

Research Article

Uncovering the Multitarget Therapeutic Mechanism of Tong-Xie-Yao-Fang on Irritable Bowel Syndrome

Xiangxue Ma^(b),¹ Jinke Huang^(b),¹ Haomeng Wu^(b),² Xia Li^(b),³ Fengyun Wang^(b),¹ and Xudong Tang^(b)

¹Institute of Digestive Diseases, Xiyuan Hospital of China Academy of Chinese Medical Sciences, Beijing, China ²Department of Gastroenterology,

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangdong Provincial Hospital of Chinese Medicine), Guangzhou, China

³Department of Spleen and Stomach Diseases of Traditional Chinese Medicine, China-Japan Friendship Hospital, Beijing, China

Correspondence should be addressed to Fengyun Wang; wfy811@163.com and Xudong Tang; txdly@sina.com

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Background. This study investigated the pharmacological mechanisms of Tong-Xie-Yao-Fang (TXYF) against irritable bowel syndrome (IBS). *Methods.* The chemical profile of TXYF was identified through UHPLC-QTOF-MS. Next, the obtained chemical profile served as the basis for network pharmacological analysis. Finally, the predictive performance of network pharmacology was validated by conducting molecular docking and animal experiment. *Results.* Seven key compounds of TXYF, namely, quercetin, ellagic acid, nobiletin, formononetin, isorhamnetin, vestitol, and licochalcone, were confirmed as the key components acting on IBS. TXYF treatment on IBS was mainly realized through the regulation of some key pathways of immune system, such as inflammatory bowel disease, cytokine-cytokine receptor interaction, HIF-1, and T cell receptor signaling pathway. NOS2, ACHE, ESR1, PTGS2, and RELA were the target genes of TXYF to improve IBS. Stable bonds between the key components and the core target genes were further verified by the results of molecular docking. *In vivo* experiments confirmed the effects of TXYF on IBS. Further Western blot analysis showed that NOS2, ACHE, and ER α were significantly upregulated in the model group in comparison with controls (P < 0.001) but then significantly downregulated after treatment with TXYF for 14 days (P < 0.001). *Conclusion.* This study applied an integrated method based on network pharmacology and experimental validation to examine the underlying "multicomponent, multitarget, and multipathway" mode of action of TXYF in treating IBS. The current findings provided indicative paradigms and new insights into exploring the multitarget therapeutic mechanism of Chinese herbal compound.

1. Introduction

Irritable bowel syndrome (IBS) refers to a functional gastrointestinal disorder that is characterized by recurrent episodes of abdominal pain with altered bowel habits [1]. IBS is classified into three types, specifically, IBS-D (diarrhea-predominant), IBS-C (constipation-predominant), and mixed or alternating IBS (IBS-M). Rome IV criteria showed that the prevalence of IBS ranges from 5% to 10% worldwide, with a gradually increasing trend [2]. IBS often causes gastrointestinal discomfort, dietary restrictions, mood disorders, and disturbances in patients' daily activities, leading to a heavy medical and financial burden [3]. In treating IBS, physicians normally focus on symptomatic drugs, the effectiveness which is far from patient's expectations [4]. Moreover, the multifactorial nature of the physiopathology of IBS could limit the development of more effective drugs [4, 5]. As a result, IBS patients begin to seek complementary and alternative therapies to relieve their discomfort [6].

Chinese herbal medicine (CHM) is gaining recognition around the world owing to increasingly reported health benefits [7]. Tong-Xie-Yao-Fang (TXYF) is one of the most famous CHM formulas employed to treat functional gastrointestinal disorders and has demonstrated its effectiveness in IBS treatment [8]. TXYF consists of 4 herbs of Baishao (Paeoniae Radix Alba), Baizhu (Atractylodes Macrocephala Koidz), Chenpi (Citrus Reticulata), and Fangfeng (Saposhnikoviae Radix), which have the advantages of multicomponents and multitargeting. However, the complexity of CHM compounds also poses great challenges to pharmacological research, as their material basis and mechanism of action are currently unclear.

