The Molecular Mechanisms of Suanzaoren Prescription for Anti-Anxiety Were Investigated Based on Network Pharmacology

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Abstract

Anxiety is a common, universal disease caused by psychological and environmental factors. There are medications available to treat anxiety disorders, but these are accompanied by problems such as addiction and withdrawal difficulties. Suanzaoren Prescription (SZRP) is often used to treat anxiety disorders in traditional Chinese medicine clinical practice. However, its therapeutic mechanism remains unclear.

This work aims to identify potential core therapeutic targets and predict major bioactive compounds by means of network pharmacology, screen compounds that may bind to anxiety targets by molecular docking methods, and validate and evaluate possible therapeutic mechanisms of compounds for anxiety in vitro cell experiments.

Database TCMSP, BATMAN-TCM, ETCM, TCMGeneDIT and TCMID was used to obtain SZRP compounds, while TCMSP, BATMAN-TCM and ETCM were used to obtain target of compounds. The targets of anxiety disorders were obtained through database TTD, OMIM, PharmgKB, CTD, DrugBank and Metacore. The network diagram is drawn by Cytoscape 3.7.2 software and STRING. Biological process and KEGG pathway analysis were conducted by MetaCore and NIMNT. AutoDock Vina software was used to conduct molecular docking. The calcium Flux assay was employed to detect the activation of compounds on CNR1 in cell line CHO-K1/CNR1/Gα15.

The 545 compounds in SZRP were collected. We found there were 1050 potential targets of these compounds in SZRP which involved in brain disease, mood disorder, endocrine system disease, bipolar disorder, etc. There were 117 potential targets of SZRP against anxiety (such as DRD2, BDNF, COMT, SLC6A4, HTR1A, etc.), which played the roles in regulation of membrane potential and synaptic transmission, nicotine addiction, retinol metabolism, GABAergic and serotonergic synapse, etc. The results of molecular docking indicated there were the biggest affinity between Jujuboside B and DRD2, BDNF, COMT, SLC6A4, Anemarsaponin B_{qt} and HTR1A, n-methylasimilobine and MAPK3. Based on cell CHO-K1/CNR1/Gα15 and calcium flux technique, we found Chrysophanol, Kaempferol and Emodin have activation effects on CNR1 with EC₅₀ 138.3μM, 52.28μM and 56.86μM respectively.

These results indicated that the effects of SZRP against anxiety were correlated with synaptic transmission, substance addiction and retinol metabolism, especially targeted on CNR1. It suggested that the therapeutic mechanism of SZRP treating anxiety might be due to the weak action of multiple compounds on multiple pathways and biological processes.

Introduction

Anxiety disorder is one of the most common mental disorders 1. It was reported that the prevalence of 12-month Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) anxiety disorder was 9.8% in 21 countries 2. And lifetime prevalence of 7.57 percent and 12-month prevalence of 4.98 percent only in China 3. It is speculated that the number of anxiety patients is also on the rise year by year with the

increase of social pressure. The benzodiazepines with sedative effects were generally used in clinical treatment. For example, diazepam quickly exert anxiolytic effects by enhancing GABAergic neurotransmission, which advantages of these drugs are rapid onset^{4,5}. However, due to poor tolerance of these drugs, long-term use in large quantities presented drug dependence⁶ and sudden withdrawal of drugs produced withdrawal reactions, such as inattention, depression, insomnia and other symptoms⁷. Excessive use could cause poisoning, manifested as unconsciousness, coma and other symptoms⁸. Therefore, there is no ideal drug for the treatment of anxiety.

Suanzaoren Prescription (SZRP) is often used to treat anxiety disorders in traditional Chinese medicine (TCM) clinical practice. SZRP also named Suanzaoren decoction, Ziziphi Spinosae Semen, Zizyphus Combination, Suanzaoren Formulae, Suan Zao Ren Tang. SZRP comes from the "Synopsis of Golden Chamber" (*Jinkui Yaolue*, the Eastern Han Dynasty, Zhang Zhong-jing). SZRP was consists of *Ziziphus Jujuba* Var. *Spinosa*, *Poria cocos* Wolf, *Anemarrhenae Rhizoma*, *Ligusticum chuanxiong* Hort., *Glycyrrhiza glabra* L. on 15:6:6:6:3 (Table 1).

Mounts of clinical studies have shown that SZRP has certain anti-anxiety effects and had no toxic side effects. A clinical study reported that 60 generalized anxiety disorder (GAD) patients (mean age 32) were randomly divided into SZRP group and control group with 30 patients in each one. After the treatment of SZRP, the Hamilton Anxiety Scale (HAMA) and Self-Rating Anxiety Scale (SAS) scores were significantly lower than those of control group⁹. Another clinical study reported that 105 GAD in the elderly patients were randomly divided into treatment group (treatment with SZRP and paroxetine, mean age 72) with 52 patients and control group (treatment with paroxetine, mean age 69) with 53 patients. After the treatment of SZRP combined with paroxetine for 1 weeks, HAMA scores were clearly lower than control group, and after 6 weeks of treatment, there was no significant difference in HAMA score. However, the incidence of adverse reactions in the treatment group was significantly lower than that in the control group¹⁰.

