Original Article

Network pharmacology study on the mechanism of the herb pair of prepared *Rehmannia* root-Chinese arborvitae kernel for anxiety disorders

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Background: Although anxiety disorders are one of the most common mental illness in population, antianxiety drugs often only have single action targets, require long-term use, and are associated with many adverse reactions and dependencies. Professor Yan Zhaojun from Shandong Provincial Hospital of Traditional Chinese Medicine (TCM) has applied the modified Renshu Powder, a TCM formula, to treat anxiety disorders, with satisfactory outcomes. Here, we investigated the mechanism of action of two core herbs (prepared *Rehmannia* root and Chinese arborvitae kernel) in the Renshu Powder in the treatment of anxiety disorders by using network pharmacology approaches.

Methods: Candidate compounds of the herb pair of prepared *Rehmannia* root-Chinese arborvitae kernel were extracted via the Traditional Chinese Medicine Systems Pharmacology (TCMSP) platform. The targets of action of the main compounds were collected using the SwissTargetPrediction database. Targets associated with anxiety disorders were retrieved from DisGeNET, Online Mendelian Inheritance in Man (OMIM), DrugBank, GeneCards, and Comparative Toxicogenomics Database (CTD) databases. The compound-target interaction network was constructed by Cytoscape 3.7.2 software, and the protein-protein interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) platform. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses the data by using Metascape.

Results: The main active compounds of the herb pair included arachidonic acid, stigmasterol, and beta-sitosterol. The key targets included Nitric Oxide Synthase 3 (NOS3), Epidermal growth factor (EGF), Prostaglandin-Endoperoxide Synthase 2 (PTGS2), Caspase 3 (CASP3), Mitogen-Activated Protein Kinase 1 (MAPK1), Peroxisome proliferator-activated receptor gamma (PPARG), RELA Proto-Oncogene, NF-KB Subunit (RELA), Estrogen Receptor 1 (ESR1), Solute Carrier Family 6 Member 4 (SLC6A4), and Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN). Anxiety disorder–related GO analysis mainly involved synaptic signaling, neurotransmitter receptor activity, and G protein–coupled neurotransmitter receptor activity. The KEGG pathways involved neuroactive ligand–receptor interaction, serotonergic synapse, PI3K/AKT/mTOR signaling pathway, and MAPK signaling pathway.

Conclusions: The mechanism of action of the prepared *Rehmannia* root–Chinese arborvitae kernel in treating anxiety disorders involves multiple ingredients, multiple targets, and pathways.

Keywords: Prepared *Rehmannia* root; Chinese arborvitae kernel; anxiety disorders; network pharmacology; mechanism of action

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Introduction

Anxiety reflects the anticipation of future threats and is a normal emotional experience; however, when this emotion is severe enough to affect behavior or cause distress, it can develop into an anxiety disorder (1). Anxiety disorders are one of the most common mental problems in the adults, with a prevalence rate of 7.3% worldwide (2) and 7.6% in China (3). Thus, it has become a social problem that cannot be ignored. According to the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-5), anxiety disorders include separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder, panic disorder, agoraphobia, generalized anxiety disorder, substance-induced anxiety, anxiety disorder due to other medical conditions, and unspecified/undefined anxiety disorder (4). The COVID-19 pandemic, which was first identified in December 2019, has been found to be associated with high incidences of psychological symptoms, such as anxiety and depression, which has raised public concern about mental health (5). Another study has shown that patients can be plagued by sleep disturbances, anxiety, or depression even 6 months after being discharged from the hospital with a cure (6).

Antianxiety drugs often only have single-action targets, require long-term use, and are associated with many adverse reactions and dependencies (7). In contrast, guided by the traditional Chinese medicine (TCM) holistic concept, the TCM prescriptions can be used to treat diseases with their multiple compounds, multiple targets of action, and multiple pathways, and share the advantages of fewer adverse reactions and higher patient compliance (8). Similarly ,TCM has certain characteristics. Many TCM drugs have shown efficacies in reducing the negative effects of anxiety symptoms and in enhancing mental health (9). The ancient Chinese formula Renshu Powder (initially seen in the *Introduction to Medicine*, Li Ting, Ming dynasty) has long been used to treat mental and physical symptoms caused by anxiety and fear. For decades, Professor Yan Zhaojun from Shandong Provincial Hospital of Traditional Chinese Medicine has applied the modified Renshu Powder to treat anxiety disorders, with satisfactory outcomes (10). The combination of prepared *Rehmannia* root and Chinese arborvitae kernel is the core drug pair in the formula.

Based on the theories of system biology, network pharmacology affords a powerful and practical research method (11). By utilizing multidisciplinary theories, it predicts the mechanism of action of a specific compound in treating a specific disease by screening the potential bioactive components, targets, and pathways. The principles of network pharmacology are consistent with TCM theories. Recent studies have shown that the possible mechanisms of COVID-19 treatment by the five recommended prescriptions are regulation of immune system, anti-inflammatory, antiviral, regulation of cell apoptosis, anti-pulmonary fibrosis, myocardial protection, etc. At the same time, β-sitosterol, quercetin and kaempferol may be involved in the treatment of COVID-19 at various stages, showing great potential anti-epidemic activity (12). Here we predicted the potential pharmacological mechanisms of the action of the herb pair of prepared *Rehmannia* root and Chinese arborvitae kernel, especially the targets and their compounds, in the treatment of anxiety disorders by using network pharmacology approaches, with a view to promote the global use of TCM-based antianxiety medications. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/apm-21-531).