Network pharmacology, which is a cutting-edge interdisciplinary discipline in the systematic study of drugs, could mechanistically link drugs and diseases as well as quantitatively represent the key aspects of overall regulatory mechanism of drug action [9]. Therefore, network pharmacology has been widely used to elucidate the pharmacological mechanisms of CHM formula [10, 11]. In this study, network pharmacology in combination with experimental validation was utilized to investigate the pharmacological mechanism of TXYF against IBS.

2. Methods

2.1. Identification of Active Ingredients and Target Genes

2.1.1. Preparation of Samples. TXYF samples were prepared by decoction. The mixture of Chen Pi (9g), Atractylodes macrocephala (18g), Paeonia lactiflora (12g) and Fang Qi (6g), Citrus Reticulata (9g), Atractylodes Macrocephala Koidz (18g), Paeoniae Radix Alba (12g), and Saposhnikoviae Radix (6g) was steeped in water 10 times for 1 hour (h), boiled for 1 h and then filtered through absorbent gauze. Similarly, the residue was extracted by steeping in water 8 times for another 0.5 h. The two filtrates were combined, concentrated in vacuo (equivalent to 1g of crude grass/ml), and finally freeze-dried.

Lyophilized powder (1.0 g) was extracted using 50 mL of methanol/water (1:1, v/v) under ultrasonication for 0.5 h. Next, the extract was centrifuged at 13,000 rpm for 10 minutes (min) at 4°C and the supernatant was filtered through a membrane filter (0.22 μ m). Finally, 1.0 μ L of the filtrate was subjected to UHPLC-QTOF-MS for further analysis.

2.1.2. Key Active Ingredient and Screening of Target Genes. The names of all compounds identified by UHPLC-QTOF-MS/MS method were entered into the TCMSP (https://www. tcmsp-e.com/) for screening key active ingredients and their corresponding target genes. Drug-like ≥ 0.18 and oral bioavailability $\geq 30\%$ were the screening criteria [12]. We used UniProt (https://www.uniprot.org/) [13] to normalize the obtained genes for gene symbols.

2.2. Identification of IBS-Related Targets. CTD (https://ctdbase.org/) [14], DisGeNET (https://www.disgenet.org/ home/) [15], and GeneCards (https://www.genecards.org/) [16] were used to screen targets related to IBS. A correlation score \geq 5 was confirmed as the screening criterion in GeneCards [16].

2.3. PPI Network Construction. Common target genes between drug and disease were defined as potential targets of TXYF against IBS and used for subsequent analysis. Potential targets were introduced into the STRING (https:// string-db.org/) [17] for PPI analysis. The species were limited to "Homo sapiens" and the minimum interaction score required was set at high confidence (0.400). PPI networks were visualized using Cytoscape 3.7.2 software [18].

2.4. GO and KEGG Enrichment Analysis. To explore the biological functions related to potential targets, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed in the DAVID platform (https://david.abcc.Ncifcrf.gov/) [19]. GO enrichment analysis was used to interpret and annotate genes from three dimensions of cellular components, molecular functions, and biological processes. KEGG database was mainly applied for pathway analysis. P < 0.05 was regarded as statistically significant.

2.5. Drug-Compound-Target Network Construction and Screening of Core Genes. A drug-compound-target network was developed to examine the relationship between the ingredients of TXYF and targets of IBS. CytoHubba, the plugin of Cytoscape [20], was used to perform the screening of core genes and key components. The degree values were calculated, and the target nodes with the top 12-degree values were taken to generate a new core network.

2.6. Molecular Docking. In the present study, to validate the compound-target correlation, molecular docking was carried out in Discovery Studio 2019. The compounds structures of TXYF and macromolecular protein target receptors related to IBS were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and RCSB PDB (https://www.rcsb.org/), respectively.