Additional, the administration of SZRP increased open arm entry (OE) and open arm time (OT) of anxiety rat model induced by elevated plus maze (EPM)¹¹⁻¹⁴. Studies indicated that the treatment of SZRP regulated the levels of neuropeptide Y¹⁵, 5-HIAA, GABA, GABA/ Glu, NE^{11,16,17}, β -endorphin (β -EP)¹⁴, increased the contents of adreno-cortico-tropic-hormone (ACTH), IL-1 β and TNF- α in serum¹⁸. Furthermore, our previous study showed that Spinosin, Jujuboside BI, Ononin and Timosaponin EI in SZRP were easy to enter the blood, especially, Spinosin, Timosaponin B-II and Ononin were found to be chemical components that could easily cross the blood-brain barrier and enter cerebrospinal fluid¹⁷. However, it remains unclear for the therapeutic mechanism of SZRP treating anxiety disorders.

In order to investigate mechanism of SZRP for anti-anxiety, we collected compounds in SZRP and searched their potential targets to explore the main diseases treated by SZRP. Secondly, the shared targets of compounds in SZRP and anxiety were achieved, their biological process and pathways were enriched and analyzed. Then the molecular docking was employed to predict the affinity between compounds in SZRP and targets with related to anxiety. Finally, based on cell CHO-K1/CNR1/G α 15 and calcium flux technique, we evaluated the activity of some compounds targeting on CNR1.

Materials And Methods

The whole workflow was illustrated in Fig. 1. First, known compounds in SZRP were collected from TCMSP, BATMAN-TCM, ETCM, TCMGenenDIT and TCMID databases. TCMSP, BATMAN-TCM and ETCM were used to obtain potential targets of compounds and conduct enrichment analysis by NIMNT and MetaCore. Secondly, the related targets of anxiety were obtained from MetaCore, OMIM, PharmGkb, TTD, CTD and Durgbank. Then the common targets of SZRP and anxiety were enriched for analysis employing NIMNT and MetaCore databases. Finally, AutoDock Vina were used to predict the compound-target group by molecular docking, and the activity of compounds was detected by the calcium flux assay.

Identification of known compounds in Suanzaoren prescription

All compounds in the five herbs of SZRP were collected from TCMSP (<https://lsp.nwu.edu.cn/>), BATMAN-TCM (<http://bionet.ncpsb.org.cn/batman-tcm/>), ETCM (<http://www.tcmip.cn/ETCM/index.php/Home/>), TCMGeneDIT (<http://tcm.lifescience.ntu.edu.tw/HanMedDBsearch.html>), TCMID (<http://119.3.41.228:8000/tcmid/>).

Identification of potential targets associated with Suanzaoren prescription and anxiety

Obtaining the potential targets of SZRP using TCMSP, ETCM and BATMAN-TCM. Targets related to anxiety were derived from six databases, TTD (<https://ttd.org/>), OMIM (<https://omim.org/>), PharmGkb (<https://www.pharmgkb.org/>), CTD (<http://ctd.mdibl.org/>), DRUGBANK (<https://go.drugbank.com/>) and MetaCore (<https://portal.genego.com/>). And the protein targets derived from TCMSP and TTD were transformed into genes using STRING version 11.0 (<https://string-db.org/>).

Construction of networks

Three networks were constructed by employing the network visualization software Cytoscape 3.7.2. These networks were herb-compound network (H-C network), compound-targets network (C-T network) and core potential targets of SZRP against anxiety. Isolated nodes do not appear in the external diagram.

The possible inter-protein interactions (PPIs) were derived from STRING database, which covered almost all functional interactions between the expressed proteins. And PPI network pairs with overall combined scores above 0.4 were selected and the isolated nodes were not shown. The PPI network results of STRING were output in TSV format, and then imported into Cytoscape 3.2.1 to screen the core potential targets.

Functional Annotation and Enrichment Analysis

Statistical analysis of functional annotation of biological process, KEGG pathway enrichment and disease enrichment analysis of the potential target for SZRP was performed by MetaCore and NIMNT (<http://www.idrug.net.cn/NIMNT/>). Biological process and KEGG pathway analysis were conducted for

the common potential targets of SZRP and anxiety by MetaCore and NIMNT. Selection of enrichment results was statistically significant (P value $\leq$ 0.05) and rank top 50 terms.

Molecular docking simulation

In order to study the active components of SZRP for the prevention and treatment of anxiety and to analyze its possible targets, this study used software AutoDock Vina for molecular docking analysis. The conformation sampling adopts the default optimization parameter, executes each operation in single-thread mode, and adopts the default scoring function using AutoDock Vina. The compounds with molecular weight < 500Da in SZRP were selected for docking using AutoDock Vina. Based on our previous studies on SZRP, we selected the core target of SZRP anti-anxiety and compounds into blood and brain for docking using AutoDock Vina.