Methods

Main active compounds and targets of the TCM drugs

The chemical composition of prepared *Rehmannia* root and Chinese arborvitae kernel was searched in the TCMSP database (http://tcmspw.com/tcmsp.php) (13), and the main active compounds and target proteins were filtered based on parameters including oral bioavailability (OB) ≥30%, drug-like properties (DL) ≥0.18, and blood-brain barrier (BBB) ≥–0.30 (14). The SMILES codes of all active compounds were confirmed through PubChem (http://pubchem.ncbi.nih.gov) (15). The targets of action of the main active components were obtained using the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) (16). The target proteins were genetically normalized using the Uniprot database (http://www.uniprot.org/) and deduplicated to obtain the gene names of the targets of

the main active compound of the drug pair of prepared *Rehmannia* root-Chinese arborvitae kernel. The main active compounds of the herb pair and their targets were imported into the Cytoscape software 3.7.2 (17) to construct a visual network of TCM Drugs-ingredients-targets. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Collection of disease-related targets

With *Anxiety Disorders* as the keyword, searching for human genes in databases including DisGeNET (https://www.disgenet.org/) (18), Online Mendelian Inheritance in Man (OMIM) (http://www.omim.org) (19), Drugbank (https://www.drugbank.ca) (20), GeneCards (https://www.genecards.org/) (21), and Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/) (22). After literature review and data deduplication/integration, the anxiety disorder-related targets were obtained. The targets of the main active compounds in the herb pair were mapped to the disease-related targets, and the intersecting genes (i.e., the predicted targets of the herb pair at which point anxiety disorder are intervention occurs) were obtained.

Protein-protein network construction and analysis

The predicted targets were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) platform (https://string-db.org/cgi/input.pl) (23) for protein-protein interaction (PPI) network construction, and the minimum required interaction score was set to 0.400. The PPI network was constructed by importing the STRING platform data files into Cytoscape software 3.7.2, and topology analysis was performed by using the Network Analyzer tool. The topology network was analyzed based on 3 parameters: *Degree*, *Betweenness Centrality* (BC), and *Closeness Centrality* (CC)". After 3 sessions of filtering (criteria: parameter values higher than the medians), the core targets were obtained.

Molecular docking validation

Receptor-ligand docking simulations were performed using Autodock Vina 1.1.2 program (Trott and Olson, 2010) for the top 3 key compounds of *Degree* and the top 3 core targets obtained from the filtering, with the docking results between the first-line therapeutic drug sertraline hydrochloride (24) and the core targets being used as a control.

Targets and pathways enrichment analyses

The predicted anxiety-intervening targets were filtered using the Metascape database (25). For targets that met the filtering criteria of $P<0.01$, Gene Ontology (GO)-Biological Process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed, and the results were visualized in Cytoscape.

Establishing the TCM drugs-ingredients-diseases-targets-pathways network

The main active ingredients, diseases, predicted targets, and major pathways of the herb pair were imported into the TCM drugs-ingredients-diseases-targets-pathways network, in order to more visually demonstrate the multitarget and multipathway characteristics of the prepared pair of *Rehmannia* root-Chinese arborvitae kernel for intervention of anxiety disorders.

Statistical analysis

The selected targets obtained in this study were analyzed by rigorous statistics. We analysed the topological data by Cytoscape version 3.6.0. Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8. The STRING platform data was imported into Cytoscape software 3.7.2 to construct the PPI network. The topology analysis was performed by using the Network Analyzer tool. All the relevant data after statistical analysis are shown in "Results" Section below.

Results

Main active compounds and targets of the TCM drugs

Six main active compounds of prepared *Rehmannia* root and Chinese arborvitae kernel were obtained by filtering via TCMSP. Among them, there were 2 active compounds in prepared *Rehmannia* root and 5 in Chinese arborvitae kernel. Beta-sitosterol was a shared active compound in these 2 herbs (*Table 1*). After gene normalization and removal of duplicate values, 102 action targets were obtained. The main active compounds of the herb pair and their targets were imported into the Cytoscape software

Table 1 The main active compounds of the herb pair of prepared Rehmannia root and Chinese arborvitae kernel

Source	Mol ID	Molecule name	OB (%)	BBB	DL	Degree value
Prepared Rehmannia root	MOL000359	Beta-sitosterol	36.91	0.87	0.75	19
Prepared Rehmannia root	MOL000449	Stigmasterol	43.83	1.00	0.76	45
Chinese arborvitae kernel	MOL000359	Beta-sitosterol	36.91	0.87	0.75	19
Chinese arborvitae kernel	MOL001439	Arachidonic acid	45.57	0.58	0.20	50
Chinese arborvitae kernel	MOL002211	11,14-eicosadienoic acid	39.99	0.76	0.20	17
Chinese arborvitae kernel	MOL003927	11,14,17-eicosatrienoic acid	44.11	0.88	0.20	18
Chinese arborvitae kernel	MOL008153	5Z-eicosenoic acid	30.70	0.89	0.20	18

OB, oral bioavailability; BBB, blood-brain barrier; DL, drug-like.

to construct a visual network of TCM drugs-ingredients-targets (*Figure 1*). The network topology analysis using the Network Analyzer plug-in showed that there were 110 nodes and 167 edges, with an average degrees of freedom of 3.036. Among these active compounds, arachidonic acid, stigmasterol, and beta-sitosterol had the highest *Degree* values, suggesting they were the key active compounds in the herb pair.

Collection of disease targets

Disease-related targets were searched in DisGeNET, OMIM, Drugbank, GeneCards, and CTD databases, and a total of 4,790 targets were obtained after removing duplicate values. The drug pair targets were mapped to the disease targets, and 70 predicted targets were obtained.