2.7. Experimental Validation

2.7.1. Animals and Experimental Protocol. Ten litters of newborn SPF SD rats (5 male offspring per dam) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co. All the experimental animals were housed in a barrier environment at the Laboratory Animal Center of Xiyuan Hospital, Chinese Academy of Traditional Chinese Medicine, at the temperature of 22–25°C with a relative humidity of 50%–60% under controlled 12-h light/dark cycle. Adequate food and water were provided for the experimental animals.

Two litters of rats were randomly selected as the control group, while the rest of the rats were used to develop IBS-D model of liver depression and spleen deficiency syndrome.

Firstly, the suckling rats were separated from the dams for 3 h a day (9 am to 12 am) from postnatal day 2 to day 14. On day 22, the suckling rats were weaned and caged separately from dams. On day 30, the body weight of all the rats was measured and randomly divided into model group, high-dose TXYF group (HL), medium-dose TXYF group (TM), and low-dose TXYF group (LH). When the rats were >6 weeks old and weighed >220 g, the enema procedure was performed using 4% acetic acid solution (1 ml) once a day for 30 seconds (s) for 2 weeks. The rats in the control group underwent the same procedure using 1 mL of normal saline. The rats were stimulated using the chronic unpredictable psychological stress method [20] starting from day 56, and another different stimulation method was selected for daily operation for 21 days. The rats were given a gavage intervention on day 77. The administered doses of TXYF were converted to TL (0.47 g/ml), TM (0.94 g/ml), and TH (1.88 g/ ml). The rats were intragastrical administered at a dose of 1 mL/100 g once a day for a total of 14 days. The other groups were given equal amounts of saline using the same method. At the end of the experiment, after fasting for 12 h, the mice were sacrificed to harvest large intestine tissues, which were stored at -80°C for subsequent studies.

2.7.2. Histological Analysis. The colonic tissues were fixed in 10% neutral-buffered formalin, and 4- μ m-thick sections were stained with hematoxylin-eosin (HE) according to standard procedures. Morphological changes of colonic mucosa were observed under light microscope (Nikon, Japan).

2.7.3. Western Blot Analysis. Tissue samples from three rats were randomly selected from each group, followed by homogenization of colonic tissue proteins with cold lysis buffer and PMSF protease inhibitor. Subsequently, the BCA protein concentration assay kit was performed to detect the protein concentration. The proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with primary antibody (rabbit anti-NOS2 (1:1000 dilution, YT3169, 131KD, Immunoway), rabbit anti-ACHE (1:500 dilution, AF5274, 67KD, Affinity), rabbit anti-Er
 α (1 : 500 dilution, BS1113, 66KD, Bioworld), and mouse anti- β -action (1:5000)dilution, YM3028, 43KD, Immunoway)) at 4°C overnight, followed by further incubation with secondary antibodies for 2h at room temperature. Bound antibodies were visualized by an enhanced chemiluminescence (ECL) system.

2.8. Statistical Analysis. The data were presented as the mean \pm standard deviation. Statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) by one-way analysis of variance for comparison. A significant difference was considered when P < 0.05.

3. Results

3.1. Active Ingredients and Target Genes. In this study, a total of 231 compounds of TXYF were identified using the UHPLC-QTOF-MS protocol. A total of 42 key active ingredients and 218 corresponding target targets were obtained after importing the identified components into the TCMSP database for screening. Figure 1 presented the compound-target network of TXYF.

3.2. Target Genes Associated with IBS. Using the available resources (GeneCards, CTD, and DisGeNET), we filtered 5273 target genes correlated with IBS. Further analysis identified 70 common target genes between the six resources by a Venn diagram (Figure 2).