Determination of compound activity

We selected five compounds in SZRP, Isomangiferin (B21543), Liquiritigenin (B20416), Kaempferol (B21126), Chrysophanol (B20238) and Emodin (B20240) (The above compounds were purchased from shanghai yuanye Bio-Technology Co., Ltd, HPLC $\geq$ 98%) to detect the effect on CNR1 receptor with calcium flux method. The positive compound was CP-55940 (Sigma, C1112). Compound was dissolved in HBSS (Gibco, 14175-095) buffer [including 20 mM HEPES (Gibco, 156030-080 2042335) pH 7.4] and diluted with DMSO (SIGMA-ALDRICH, D2650). The cell line CHO-K1/CNR1/Gα15 expressing CNR1 receptor (GenScript, M00299, R10011904-2) was used to investigate the activity of compound on CNR1 receptor. The cell CHO-K1/CNR1/Gα15 was cultured in medium Ham's F12 (Gibco, R8142) (added 10% FBS, 100 µg/ml HygromycinB, 200 µg/ml Zeocin), and incubator with 37°C, 5%CO₂. The CHO-K1/CNR1/Gα15 cells were seeded in a 384-well black-wall (Corning, 3764), clear-bottom plate (Corning, 3656) at a density of 10,000 cell per well in 20 µL growth medium at 18 hours prior to the day of calcium assay and maintained at 37 °C/ 5% CO₂. For agonist assay, 20 µL dye-loading solution (Ham's F12, 10% FBS) was added into the wells. Then the plate was placed into a 37°C incubator for 60 minutes, followed by a 15 minutes' incubation (Thermo, 3111) at room temperature. At last, 10 µL compound was added into well of the assay plate during reading in FLIPR^{tetra} (Molecular Devices). For FLIPR reading, the plate containing 5× compound solution was placed in FLIPR. Solutions were added into the cell plate automatically at the 20 seconds and the fluorescence signal was monitored for an additional 100 seconds (21 sec to 120 sec.). Data were recorded by ScreenWorks (version 3.1) as FMD files with FLIPR. Data acquisition and analyses was performed using ScreenWorks (version 3.1) program and exported to Excel. The average value of the first 20 seconds' reading was calculated as the baseline and the relative fluorescent units (ΔRFU) intensity values were calculated by subtracting the average value of baseline from the maximal fluorescent units (21s to 120s).

The % stimulation was calculated with the following equation: % Stimulation = (ΔRFUCompound-ΔRFUBackground)/(ΔRFUAgonist control - ΔRFUBackground) *100 %.

Dose response curves were fitted with four-parameter-logistic-equation by the software GraphPad Prism 8. The four parameters logistic equation was: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X) * \text{HillSlope}})$

X is the logarithm of concentration. Y is the response.

Results

In order to collect compounds in SZRP and potential targets of SZRP as much as possible, we used the Chinese name, botanical name and common name of herbs in SZRP to search databases and literatures (Table1).

Ingredients in Suanzaoren prescription

Based on TCMSP, BATMAN-TCM, ETCM, TCMGenenDIT and TCMID databases, we collected 64 compounds in *Ziziphi Spinosa Semen* (Suanzaoren), 103 compounds in *Poria Cocos Wolf.* (Fuling), 123 compounds in *Anemarrhenae Rhizoma* (Zhimu), 292 compounds in *Ligusticum chuanxiong Hort.* (Chuanxiong), and 28 compounds in *Glycyrrhiza.glabra L.* (Zhigancao). Combining the components in the five herbs and removing the duplicate components of the same name, a total of 545 compounds in the SZRP were obtained (Table 2) (Additional file1).

We established the herb-compound network for SZRP (Fig. 2) (Additional file 2). This herbal-compound network is made up of 5 herbs and 545 compounds. This network had 550 nodes and 610 edges (herb-compound). This H-C network had the average shortest path length 3.08, average degree 2.22 and average neighborhood connectivity 196.43.

Potential targets of Suanzaoren prescription

The 305 potential target proteins were obtained from the database TCMSP, after mapped using the STRING database and removed the duplicates, there were 111 potential gene targets for SZRP. For each component in herbs, database BATMAN-TCM ranks its predicted candidate targets based on the order of the reduced scores given by the target-predictive algorithm for herb-target interaction prediction. There were potential 847 gene targets for SZRP with gene score ≥ 20 from BATMAN-TCM. We collected 408 potential target genes for the compounds in SZRP from database ETCM. The potential targets of 330 compounds were found. Combining targets of these 3 databases, SZRP had a total of 1050 potential targets were obtained after eliminating duplicates (Table 3) (Additional file 3).

A network of compound-targets (H-T network) for SZRP was constructed (Fig. 3). This C-T network had 1380 nodes (330 compound nodes and 1050 target nodes) and 6423 edges. This H-T network had the average shortest path length 3.88, average closeness is 0.07 and average degree 9.31 (Additional file 4). Among them, compounds with the degree 3 included FER, N-Trans-Feruloyltyramine, 6-O-E-Feruloylajugol, B-Sitosterol-B-D-Glucoside, palmitic acid, Choline, Chrysanthemaxanthin. There were 51 compounds with the degree 2, for example mairin, ceanothic acid, Jujuboside A and Jujuboside B, etc

Disease enrichment analysis potentially treated by Suanzaoren prescription

The disease enrichment analysis of 1050 potential targets of SZRP were performed using NIMNT and MetaCore databases in order to investigate disease potentially treated by SZRP.

NIMNT-based enrichment analysis showed that the top 10 (P value < 0.001 and from small to large) diseases potentially treated by SZRP included brain disease, Alzheimer's disease, tauopathy, migraine, nutrition disease, pre-eclampsia, coronary artery disease, overnutrition, mood disorder, obesity (Fig. 4) (Additional file 5).

The results based on MetaCore database showed the top 10 (P value < 0.001 and from small to large) diseases potentially treated by SZRP were chemically-induced disorders; pathological conditions, signs and symptoms; mental disorders; nutritional and metabolic diseases; psychiatry and psychology; metabolic diseases; movement disorders; genetic diseases, inborn; congenital, hereditary, and neonatal diseases and abnormalities; bipolar and related disorders (Fig. 4) (Additional file 5).