PPI network construction and analysis

These 70 predicted targets were loaded into the STRING platform to obtain the predicted PPI relationship network, which had 70 nodes and 358 edges, with a mean *Degree* value of 10.2. The data were imported into Cytoscape for topological analysis, and the PPI network yielded from this is presented in *Figure 2*. The core targets were identified on the basis of the *Degree* value, BC, and CC and arranged according to the *Degree* value (*Figure 3*). The top 10 targets were *NOS3*, *EGF*, *PTGS2*, *CASP3*, *MAPK1*, *PPARG*, *RELA*, *ESR1*, *SLC6A4*, and *PTEN*, indicating these targets play important roles in the treatment of anxiety disorders.

Molecular docking validation

The top 3 active compounds ranked by *Degree* value were selected to dock with the top 3 core targets, and sertraline hydrochloride was used as a positive control. The results show that the binding energies of all molecules to proteins are less than 0, which indicates that the ligand can spontaneously bind to the receptor; the binding energies of stigmasterol and beta-sitosterol to the core target were less than −5.0 kJ/mol, and their binding energies for docking with sertraline hydrochloride were quite similar. Thus, the proper core targets and ligands were selected. Stigmasterol and beta-sitosterol had the optimal binding energies to NOS3, as shown in *Table 2* and *Figures 4* and *5*, in which the red dashed lines represent hydrogen bonds, indicating that stigmasterol forms hydrogen bonding with NOS3 at the active site ARG-365 and beta-sitosterol forms hydrogen bonding with NOS3 at the active site TRP-356.

Targets and pathways enrichment analyses

With a filtering condition of $P<0.01$, the predicted targets underwent enrichment analyses in Metascape software. A total of 53 GO terms were obtained, consisting of 20 biological process (BP) terms, 14 cell composition (CC) terms, and 19 molecular functions (MF) terms, as shown in *Figure 6*. Enrichment analysis of KEGG pathway showed 80 KEGG pathways, and the top 20 pathways are visualized with a bar graph (*Figure 7*).

Establishing TCM drugs-ingredients-diseases-targets-pathways network

The herb pair and its main active compounds, diseases, predicted targets, and main pathways were imported into Cytoscape software to draw the TCM drugs-ingredients-diseases-targets-pathways network, with an attempt

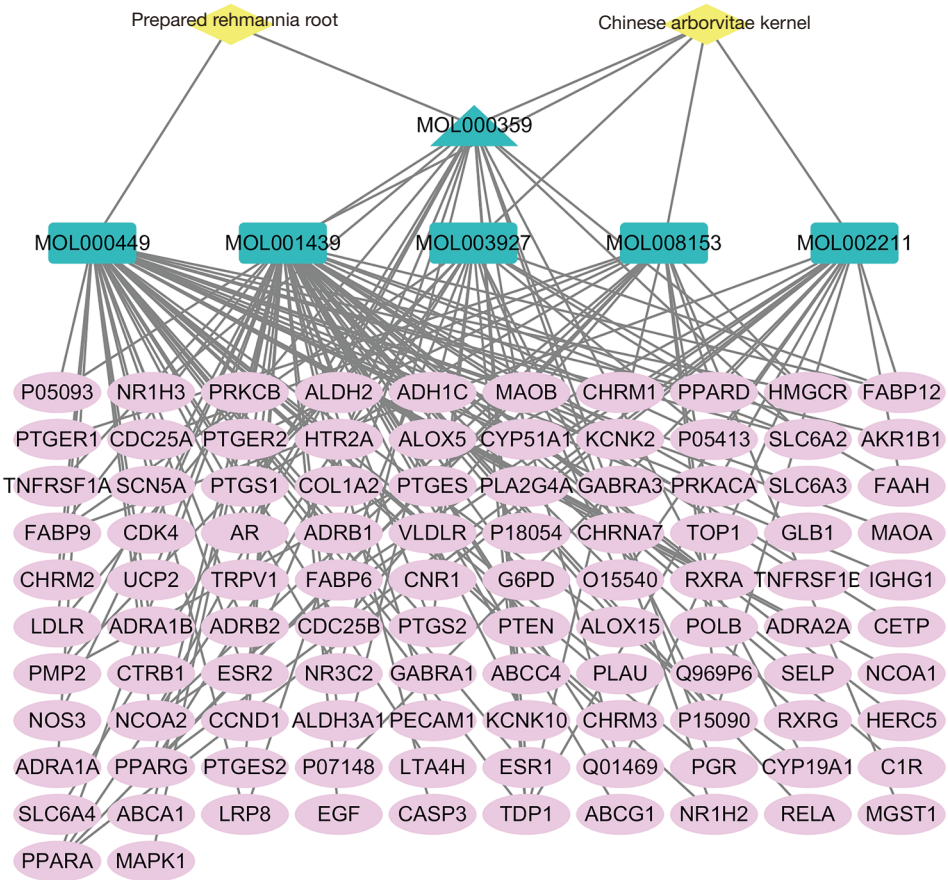

Figure 1 TCM drugs-ingredients-targets network. TCM, Traditional Chinese Medicine.

to demonstrate the multicompound, multitarget, and multipathway characteristics of the herb pair. The results are shown in *Figure 8*.

Discussion

Experimental and clinical studies have demonstrated that both genetic and environmental factors contribute to the risk of developing an anxiety disorder (26). The common triggers for anxiety disorders include neurotransmitters, neuroendocrine function, neuroanatomy, immune disorders, stressful psychological events, and drug addiction and withdrawal (27). These influencing factors are also involved in the targets and pathways by which prepared *Rehmannia* root and Chinese arborvitae kernel treat anxiety disorders.