3.3. PPI Network and Core Gene Analysis. The Venn diagram revealed (Figure 3) 24 overlapping genes between the disease targets and the drug targets. Subsequently, the 24 targets were imported into the String database for PPI analysis, and we generated a network (Figure 4) containing 24 nodes and 312 edges.

3.4. GO and KEGG Enrichment Analysis. Figures 5 and 6 present the results of GO and KEGG enrichment analysis, respectively. In GO analysis, biological processes mainly enriched in positive regulation of gene expression, response to drug, and positive regulation of ERK1 and ERK2 cascade, negative regulation of apoptotic process. In KEGG analysis, inflammatory bowel disease, cytokine-cytokine receptor interaction, HIF-1 signaling pathway, T cell receptor signaling pathway, and pathways in cancer were the significantly enriched pathways.

3.5. Construction of Drug-Compound-Target Network and Identification of Core Targets. A drug-compound-target network (Figure 7) containing 34 compounds, 24 targets, 58 nodes, and 121 edges was developed. Using Cytoscape plugin cytoHubba, the core active compounds and core targets were identified according to the top 12-degree values. The core targets were NOS2, ACHE, ESR1, PTGS2, and RELA, and the top active compounds were quercetin, formononetin, ellagic acid, isorhamnetin, vestitol, licochalcone, and nobiletin (Figure 8).

3.6. *Molecular Docking Analysis*. According to the results of molecular docking, all the LibDock scores were over "50" (Figure 9), suggesting that all the key active ingredients were well docked to the corresponding target genes. 3D and 2D molecular docking model of the key active ingredients with core target genes are presented in Supplementary Figures 1 and 2.



FIGURE 1: The compound-target network of TXYF. Violet represents ingredients of TXYF, and green represents targets of IBS.



FIGURE 2: Venn diagram of targets related to IBS. Blue circles indicate targets from DisGeNET, pink circles indicate targets from CTD, and orange circles indicate targets from GeneCards.

3.7. Experimental Validation

3.7.1. Effect of TXYF on IBS-D. The experimental protocol is shown in Figure 10. As shown in Figure 11, the body weight, daily food intake, abdominal withdrawal reflex, and fecal water content were determined to evaluate the effects of TXYF on IBS-D. Before medication, the body weight, daily food intake, and abdominal withdrawal reflex were lower in the model group compared with the control group (P < 0.05). Interestingly, the three indexes were significantly



FIGURE 3: Venn diagram of targets from TXYF and IBS. Pink circles indicate targets that can be targeted by TXYF, and blue circles indicate targets of IBS.

increased in the rats treated with different doses of TXYF for 14 days compared to the model group (P < 0.05). Similarly, fecal water content was higher in the model group than in the control group (P < 0.05), but it was significantly decreased in rats treated with different doses of TXYF for 14 days (P < 0.05).

According to the results of HE (Figure 12), no loss of epithelial structures and normal glands was observed in the colonic tissues of the control rats, while a small amount of inflammatory cell infiltration, submucosal edema, and gap enlargement were seen in the mucosa of the model group. It is noteworthy that the degree and extent of damage to the colonic tissues of the rats were alleviated by the intervention of TXYF, especially in the rats that received a high dose of TXYF.







FIGURE 5: Results of GO analysis. Biological processes are colored purple, cellular components are blue, and molecular functions are red. The larger the shape, the greater the number of genes enriched.

3.7.2. Western Blot Analysis. Three potential targets (NOS2, ACHE, and ESR1) predicted by network pharmacology were further validated. As shown in Figure 13, NOS2, ACHE, and ER α were significantly higher-expressed in the model group compared with the control group (P < 0.05). Interestingly, NOS2, ACHE, and ER α were significantly low-expressed in rats treated with different doses of TXYF for 14 days than the model group (P < 0.05). These results all indicated that TXYF could be beneficial to IBS-D through NOS2 and ACHE as well as ER α among other potential targets.