We conducted a comparative analysis of the top 50 diseases potentially treated by SZRP based on databases NIMNT and MetaCore. There were 11 diseases with overlap (Table 4). They were brain disease, mood disorder, endocrine system disease, bipolar disorder, etc.

Anxiety disorders, the most prevalent mental or mood disorders, is considered a polygenic, multifactorial trait wherein the continuum of physiological anxiety up to psychopathology is likely to be shaped by the interplay of central nervous system, endocrine system and immune system. This disease enrichment analysis suggested that SZRP may be used to treat anxiety.

Targets related to anxiety

We collected targets related to anxiety from six database (Table 5). A total of 67 gene targets related to anxiety were found in OMIM. 7 gene targets for anxiety were found in PharmGkb. The 41 therapeutic targets for anxiety disorders from TTD were converted by STRING. The 50 genetic targets for anxiety disorders were found in CTD. Durgbank has 73 protein targets for anxiety disorders, which are converted to 73 by STRING. The 133 genes associated with anxiety were found in MetaCore. After integrating targets from the six databases and removing duplicates, there were 249 targets related to anxiety (Table 5) (Additional file 6).

We collected 1050 potential targets for compounds in SZRP and 249 targets associated with anxiety disorders. There were 117 targets after overlap between these types of targets (Table 6) (Additional file 7).

We constructed the PPI network related to SZRP for anti-anxiety employing 117 common potential targets (Fig. 5) (Additional file 8). This PPI network had the average shortest path length 2.06, average degree 23.03 and average neighborhood connectivity 29.24. The target with the highest degree is DRD2, whose degree was 54. Through the double median of Betweenness, Closeness and Degree three

parameters, 11 core targets were obtained after four screening. The 5 targets were ranged from the largest to the smallest in order of Degree value, and were DRD2, BDNF, COMT, SLC6A4, HTR1A.

Functional annotation of biological process

The results of NIMNT-based GO-BP enrichment analysis showed that the biological processes of the top 10 (P value < 0.05 and from small to large) included, regulation of postsynaptic membrane potential, regulation of membrane potential, monoamine transport, G protein-coupled receptor signaling pathway coupled to cyclic nucleotide second messenger, response to ammonium ion, response to xenobiotic stimulus, modulation of chemical synaptic transmission, regulation of trans-synaptic signaling, catecholamine transport, regulation of amine transport (Fig. 6) (Additional file 9).

The enrichment analysis results of Process Networks based on MetaCore showed that the biological processes of the top 10 (P value < 0.05, arranged from small to large) (Fig. 6) (Additional file 9) include, chemical synaptic transmission, anterograde trans-synaptic signaling, trans-synaptic signaling, synaptic signaling, regulation of postsynaptic membrane potential, cell-cell signaling, regulation of membrane potential, response to organic cyclic compound, system process, regulation of biological quality.

After comparative analysis of the biological processes of the top 20 (P value < 0.05, arranged from small to large) in the two databases, there is 3 biological process after taking the intersection. They were regulation of postsynaptic membrane potential, regulation of membrane potential, and synaptic transmission, GABAergic.

Enrichment analysis of KEGG pathway

NIMNT-based KEGG enrichment analysis results showed that the top 10 pathways (P value < 0.05, arranged from small to large) included, neuroactive ligand-receptor interaction, nicotine addiction, serotonergic synapse, morphine addiction, GABAergic synapse, drug metabolism-cytochrome P450, retrograde endocannabinoid signaling, chemical carcinogenesis, calcium signaling pathway, taste transduction (Fig. 7) (Additional file 10).

MetaCore based Pathway enrichment analysis results showed that the top 10 (P value < 0.05, arranged from small to large) (Fig. 7) (Additional file 10) pathways included, tinnitus-associated changes in auditory pathway, protein folding and maturation_insulin processing, mediated direct regulation of xenobiotic metabolizing enzymes, mediated direct regulation of xenobiotic metabolizing enzymes, mediated direct regulation of xenobiotic metabolizing enzymes, mediated direct regulation of xenobiotic metabolizing enzymes, nicotine signaling (general schema), retinol metabolism, estradiol metabolism, serotonin modulation of dopamine release in nicotine addiction.

There were 4 pathways in total after taking the intersection of the top 20 (P value < 0.05, arranged from small to large) pathways in the two databases. The common pathways were nicotine addiction, retinol metabolism, GABAergic and serotonergic synapse.

Molecular docking simulation

According to our previous studies, the components of SZRP into blood and brain were Spinosin, Ononin, Timosaponin EI, Timosaponin B-II and Jujuboside BI 17. According to the chemical structure of these 5 compounds, there were other 7 compounds with the same skeleton structure in SZRP. Among these 12 compounds, namely Anemarsaponin B, Anemarsaponin B_{qt}, Anemarsaponin E, Anemarsaponin E_{qt}, Jujuboside B, Jujuboside B_{qt}, ononin, Spinosin, Timosaponin B III, Timosaponin B III_{qt}, Timosaponin BII, Timosaponin BII_{qt}. We performed molecular docking analysis of 5 core targets with these 12 compounds. Results showed that the highest score of docking were DRD2-Jujuboside B, BDNF-Jujuboside B, COMT-Jujuboside B, SLC6A4-Jujuboside B, HTR1A- Anemarsaponin B_{qt} (Fig. 8) (Table 7) (Additional file 11).