It has been confirmed that prepared *Rehmannia* root can exert its anxiolytic effect through enhancing GABAAR1 expression, increasing central γ-aminobutyric acid (GABA) level, and reducing N-methyl-D-aspartate receptor 1 expression by decreasing glutamate level (28). Filtering in our current study revealed that arachidonic acid, stigmasterol, and beta-sitosterol were the key active compounds in the herb pair prepared Rehmannia root and Chinese arborvitae kernel. Arachidonic acid (AA) is one of the most abundant and widely distributed polyunsaturated fatty acids (PUFA) in mammals and also one of the essential fatty acids in humans. It is catalyzed by phospholipase A2 (PLA2) and can generate various metabolites through cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) pathways, playing a wide range of important physiological roles; in particular, it has unique biological activities in lowering blood lipids; inhibiting platelet aggregation; fighting against inflammation, cancer, and lipid oxidation; and promoting the development of brain tissue (29). Research has shown that stigmasterol exerts its neuroprotective effects by downregulating

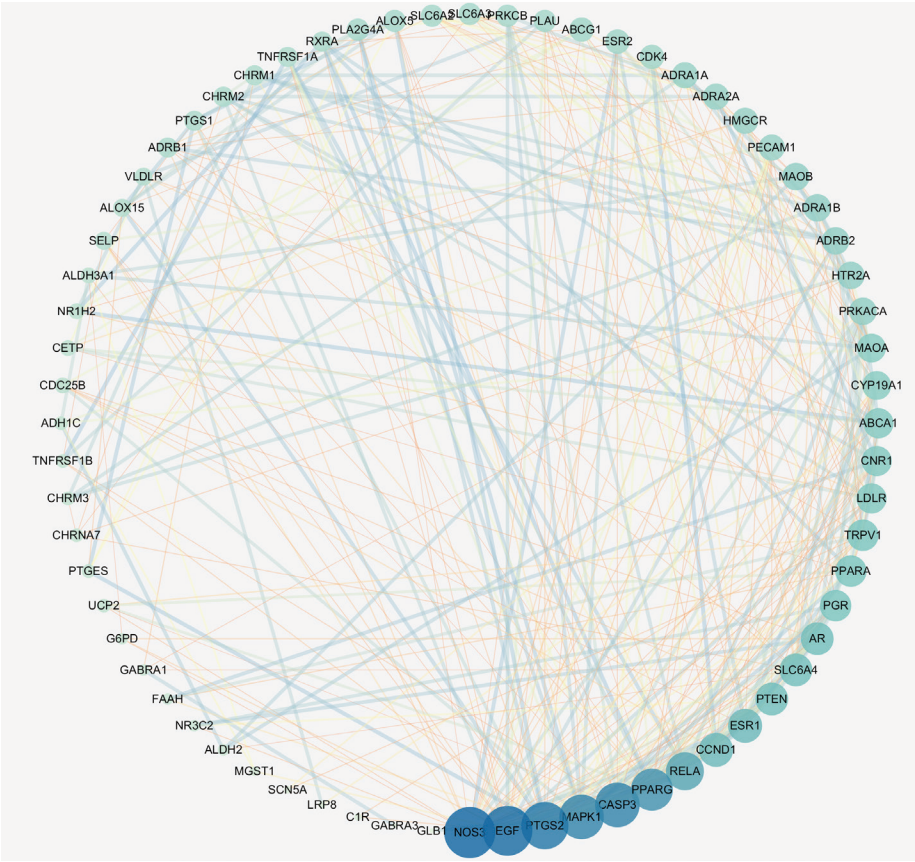

Figure 2 PPI network. PPI, protein-protein interaction.

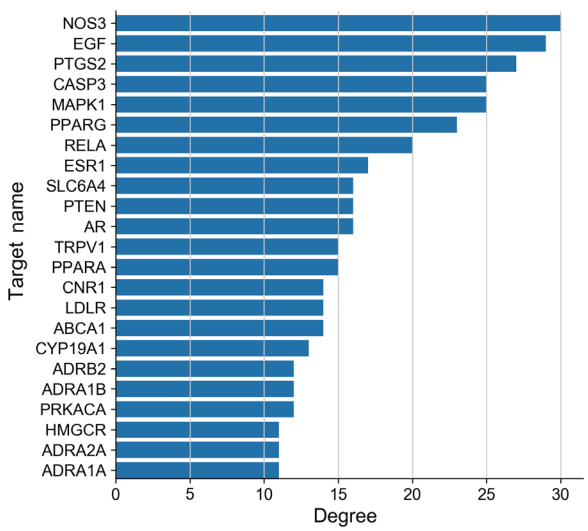

Figure 3 Core targets.

GluN2B-mediated excitotoxicity and reducing oxidative stress, showing therapeutic promise for cerebrovascular and neurodegenerative diseases (30). Beta-sitosterol has sedative and anxiolytic effects (31), and its mechanism of action may be related to the GABA/benzodiazepine receptor complex (32). Monoamine neurotransmitters, such as 5-HT, norepinephrine (NE), and dopamine (DA), have been suggested to play important roles in the pathogenesis of neurological and psychiatric disorders, and β-sitosterol may also exert its anxiolytic effect by mediating DA, 5-HT, and NE (33).

In our current study, topological analysis revealed that the key targets of the herb pair of prepared *Rehmannia* root and Chinese arborvitae kernel for anxiety disorders were *NOS3*, *EGF*, *PTGS2*, *CASP3*, *MAPK1*, *PPARG*, *RELA*, *ESR1*, *SLC6A4*, and *PTEN*. The nitric oxide synthase 3 (*NOS3*) gene is located in chromosome region 7p35-p36. Physiologically, nitric oxide (NO) in blood vessels is mainly synthesized by NOS3. NOS is mainly expressed in neurons

Table 2 Docking of the main active compounds with the core target protein molecules

Active compound	Target protein	PDB (Protein Data Bank) ID	Binding energy (kJ·mol^{-1})
Arachidonic acid	NOS3	4D1P	–6.3
	EGF	1NQL	–4.2
	PTGS2	5F19	–6.7
Stigmasterol	NOS3	4D1P	–9.2
	EGF	1NQL	–5.9
	PTGS2	5F19	–8.7
Beta-sitosterol	NOS3	4D1P	–9.2
	EGF	1NQL	–5.5
	PTGS2	5F19	–7.1
Sertraline	NOS3	4D1P	–9.0
	EGF	1NQL	–5.2
	PTGS2	5F19	–7.5