4. Discussion

TXYF has been widely used in the treatment of IBS and has shown great efficacy. However, complex characteristics of CHM compound pose great challenges to the study of its mechanism of action. In this study, to investigate the pharmacological mechanisms of TXYF against IBS, network pharmacology in combination with experimental validation was performed. Network pharmacology screened a total of 34 active ingredients of TXYF acting on 24 IBS-related



FIGURE 6: Results of KEGG pathway analysis. The larger the shape, the greater the number of genes enriched.



FIGURE 7: Drug-ingredient-target genes network of TXYF. Violet represents ingredients of TXYF and green represents targets of IBS.

targets. Specifically, the key active compounds were quercetin, formononetin, ellagic acid, isorhamnetin, vestitol, licochalcone, and nobiletin, and the core targets were ESR1, NOS2, ACHE, PTGS2, and RELA. Animal experiments validated the key results of network pharmacology.

In the present study, a total of 231 compounds of TXYF were identified using the UHPLC-QTOF-MS protocol and served as the basis for further network pharmacological analysis. Finally, key active compounds, namely, quercetin,

ellagic acid, nobiletin, formononetin, isorhamnetin, vestitol, and licochalcone, were screened based on the predictions from network pharmacology. Oxidative stress is closely related to the pathological mechanism of IBS, and the mechanism lies in the excessive reactive oxygen species (ROS) production produced by oxidative stress, which in turn damages gastrointestinal epithelial cells [21]. Quercetin is a subclass of flavonoids with a variety of pharmacological activities such as anti-inflammation, antioxidation,



FIGURE 8: Core active compounds and core targets located in the top 12-degree values. Quercetin, formononetin, ellagic acid, isorhamnetin, vestitol, licochalcone, and nobiletin are the core active compounds. NOS2, ACHE, ESR1, PTGS2, and RELA are the core targets.



FIGURE 9: Results of libdock score of molecular docking.

anticarcinogenesis, and antivirus [22]. The addition of quercetin to diets has been reported to be able to improve antioxidant capacity and alleviate oxidative damage to the intestinal mucosa [23]. *In vitro* studies have also observed that quercetin can inhibit ROS production in

gastrointestinal epithelial cells, thereby preventing damage resulted from oxidative stress [24]. Additionally, quercetin could contribute to the antioxidant capacity of the body through promoting the protein abundance of Nrf2 and regulating GSH-related redox homeostasis in enterocytes



FIGURE 11: TXYF ameliorated the symptoms in IBS-D rats (n = 10). (a) Body weight, (b) daily food intake, (c) abdominal withdrawal reflex, (d) fecal water content. ${}^{\#}P < 0.01$ compared to the controls before medication. ${}^{\&\&}P < 0.01$ compared to the controls after medication. ${}^{**}P < 0.01$ compared to the models after medication.

[23]. Study also found that quercetin can significantly downregulate the colonic expression of ngn3, TPH, and pdx1, increase pain threshold pressure, and reduce visceromotor responses in IBS animals [25]. Ellagic acid is a strong antioxidant with various properties such as antioxidation, reduction of inflammation, and apoptosis [26]. Receiving ellagic acid improves sleep quality and gastrointestinal symptoms in IBS patients, which may be realized through antioxidation and moist properties of ellagic acid [27]. Other key components have also been discovered to have antioxidant effects by earlier studies [28–32], but studies on their

effects on IBS are highly limited. The present network pharmacology revealed their importance and can provide direction for their further research in IBS.

PPI network was developed, and NOS2, ACHE, ESR1, PTGS2, and RELA were identified as the core targets of TXYF against IBS. NOS2 is a reactive free radical functioning as a biological mediator in several biological processes, including neurotransmission and antibacterial and antitumor activities. Recent studies have detected a close relationship between NOS2 and inflammation and visceral hypersensitivity [33]. NOS2 overactivation can result in



FIGURE 12: Representative microscopic pictures of H&E staining (amplification, ×200).