There were many studies 19-21 indicated that cannabinoid receptor 1 (CNR1) and mitogen-activated protein kinase 3 (MAPK3) played the key role in the pathophysiological mechanism of anxiety. Therefore, we executed molecular docking of compounds with molecular weight ≤ 500 Da in SZRP employing AutoDock Vina. Results showed there were the biggest affinity between CNR1 and Isomangiferin, MAPK3 and n-methylasimilobine (Fig. 8) (Table 7) (Additional file 11).

Cell experiments for validating binding affinities of compounds and targets

According to the results of molecular docking, the mean absolute value of the combined score of the three subtypes of CNR1 (5tgz, 5xra, 5xr8) with compounds was greater. And based on compounds being purchased and reported or not, we selected five compounds to verified the activity of targeting on CNR1 by experiments. These compounds were Liquiritigenin, Isomangiferin, Kaempferol, Emodin and Chrysophanol. The calcium Flux assay was used to detect the activation of these 5 compounds on CNR1 (CHO-K1/CNR1/Gα15 cell). The results showed the EC₅₀ of positive compound CP-55940 targeting on CNR1 was 49.44μM. The EC₅₀ of Isomangiferin, Liquiritigenin, Kaempferol, Chrysophanol and Emodin activating on CNR1 were 5.271×10^{-5} M, 5.686×10^{-5} M, 5.228×10^{-5} M, 1.383×10^{-4} M and 5.437×10^{-5} M, respectively (Fig. 9) (Additional file 12). Chrysophanol and Kaempferol had the maximum effective rate of 81% and 68%.

Discussion

In this study, we found that 545 chemical constituents in herbs of SZRP might target on 1050 biomolecules, and play a role in the treatment of brain disease, mood disorder, etc. The effects of SZRP on anti-anxiety may be via regulating membrane potential and synaptic transmission, etc., which involve in 117 biomolecules, such as DRD2, BDNF, COMT, SLC6A4, HTR1A etc. Especially, we found that Chrysophanol, Kaempferol and Emodin activated CNR1 with EC₅₀ 1.383×10^{-4} M, 5.228×10^{-5} M and 5.686×10^{-5} M respectively.

Endocannabinoid is found in the neuro-immune systems of both animals and humans. Cannabinoid receptors are considered to be key regulators of anxiety, neurodegenerative diseases and other diseases 22. Cannabinoid receptors are divided into CNR1 and CNR2, which are involved in pain perception, stress suppression, memory, motor function regulation, cognition and emotional response 23-25. A large number of pharmacological studies have shown that CNR1 acted as an important regulator of anxiety-like behavior. For example, in the elevated plus-maze and the light/dark avoidance task, the complete CNR1 null-mutant mice showed an anxiogenic-like phenotype, in contrast, the conditional mutant mice lacking CNR1 expression specifically in cortical glutamatergic neurons failed to show a similar phenotype 26. In addition, study showed that exploration was increased when CNR1 receptor was deleted in cortical and striatal GABAergic neurons, however, deletion of CNR1 receptor from cortical glutamatergic neurons caused a decreased exploration in these task 27. And in mice lacking the CNR1 receptor either in cortical glutamatergic or in GABAergic neurons, CNR1 receptors expressed in cortical glutamatergic neurons favors novelty seeking, whereas CNR1-dependent control of inhibitory GABAergic neurons favors behavioral inhibition by novelty-induced behavioral inhibition test 28. Thus, CNR1 on cortical glutamatergic and GABAergic neurons exert opposing on anxiety-like behavior, but this function seems to operate within specific limits of neuronal activity 29. Numerous pharmacological studies support the view of bidirectional regulation of anxiety behavior by CNR1. Interestingly, experimental studies have shown that anti-anxiolytic-like effects in the low dose of cannabinoids via the CNR1 receptor on cortical glutamatergic terminals, on the contrary, the CNR1 receptor on the GABAergic terminals induce an anxiogenic-like effect under a high-dose treatment 30. Experimental studies have also found, while a mild activation of CNR1 receptors in the prefrontal cortex ventral hippocampus attenuates anxiety and a slight CNR1 receptor stimulation in the amygdala results in an anxiogenic-like response 31. Taken together, although the regulation of anxiety behavior by CNR1 showed bidirectional in different brain regions and different types of neurons, the complete CNR1 null-mutant body showed anxiety. Therefore, it might be effective or therapeutic for anxiety to activate CNR1 moderately. In this study, we found Chrysophanol, Kaempferol and Emodin in SZRP activated CNR1 with EC_{50} 138.3, 52.28 and 56.86 μ M respectively.

Chrysophanol (molecular weight 254.2 g/mol, $C_{15}H_{10}O_4$) belonged to anthraquinones and was yellow powder and soluble in methanol and ethanol. In a study of seahorses, compound Chrysophanol has been found that it relieved the anxious behavior of mice induced by chronic unpredictable mild stress (CUMS), which were correlated with reducing IL-1 β and reactive oxygen species (ROS), increasing neurotransmitter (NE, DA, 5-HT) concentrations and BDNF expression 32.