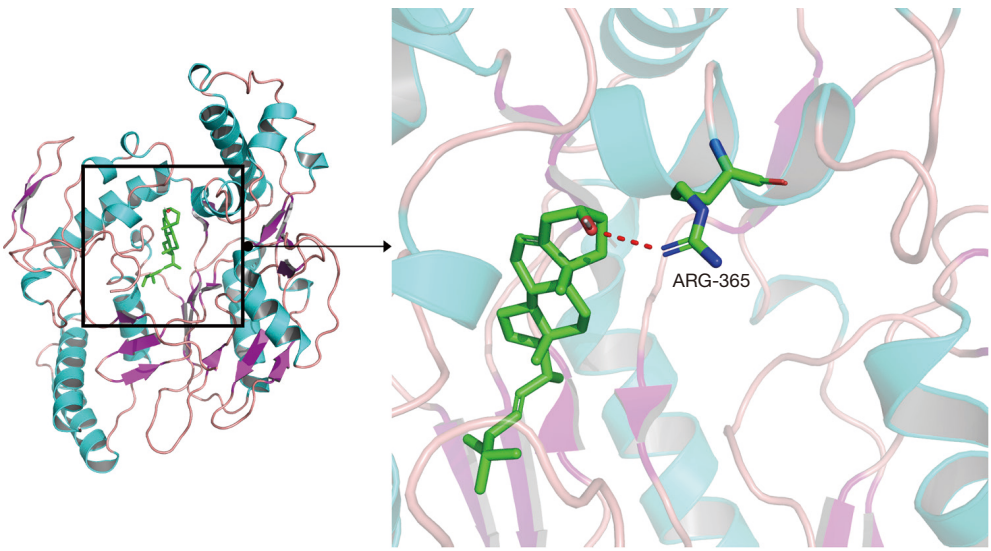

Figure 4 Molecular docking of stigmasterol with NOS3.

and differentially distributed in different regions of the brain; abnormal *NOS3* gene expression induces neuronal and glial degeneration in the brain (34). Malformations of the cerebral vasculature are associated with reduced *NOS3* expression. Decreased NOS3 expression in cerebral vasculature is associated with the risk of vascular lesions, vascular smooth muscle cell apoptosis, and amyloid plaque formation (35). As a physiologically active low-molecular-weight polypeptide, epidermal growth factor (*EGF*) plays a special role in regulating cell growth, proliferation, and differentiation. Its pathophysiological roles in various diseases have increasingly been recognized. *EGF* is closely related to cognitive function, as it is a potent mitogen that promotes the growth and development of the nervous system while protecting and nourishing neurons and dopamine when oxidative damage to neurons occurs (36). The *PTGS2* gene is a core enzyme in prostaglandin biosynthesis and has been shown to play

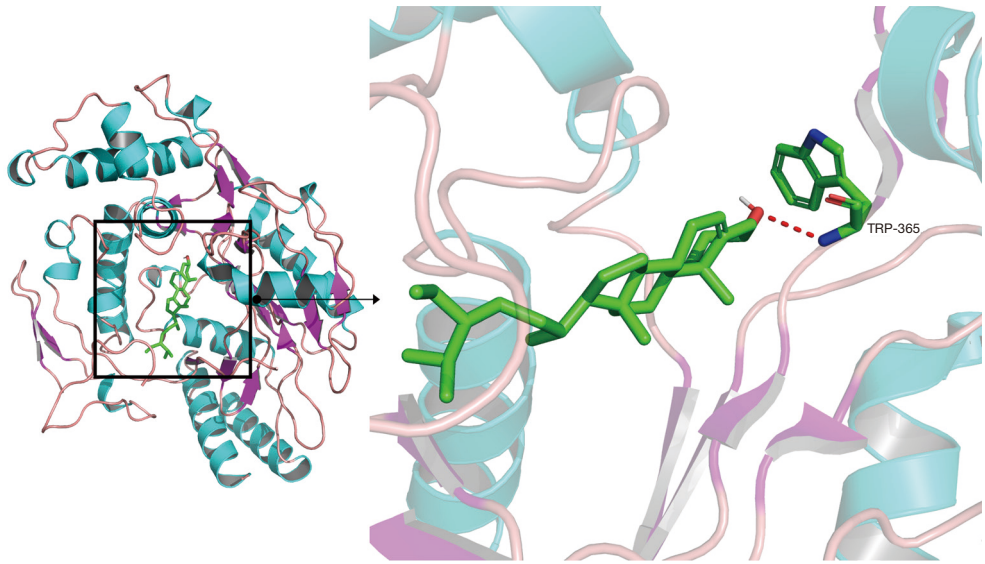

Figure 5 Molecular docking of beta-sitosterol with *NOS3*.