FIGURE 13: (a–d) Western blot analysis for the expression of NOS2, ACHE, and ESR1 in colonic tissues of the IBS-D rat (n = 3). ^{##}P < 0.001 compared to the controls. ^{**}P < 0.001 compared to the models.

a variety of inflammatory diseases, and hypersensitivity can be controlled by modulating NOS2-associated inflammation [33]. In this study, animal experiments revealed that the level of NOS2 in the colonic tissue of rats in the IBS model group was significantly elevated compared to the control group but was then significantly decreased after TXYF treatment for 14 days. ACHE hydrolyzes the neurotransmitter acetylcholine at the neuromuscular junction and brain cholinergic synapses, thereby terminating signal transmission. ACHE is present in a variety of molecular forms with similar catalytic properties, but differs in the way how its oligomers assemble and cells attach to the cell surface. Conditions associated with ACHE include colonic pseudo-obstruction. With deepening understanding of the brain-gut axis in the pathogenesis of IBS [34], more attention should be paid to the role of ACHE in IBS [35]. The present study detected a significant increase in ACHE level in the IBS model in comparison to the control group, but interestingly, it was decreased after implementing 14 days of TXYF treatment. A growing body of data suggested that sex hormones may play a crucial role in the pathogenesis of IBS [36]. ESR1 is a protein-coding gene that encodes an estrogen receptor and ligand-activated transcription factor. Concurrent alterations in immunomodulatory cytokines and microRNAs have been observed in IBS patients, indicating that dysregulation of local immune responses may be realized in an estrogendependent manner [37]. The results of in vivo experiments confirmed that TXYF had certain effects on IBS, and Western blot analysis revealed that the ER α level was significantly higher in the colon tissue of the IBS model group than that in the control group, but it was decreased significantly after 14 days of TXYF treatment. PTGS2 is a PTGS that influences the development of chronic inflammatory diseases through producing inflammatory mediator prostaglandin E-2 (PGE (2)) and is a potential therapeutic target for IBS [38, 39]. RELA, also known as NF- κ B, is a ubiquitous transcription factor involved in several biological processes. Previous studies have confirmed that TXYF can effectively improve intestinal permeability and enhance intestinal mucosal barrier function in IBS via the NF- κ B pathway [40]. Furthermore, molecular docking analysis demonstrated that TXYF can effectively bind to specific proteins in IBS target genes, further validating the predictive results of network pharmacology.

However, the limitations of the current study should be equally acknowledged. IBS is classified into IBS-D, IBS-C, and IBS-M, but we only used IBS-D rats to verify the results of network pharmacology. Hence, whether the experimental results from IBS-D were applicable to IBS-C and IBS-M should to be further verified.

5. Conclusion

The present study applied an integrated method based on network pharmacology and experimental validation to uncover the underlying "multicomponent, multitarget, and multipathway" mode of action of TXYF for the treatment of IBS. This study provided indicative paradigms and new insights into the exploration of the multitarget therapeutic mechanism of Chinese herbal compound.

Abbreviations

TXYF:	Tong-Xie-Yao-Fang (TXYF)
IBS:	Irritable bowel syndrome (IBS)
UHPLC-QTOF-	Quadrupole time-of-flight mass
MS:	spectrometry
IBS-D:	Diarrhea-predominant
IBS-C:	Constipation-predominant
IBS-M:	Mixed or alternating IBS (IBS-M)
CHM:	Chinese herbal medicine

GO:	Gene Ontology
KEGG:	Kyoto Encyclopedia of genes and
	genomes.

Data Availability

All the data obtained or analyzed during this work were available on request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiangxue Ma designed the study and drafted the manuscript. Jinke Huang, Haomeng Wu, and Xia Li helped with the implementation of this work. Fengyun Wang and Xudong Tang contributed to the review and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

3D and 2D molecular docking model of the key active ingredients with core target genes. (*Supplementary Materials*)

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