Flavonoids Kaempferol (molecular weight 286.2g/mol, $C_{15}H_{10}O_6$) was yellow powder and slightly soluble in water, soluble in hot ethanol, ether or alkali solution. Some studies indicated Kaempferol had effects on anxiety. Kaempferol can enhanced number of entries and time spent in the open arm in EPM test, which demonstrated anxiolytic activity 33. Kaempferol from the leaves of *Apocynum venetum* possesses anxiolytic activities in the EPM test in mice, and benzodiazepine antagonist flumazenil was partially antagonized the anxiolytic activity of kaempferol 34. Kaempferol from *Tagetes* genus performed anxiolytic effects in open-field, exploration cylinder, hole-board task, and this anxiolytic effect was

significantly inhibited in the presence of the receptor antagonist 5-HT_{1A} (WAY 1006355) 35. Using light/dark model and hole-board experiment to evaluate whether Kaempferol has anti-anxiety activity in mice model, results showed Kaempferol had effects on anxiety and at a dose up to 2000 mg/kg body weight showed no mortality 36.

Anthraquinones Emodin (molecular weight 270.2g/mol, C₁₅H₁₀O₅) was yellow powder and soluble in ethanol, slightly soluble in ether, chloroform, benzene, insoluble in water. It has the role of anti-inflammatory 37-39, neuroprotective 40 and cardiovascular protective ^{41, 42}. No reports were found about Emodin effecting on anxiety.

Based on protein-protein interaction network related to Suanzaoren prescription for anti-anxiety, we found some possible important biomolecules regulated by Suanzaoren prescription, such as DRD2, BDNF, COMT, SLC6A4, HTR1A, etc.

Dopamine receptor D2 (DRD2) belonged to the G protein-coupled receptors. DRD2 receptor is the common drug target for anti-psychotics 43. Some studies showed DRD2 played a role in anxiety disorders, for example, anxiety disorders in children and adolescents 44. The expression of DRD2 significantly increased in the anxiety mouse models established with constrain stress and social isolation 45. And nicotine-dependent smokers enhanced prefrontal DRD2, might influence vulnerability for anxiety disorders 46.

The brain derived neurotrophic factor (BDNF) is a critical contributor to neuronal growth, development, learning, and memory 47. BDNF expression associated with the pathophysiology of anxiety ^{48, 49}. Clinical studies have revealed that GAD has been showed lowered plasma BDNF levels 50-52. BDNF also may play a central role in the pathogenesis of bipolar disorder 53. In our previous experimental study of SZRP, it was found that SZRP can improve the BDNF mRNA expression level in insomnia rat, thus improving neuronal damage, improve the symptoms of insomnia ^{54, 55}. And SZRP can also up-regulate the expression of BDNF genes, and has an anti-depressant effect 56.

Catechol-O-methyltransferase (COMT) inactive catecholamine neurotransmitters and catechol hormones, also shortens the biological half-lives of certain neuroactive drugs. Some findings demonstrated that COMT Val (158) Met polymorphism is associated with risk of GAD via reduced resting parasympathetic nervous control 57 and COMT SNPs also modulated the association between antenatal maternal anxiety 58. Experimental research found that COMT KO mice showed increased anxiety 59.

Sodium-dependent serotonin transporter (SLC6A4) is sodium-dependent serotonin transporter, whose primary function in the central nervous system involves the regulation of serotonergic signaling. SLC6A4 are associated with vulnerability to affective disorders. Clinical case analysis found that, the serotonin transporter gene SLC6A4 involved in the etiology of anxiety-related ^{60, 61}. It was found in some experimental studies that serotonin transporter expression was associated positively with anxiety-like behavior ^{62, 63}.

5-hydroxytryptamine receptor 1A [HTR1A] was G-protein coupled receptor for 5-hydroxytryptamine (serotonin) and also function as a receptor for various drugs and psychoactive substances. HTR1A played a role in the regulation of dopamine and 5-hydroxytryptamine levels in the brain, and thereby affect neural activity, mood and behavior. The study showed that mice with 5-HT1AR gene knockout showed anxiety-like behavior 64. 5-HT1AR neurotransmission-related dysfunction has been associated with several psychiatric disorders, including anxiety 65, in 5-HT neurons to repress HTR1A expression and drive adult anxiety-like behaviors 66. SZRP can reduce time for voluntary activities and regulate the expression of 5-HT1AR in the hippocampus of rats, thus improving the sleep state of sleep-deprived rats 67-69.

Mitogen-activated protein kinase 3 [MAPK3] is mitogen-activated protein kinase 3, which acts as an essential component of the MAP kinase signal transduction pathway, and thus suppress cancer cells 70. MAPK3 is also associated with mental illness, such as autism spectrum disorder 71.

Triterpenoids Jujuboside B (molecular weight 1045.2 g/mol, $C_{52}H_{84}O_{21}$) was easier to get into the blood and white crystalline powder, soluble in methanol, almost insoluble in ether. It has activities of inhibiting leukemia cell growth 72, anti-inflammatory 73, ameliorate cognitive deficiency 74, antiplatelet 75 and etc. Based on molecular docking, we found Jujuboside B in SZRP might target on DRD2, BDNF, COMT and SLC6A4.

Steroids Anemarsaponin B_{qt} (molecular weight 416.71 g/mol, $C_{45}H_{74}O_{18}$) was easy to cross the blood-brain barrier 17 and white powder, soluble in benzene, pyridine and chloroform, soluble in ethanol, methanol and acetone, slightly soluble in water. Pharmacological action of Anemarsaponin B_{qt} has not been reported. But the pharmacological effects of similar compounds have been reported. Anemarsaponin BII mainly increased bone formation 76, Anemarsaponin B has anti-inflammatory 77 and inhibit platelet aggregation 78. In this study, we found Anemarsaponin B_{qt} in SZRP might target HTR1A.