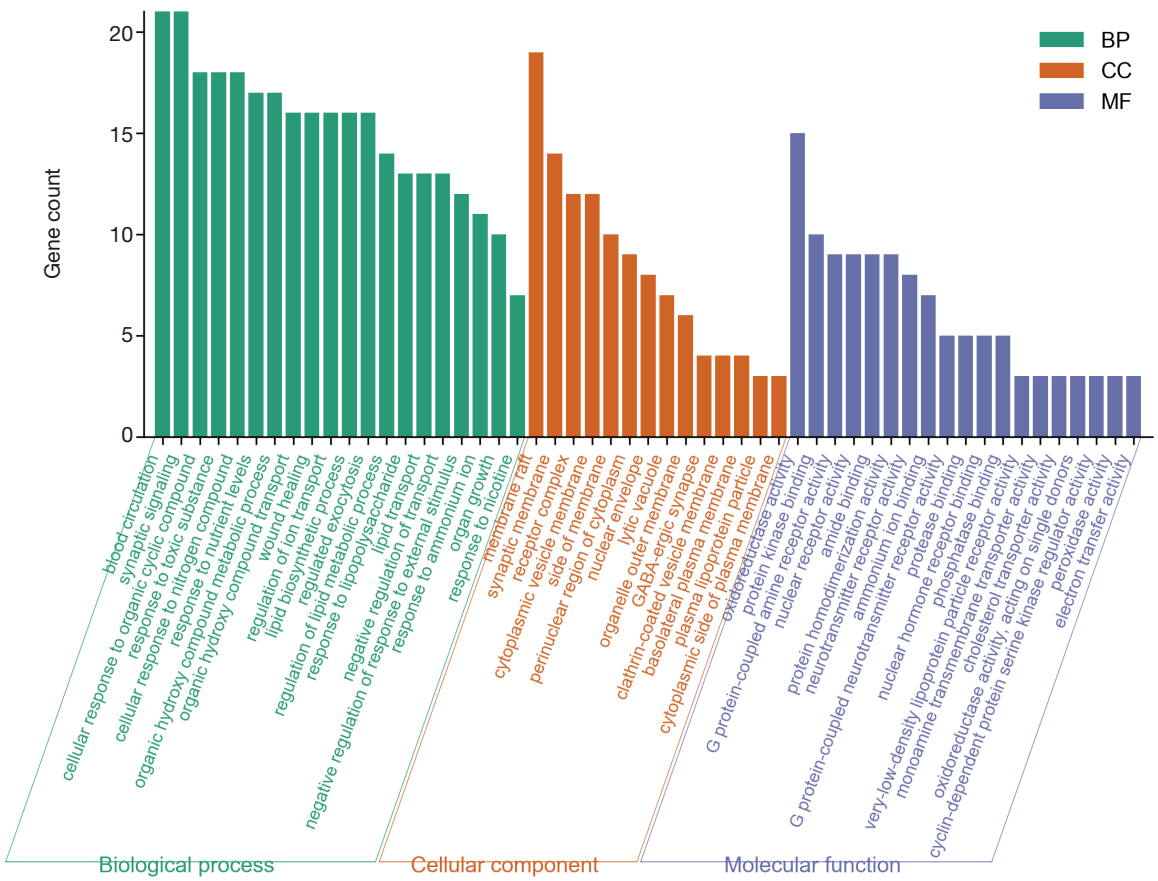

Figure 6 Gene Ontology enrichment analysis-biological process.

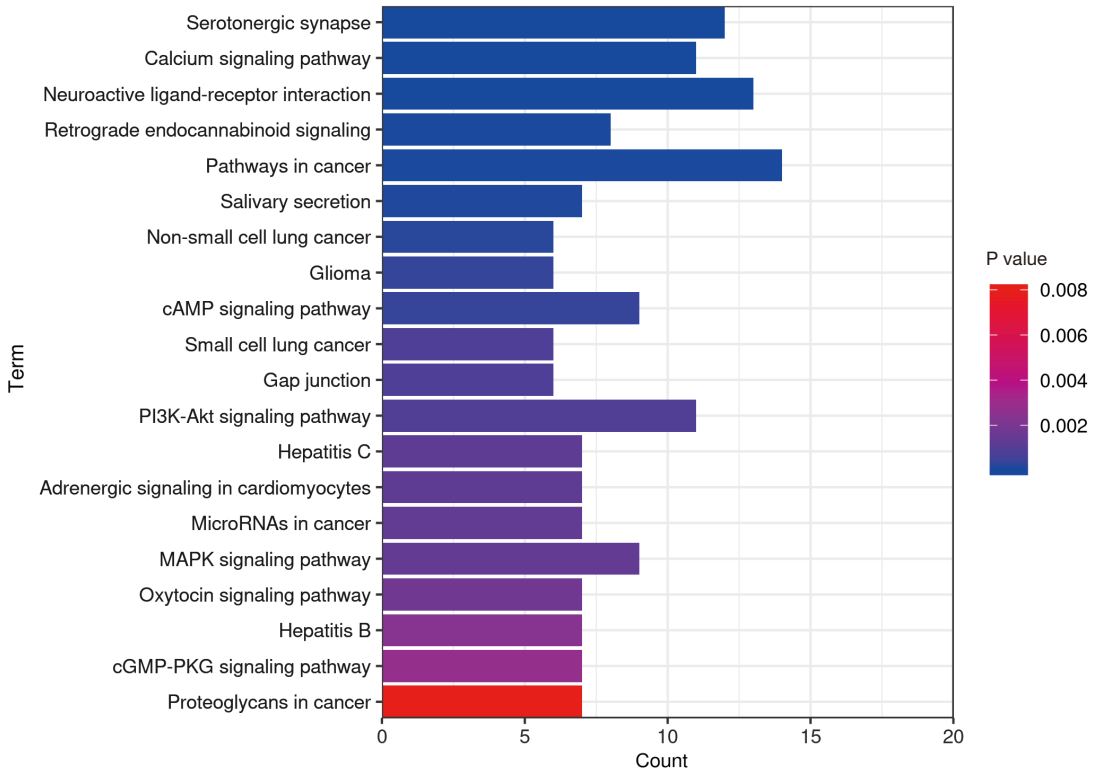

Figure 7 KEGG pathway enrichment analyses. KEGG, Kyoto Encyclopedia of Genes and Genomes.

