagen-Induced Arthritis. J. Tradit Chin. Med. 40 (3), 401–406. doi:10.19852/j.cnki.jtcm.2020.03.006

Wang, P., Tang, S. H., Su, J., Zhang, J. Q., Cui, R. Y., Xu, H. Y., et al. (2018). [Modern Research Progress of Traditional Chinese Medicine Based on Integrative Pharmacology]. Zhongguo Zhong Yao Za Zhi 43 (7), 1297–1302. doi:10.19540/j.cnki.cjcmm.2018.0052

Wang, W. P., Shi, S. M., Liu, J., and Zhang, Q. Q. (2010). Treatment of 31 Cases of Rheumatoid Arthritis of Qi-Blood Deficiency by Internal and External Administration of Modified “Huangqi Guizhi Wuwu Decoction”. Shanghai J. Tradit Chin. Med. 44 (5), 43–45. doi:10.16305/j.1007-1334.2010.05.014

Wilson, M. S., Ramalingam, T. R., Rivollier, A., Shenderov, K., Mentink-Kane, M. M., Madala, S. K., et al. (2011). Colitis and Intestinal Inflammation in IL10−/− Mice Results from IL-13Ra2-Mediated Attenuation of IL-13 Activity. Gastroenterology 140 (1), 254–264. doi:10.1053/j.gastro.2010.09.047

Wu, J. W., and Shen, T. (2011). Clinical Study on the Treatment of Rheumatoid Arthritis with Wangbi Tablet. Liaoning J. TCM 38 (12), 21–23. doi:10.13192/j.ljtcm.2011.12.90.wujw.032

Xu, H.-Y., Zhang, Y.-Q., Liu, Z.-M., Chen, T., Lv, C.-Y., Tang, S.-H., et al. (2019). ETCM: an Encyclopaedia of Traditional Chinese Medicine. Nucleic Acids Res. 47 (D1), D976–D982. doi:10.1093/nar/gky987

Xu, H. Y., Liu, Z. M., Fu, Y., Zhang, Y. Q., Yu, J. J., Guo, F. F., et al. (2017). [Exploiture and Application of an Internet-Based Computation Platform for Integrative Pharmacology of Traditional Chinese Medicine]. Zhongguo Zhong Yao Za Zhi 42 (18), 3633–3638. doi:10.19540/j.cnki.cjcmm.2017.04.141

Xu, H. Y., and Yang, H. J. (2014). [Integrative Pharmacology: New Paradigm of Modernization of Chinese Medicine]. Zhongguo Zhong Yao Za Zhi 39 (3), 357–362. doi:10.4268/cjcmm20140302

Yang, R., Liu, Z., Shang, S., Qin, X., and Xia, P. (2016). Quantification of Neomangiferin in Rat Plasma by Liquid Chromatography-Tandem Mass Spectrometry and its Application to Bioavailability Study. J. Pharm. Anal. 6 (5), 335–340. doi:10.1016/j.jspa.2016.03.005

Yang, M., Ji, H. W., Cao, X. J., Luo, Q., Yi, L., Lei, P., et al. (2009). Clinical Study of Wangbi Pills in the Treatment of Rheumatoid Arthritis (Yin Deficiency in Liver and Kidney, Blood Stasis Stagnation Syndrome), Mod. Tradit Chin. Med. 29, 21–23.

Yu, X. E., Qin, J. P., Li, J. C., Huang, W. Z., Wang, Z. Z., and Xiao, W. (2016). [Rapid Analysis on Chemical Constituents in Yinyanghuo Zonghuangtong Capsule by UPLC/Q-TOF-MS/MS]. Zhongguo Zhong Yao Za Zhi 41 (24), 4587–4597. doi:10.4268/cjcmm20162417

Zhang, X. M. (2016). Wangbi Tablets Combined with Methotrexate and Leflunomide in the Treatment of Rheumatoid Arthritis for 17 Cases. Chin. Med. Mod. Distance Education China 14 (1), 90–91. doi:10.3969/j.issn.1672-2779.2016.01.047

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

Copyright © 2021 Jiao, Xu, Chen, Guo, Deng, Zhang, Zhang, Shi and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.