Alkaloids n-methylasimilobine (molecular weight 281.3 g/mol, $C_{18}H_{19}NO_2$) was yellow powder and soluble in methanol, ethanol, DMSO and other organic solvents. It has antioxidative activity 79, significant cytotoxic activities against carcinoma cell line 80 and inhibition of platelet aggregation 81. Based on molecular docking, this study showed that n-methylasimilobine in SZRP might target on MAPK3.

In this study, biological process enrichment analysis of potential target for shared target indicated that the regulation of membrane potential and GABAergic synaptic transmission might be some mechanism of SZRP anti-anxiety. Regulation of membrane potential refers to the regulation of the potential difference between the two sides of the membrane. Regulation of membrane potential have been implicated in diverse pathophysiological processes, including mood regulation etc. According to experimental research reports, the medial habenula (an epithalamic structure implicated in anxiety-like behavior) , neurons are capable of firing transient, high-frequency action potential bursts mediated by T-type channels in C57BL/6J mice 82. But an impaired maintenance of long-term potentiation in the hippocampus and

display enhanced anxiety-like behavior of Np65 KO mice 83. However, membrane potential increased and anxiety-like behavior were significantly relieved after the treatment of G-1 (the GPER agonist) 84. Similarly, postsynaptic membrane potential and GABAergic synaptic transmission are closely related to anxiety disorders. Mechanisms underlying the anxiolytic effects of metformin by up-regulated the surface expression of GABAA receptors and increased miniature inhibitory postsynaptic currents 85. The study showed that in EPM model rats treated by SZRP, the OE and OT were significantly increased, and the content of GABA was extremely significantly elevated 86 and the mRNA expression level of GABAA receptor was up-regulated 14. After being treated by SZRP, the content of GABA in hypothalamus, brainstem and brain tissue increased in insomnia model rat with chronic sleep deprivation, insomnia model rat with liver and blood deficiency and elderly insomnia model rats 87-90. In insomnia rats with fatigue model, SZRP could down-regulate the levels of GABA in the cerebral cortex and hypothalamus 91. It is not reported that SZRP may regulate membrane potential in anxiety.

In additional, pathways enrichment analysis of potential target for shared target of SARP and anxiety suggested that pathway nicotine addiction, retinol metabolism, serotonergic synapse might be regulated by SZRP. Nicotine addiction that means nicotine dependence and addictive behavior. However, nicotine addiction and nicotine withdrawal can lead to anxiety-like behavior 92-94. Experimental research report that activation of the neuronal PPARgamma [the receptor for the thiazolidinedione class of medications] prevents the expression of signs of nicotine withdrawal 95 and increased expression of CRF (Corticotropin-Releasing Factor) and CRF-receptors in certain areas of the brain after the development of intraperitoneal nicotine administrations in rats 96. Interestingly, the hippocampus seems to be closely linked to nicotine 97-99 and is also known to be associated with anxiety 100-102. Retinol (vitamin A) metabolism refers to retinol comes out of the liver and binds to retinol-binding proteins, which are transported to various tissues of the body through the blood for normal metabolism. Retinol metabolism is involved in oxidative processes 103-104 in body, and is also associated with anxiety. Retinol induced anxiety-like behavior in adult rats chronically subjected to vitamin A supplementation 104-105. Serotonin (5-HT) is an important neurotransmitter, which is high in the cerebral cortex and synapses. Serotonin in the brain controls emotional regulation, cognitive function and other functions. Neurotransmitters such as serotonin and synapses are known to play an important role in the development of anxiety 106. It was found that the integrity of serotonergic synapses and anxiety related phenotype in mice 107. It was worth noting that only a few studies had considered relationship between pre-and post-synaptic serotonergic transmission. Anxiety caused by serotonergic synapse was due to an imbalance of serotonergic synapse regulated mRNAs expression, such as 5-HTR_{2A} is upregulated 108, 5-HTR_{1A} down-regulated 109.

It was found that SZRP exhibited binding affinity for serotonin receptors on the basis of Ultra-performance liquid-chromatography/electrospray-ionization synapt high-definition mass spectrometry (UPLC/ESI-SYNAPT-HDMS) combined with pattern recognition approaches 110. A clinical study reported that the treatment of SZRP increased the serum 5-HT content in patients with liver blood deficiency insomnia 111. In the EPM rat model of anxiety, the content of 5-HIAA in the hippocampus and thymus of

SZRP group was significantly higher than that of the model group 11·16·86. And the content of 5-HT and 5-HIAA in serum, hypothalamus, hippocampus, cerebral cortex and brain stem increased in model rats of insomnia and sleep deprivation rats treated by SZRP 68·89-91·112-115. And in Alzheimer's disease rats induced by D-galactose and A β 1-42, serum 5-HT concentration in SZRP group was significantly higher than that in model group 116. Other experimental reported that chronic stress induced depression in rat, compared with model group, SZRP significantly increased the content of 5-HT in both hippocampus and cerebral cortex 117. The administration of SZRP regulated the expression of 5-HT_{1A}R and 5-HT_{2A}R in hippocampus, hypothalamus, and thymus of rat with chronic sleep deprivation 67·69. However, there was no reports about the treatment of SZRP regulating serotonergic synapse in anxiety. While SZRP is involved in the therapy of anxiety by way of the nicotine addiction, retinol metabolism, serotonergic synapse, which we first discovered.