an important role in the development of Alzheimer's disease (37). *PTGS2* may increase the production of inflammatory chemokines and cytokines by activating its downstream pathways (e.g., PI3-K/AKT and PKA/CREB pathways), thus promoting neuronal apoptosis and inflammation and inhibiting neurite outgrowth (38). Mitogen-activated protein kinase 1 (*MAPK1*), an effector kinase of the MAP kinase signaling pathway, is controlled by cell proliferation, differentiation, apoptosis, and angiogenesis (39). Anxiety disorder comorbidity, as a risk factor for suicide, was found to be significantly associated with variations in *MAPK1* polymorphisms (40). High levels of peroxisome proliferator-activated receptors (PPAR) have been found in the hippocampus and amygdala of the brains of patients with anxiety disorders. PPAR group includes three isoforms encoded by the *PPARG*, *PPARA*, and *PPARD* genes, with the gamma type (i.e., *PPARG*) showing the highest expression in the central nervous system; studies have identified at least 14 PPAR-regulated proteins associated with anxiety disorders, suggesting that PPAR might be involved in neuroinflammatory protection (41). Estrogen is thought to play a key role in anxiety. Estrogen receptor 1 (*ESR1*) polymorphisms are associated with the risk of anxiety disorders (42), and there is a strong association between *ESR1* variants and cognitive outcomes (43). Alterations in serotonin 5-hydroxytryptamine (5-HT) neurotransmission and peripheral immune activation have been shown to be strongly associated with a variety of psychiatric disorders (44). *SLC6A4* is a serotonin transporter gene. Studies have shown that there are many predisposing factors for affective disorder, such as epigenetic processes including SLC6A4 promoter methylation and microRNA silencing. (45). The tumor suppressor PTEN is a vital homeostatic regulator, by virtue of its lipid phosphatase activity against phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], which downregulates the PI3K/AKT/mTOR prosurvival signaling (46). *PTEN* plays an important role in the development of neurons. In addition, it also plays a role in synaptic plasticity in adulthood. Studies have shown that the synaptic function of *PTEN* in amygdala is closely related to specific behavioral characteristics (47).

In our current study, GO enrichment analysis was performed to describe the intracellular properties of core

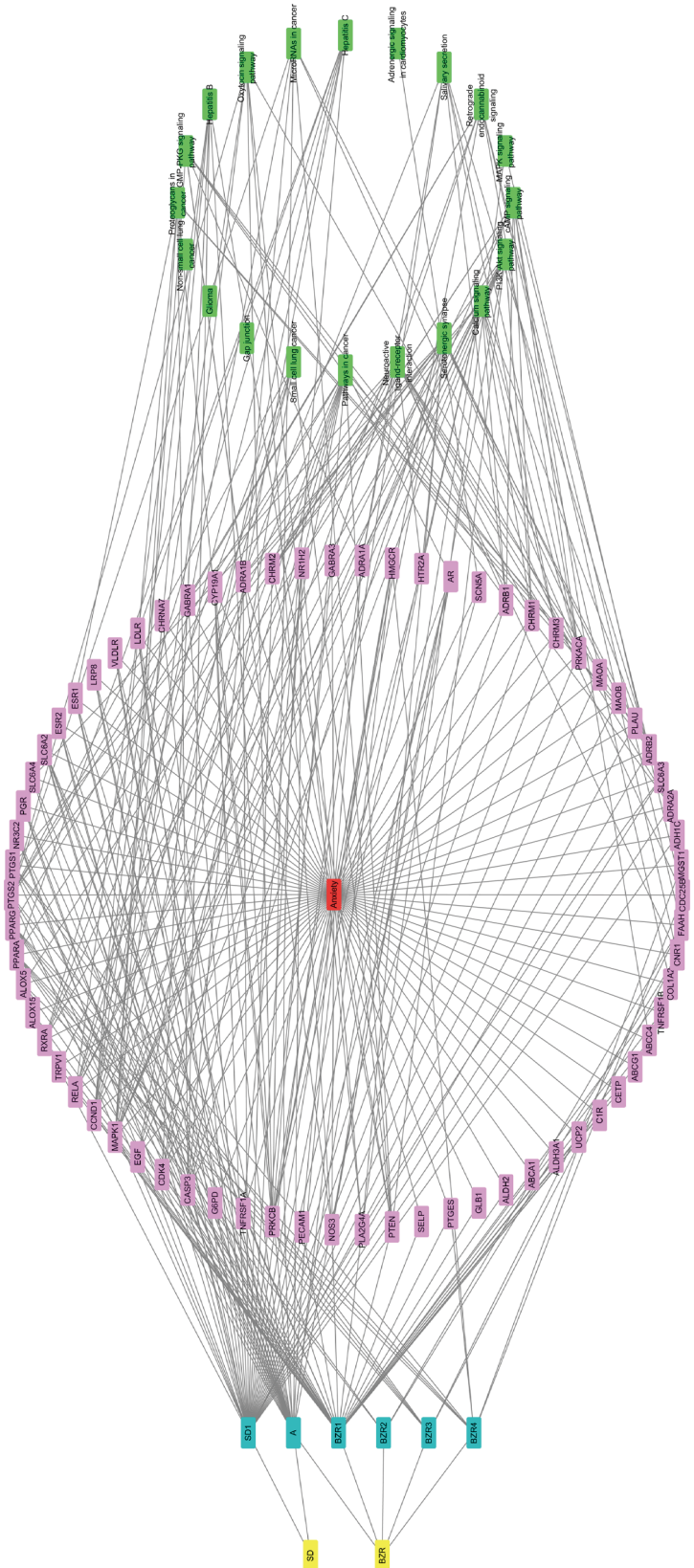

Figure 8 The TCM drugs-ingredients-diseases-targets-pathways network. TCM, Traditional Chinese Medicine.

target proteins, where biological processes, including blood circulation, synaptic signaling, cellular response to organic cyclic compound, response to toxic substance, cellular response to nitrogen compound, response to nutrient levels, and organic hydroxy compound metabolic process, were found to be involved in the therapeutic effects of prepared *Rehmannia* root and Chinese arborvitae kernel on anxiety disorders. The related molecular functions, including oxidoreductase activity, protein kinase binding, G protein–coupled amine receptor activity, nuclear receptor activity, amide binding, protein homodimerization activity, neurotransmitter receptor activity, and ammonium ion binding, might enable the herb pair to exert its antianxiety activity. Furthermore, cellular localization showed that the mechanism sites were the membrane raft, synaptic membrane, receptor complex, cyptoplasmic vesicle membrane, side of membrane, perinuclear region of the cytoplasm, and other cellular environments.