Conclusions

In this study, the therapeutic components and mechanism of SZRP for anxiety disorders were investigated by using network pharmacology combined with experimental verification. The main findings were as follows: (1) the 525 compounds and 1050 potential targets in SZRP were collected, and SZRP might be used to treat brain disease, mood disorder, endocrine system disease, bipolar disorder, etc. (2) the 249 potential targets of anxiety were obtained, and 117 possible targets of SZRP against anxiety were found, for example, DRD2, BDNF, COMT, SLC6A4, HTR1A and other biological molecules. (3) the 117 potential targets involved in some biological process, including of regulation of membrane potential, and synaptic transmission, GABAergic, etc. (4) the 117 potential targets played roles in many pathways, such as nicotine addiction, retinol metabolism, GABAergic and serotonergic synapse. (5) based on molecular docking, the biggest affinity were found between Jujuboside B and DRD2, BDNF, COMT, SLC6A4, Anemarsaponin B_{qt} and HTR1A, n-methylasimilobine and MAPK3. (6) Chrysophanol, Kaempferol and Emodin have activation effects on CNR1 with EC₅₀ 138.3 μ M, 52.28 μ M and 56.86 μ M respectively. However, there are limitations to this study. The ingredients in SZRP and anxiety-related genes may not be global or full. We did not verify all the affinity between compounds and targets employing experiments. In conclusion, this study provided theoretical basis and clues for the study on the pharmacological mechanism of SZRP against anxiety.

Abbreviations

SZRP	Suanzaoren prescription
CNR1	cannabinoid receptor 1
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
TCM	traditional Chinese medicine
GAD	generalized anxiety disorder
HAMA	Hamilton Anxiety Scale
SAS	Self-Rating Anxiety Scale
OE	open arm entry
OT	open arm time
EPM	elevated plus maze
β-EP	β-endorphin
ACTH	adreno-cortico-tropic-hormone
H-C network	herb-compound network
C-T network	compound-targets network
CUMS	chronic unpredictable mild stress
ROS	reactive oxygen species
DRD2	Dopamine receptor D2
BDNF	brain derived neurotrophic factor
COMT	Catechol-O-methyltransferase
SLC6A4	Sodium-dependent serotonin transporter
HTR1A	5-hydroxytryptamine receptor 1A
BP	Biological Process

Declarations

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Conflicts of interest The authors declare that there is no conflict for publication of this paper.

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Tables

Due to technical limitations, table 1-7 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The workflow of network pharmacology approach to investigate the mechanisms of Suanzaoren prescription against anxiety.

Figure 2

The network of compound and herb in Suanzaoren prescription. This network comprised 550 nodes and 610 edges (herb-compound). The triangle represents traditional Chinese medicine and the circle represents compounds. Suanzaoren (SZR) and its compounds are shown in red, Fuling (FL) and its compounds in green, Zhimu (ZM) and its compounds in yellow, Chuanxiong (CX) and its compounds in brown, zhigancao (ZGC) and its compounds in blue, pink nodes represent common compounds.

Figure 3

The network of compounds in herbs and potential targets in Suanzaoren prescription. Yellow node represents compounds, green node represents targets, and the line represents the interaction between compounds and potential targets. Isolated nodes are not shown in the diagram.

Figure 4

The top 50 diseases enriched for the potential targets of the Suanzaoren prescription. A. Diseases enrichment from NIMNT; B. Diseases enrichment from MetaCore; Select the top 50 for visualization. X-axis shows the -lg (P value) of the terms; Y-axis shows the different diseases (P<0.001). * mean that the disease is related to nervous system or emotional disorder.

Figure 6

Top 50 biological process enrichment analysis of potential target for shared target. A. Biological processes enrichment from NIMNT; B. Biological process enrichment from MetaCore. X-axis shows the name of the biological processes, the left Y axis shows gene number and the right Y axis shows the -lg (P value) of the terms ($P < 0.001$).

Figure 7

Top 50 pathways for gene target enrichment of potential target for shared target. A. Pathway enrichment come from NIMNT, X-axis shows the -lg (P value) of the terms, the left Y axis shows the different pathway (P<0.05). B. Pathway enrichment come from Metacore, X-axis shows the -lg (P value) of the terms, the left Y axis shows the different pathway (P<0.01).

Figure 8

The molecular docking for compound in Suanzaoren prescription and core targets with related to anti-anxiety. * DRD2: Dopamine receptor D2; BDNF: brain derived neurotrophic factor; COMT: Catechol-O-methyltransferase; SLC6A4: sodium-dependent serotonin transporter; HTR1A: G-protein coupled receptor for 5-hydroxytryptamine; CNR1: Cannabinoid receptor 1; MAPK3: mitogen-activated protein kinase 3. (The dotted yellow line in the figure represents hydrogen bonds, and the number represents the bonding force between hydrogen bonds).

Figure 9

The EC₅₀ of compound in Suanzaoren prescription against the target of Cannabinoid receptor 1. A. CP-55940 - CNR1; B. Chrysophanol - CNR1; C. Kaempferol - CNR1; D. Emodin - CNR1; E. Isomangiferin - CNR1; F. Liquiritigenin - CNR1. Mean ± S.E.M., n=3.

Supplementary Files

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