KEGG enrichment pathways mainly involved signaling pathways (e.g., PI3K-Akt signaling pathway, mTOR signaling pathway, cAMP signaling pathway, MAPK signaling pathway, TNF signaling pathway, and VEGF signaling pathway), neurological system (e.g., neuroactive ligand-receptor interaction and serotonergic synapse), endocrine system (e.g., thyroid hormone signaling pathway, estrogen signaling pathway, and estrogen signaling pathway), and drug dependence (e.g., cocaine addiction and amphetamine addiction); in addition, the immune system, cellular processes, cancer, and amino acid metabolism were also involved.

Neuroactive ligand-receptor interaction signaling pathways are closely related with learning and memory and play key roles in neuronal processes, such as neural plasticity and synaptic function (48). In psychological stress-induced susceptible mice versus resilient mice, the differentially expressed genes (DEGs) were upregulated in neuroactive ligand-receptor interaction, PI3K-Akt, VEGF, Ras, and chemokine pathways, and downregulated in cGMP-PKG, B cell receptor, and NOD-like receptor pathways (49). The PI3K/AKT/mTOR signaling pathway is one of the classical pathways regulating the cell cycle. It is widely present in neuronal cells and plays an important role in the regulation of cell division, differentiation, and survival, as well as tumorigenesis. It is closely associated with a variety of mood disorders and neurological diseases. The PI3K/Akt signaling pathway is a downstream signaling pathway of BDNF/TrkB and is involved in disease pathogenesis through processes such as neurotrophic factor release, glutamatergic system, hippocampal neuronal apoptosis, mitochondrial function, glucose/lipid metabolism, and cerebral angiogenesis (50-52). The mTOR signaling pathway involves the proliferation of neural stem cells, regulation of neural circuits, and regulation of circadian rhythms. It regulates memory and learning behaviors through synaptic plasticity (53). Recent studies have confirmed that ketamine is a glutamatergic N-methyl-D-aspartate receptor (NMDA-R) antagonist, which has the characteristics of fast onset and lasting effect in the process of antidepressant. It is therefore assumed that depression is closely associated with the mTOR pathway, and the mTOR pathway may play a key role in depression treatment (54). Another experiment confirmed that early-life stress alters synaptic plasticity by inhibiting the hippocampal mTOR pathway, which may result in increased anxiety-like behaviors and impaired cognitive function (55); anxiety disorders are associated with inflammation. Long-term stress environment can destroy the function of hypothalamus pituitary adrenal axis and the autonomic nervous system, which may induce systemic proinflammatory diseases. Systemic inflammation can enter the brain and increase levels of pro-inflammatory cytokines, which have been shown to promote both direct and indirect neurotoxic effects (56). thus, the mTOR pathway is closely related to anxiety disorders and may be a new direction for antianxiety drug research. The MAPK/ERK signaling pathway is a mechanism that mediates the transmission of extracellular signals to their intracellular targets. It affects the emotional function, social behavior, and spatial memory in humans and is a key element of the neuroinflammatory pathway triggered by glial cells during the development of neurodegenerative diseases (57). Acute local blockade of this MAPK signaling pathway in hypothalamic paraventricular nucleus (PVN) may cause a profound anxiogenic phenotype (58). Phosphodiesterase (PDEs) is an enzyme involved in camp and cGMP (two key second messengers) homeostasis. Phosphodiesterase 2 (PDE2) is involved in cGMP PKG signal transduction, which is highly expressed in limbic brain regions (including hippocampus and amygdala), which may become a breakthrough in the treatment of depression and anxiety (59,60).

Alterations in various behaviors (including appetite, mood, sleep, and cognitive function) in patients with anxiety disorders have been associated with the serotonergic system, and a growing body of research supports a role for dysfunction of serotoninergic, noradrenergic, and dopaminergic systems in the neurobiological processes involved in major depression disorder and anxiety

disorders (61). Estrogen promotes synapse formation, induces growth factor production, protects against oxidative stress, regulates cognition- and mood-related neurotransmitters such as 5-HT, norepinephrine (NE), and acetylcholine, and is associated with learning-, memory-, and anxiety-related behaviors (43). The amygdala is an important structure for anxiety and fear responses; research has confirmed that oxytocin can influence anxiety levels by regulating amygdala activation (62). Thyroid hormones can facilitate intracellular pathways of signaling and affect the neurotransmission of 5-HT and norepinephrine, thus participating in mood and behavior regulation processes (63).

In summary, our current study preliminarily predicted that the mechanism of action of the herb pair of prepared *Rehmannia* root-Chinese arborvitae kernel in treating anxiety disorders involves multiple ingredients, multiple targets, and multiple pathways. It was found that the herb pair may exert its anxiolytic effect via biological processes such as neuroplasticity, signal transduction, hormone regulation, and inflammatory response through neuroactive ligand-receptor interaction, serotonergic synapse, PI3K/AKT/mTOR signaling pathway, MAPK signaling pathway, oxytocin signaling pathway, estrogen signaling pathway, and PPAR signaling pathway.

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Footnote

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