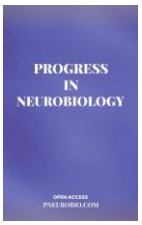

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Research Article

Potential Mechanisms and Material Basis of Suanzaoren Prescription on Anxiety

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ABSTRACT

Background: Anxiety is a common, universal disease caused by psychological and environmental factors. There are medications available to treat anxiety disorders, but are accompanied by problems such as addiction and withdrawal difficulties. Suanzaoren prescription (SZRP) is used to treat anxiety disorders in traditional chinese medicine clinical practice. However, its therapeutic mechanism remains unclear. This study aimed to clarify the effects of SZRP on anxiety through *in vivo* experiments, then we predicted potential mechanisms of SZRP by network pharmacology. Molecular and *in vitro* experiments were conducted to reveal material basis of anti-anxiety function. **Methods:** The anxious-like behavior of ICR mice was established by single intraperitoneal injection of PTZ (30 mg/kg), and effects of SZRP on anxiety-like behavior was evaluated by elevatd plus maze, dark box and open field experiment. We conducted network pharmacology techniques to predict potential pathways and targets of SZRP on anxiety. AutoDock vina software was used to conduct molecular docking. The calcium flux assay was employed to detect the activation of compounds on CB1 in cell line CHO-K1/CB1/Gα15, and anti-anxiety effects of chrysophanol, kaempferol and emodin were also verified by elevated plus maze and open field experiment. **Results:** SZRP (43.4 g/kg/d) could improve anxiety-like behavior induced by PTZ (30 mg/kg). Furthermore, pathway enrichment analysis revealed the potential significance of GABAergic and cannabinoid receptor signaling pathways. Asperglaucide, isomangiferin, chrysophanol, perlolyrine, and anemarcoumarin a might be key components of SZRP for anxiety. Based on cell CHO-K1/CB1/Gα15 and calcium flux technique, we found chrysophanol, kaempferol and emodin have active effects on CB1 with EC_{50} 138.3μM, 52.28μM and 54.37μM respectively. Among them, kaempferol and chrysophanol showed better anti-anxiety effects on anxiety-like behavior model. **Conclusion:** This study demonstrated that SZRP may be involved in GABAergic and cannabinoid receptor signaling pathways, thereby improving behavioral disorders of anxiety, which is most effective kaempferol and chrysophanol, and this may be key compounds for SZRP in the treatment of anxiety.

1. Introduction

Anxiety disorder is one of the most common mental disorders [1]. It's 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,

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5]. However, due to poor tolerance of these drugs, long-term use in large quantities presented drug dependence [6] and sudden withdrawal of drugs produced withdrawal reactions, such as inattention, depression, insomnia and other symptoms [7]. Excessive use could cause poisoning, manifested as unconsciousness, coma and other symptoms [8]. Therefore, there is no ideal medicine for the treatment of anxiety.

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 [9]. Cannabinoid receptors are divided into CB1 and CB2, which are involved in pain perception, stress suppression, memory, motor function regulation, cognition and emotional response [10-12]. A large number of pharmacological studies have shown that CB1 acted as an important regulator of anxiety-like behavior [13, 14]. For example, in the elevated plus-maze and the light/dark avoidance task, the complete CB1 null-mutant mice showed an anxiogenic-like phenotype, in contrast, the conditional mutant mice lacking CB1 expression specifically in cortical glutamatergic neurons failed to show a similar phenotype [15]. Taken together, although the regulation of anxiety-like behavior by CB1 showed bidirectional in different brain regions and different types of neurons, the complete CB1 null-mutant body showed anxiety. Therefore, it might be effective or therapeutic for anxiety to activate CB1 moderately.

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.* (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 group [16]. 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. However, the incidence of adverse reactions in the treatment group was significantly lower than that in the control group [17].

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) [18-21]. Studies indicated that the treatment of SZRP regulated the levels of neuropeptide Y [22], 5-HIAA, GABA, GABA/Glu, NE [21, 23, 24], β-endorphin (β-EP) [18], increased the contents of adreno-cortico-tropic-hormone (ACTH), IL-1β and TNF-α in serum [25]. 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 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/CB1/Gα15 and calcium flux technique, we evaluated the activity of some compounds targeting on CB1and identified compounds that treat anxiety.

2. Materials and Methods

The whole workflow was illustrated in (Figure 1).

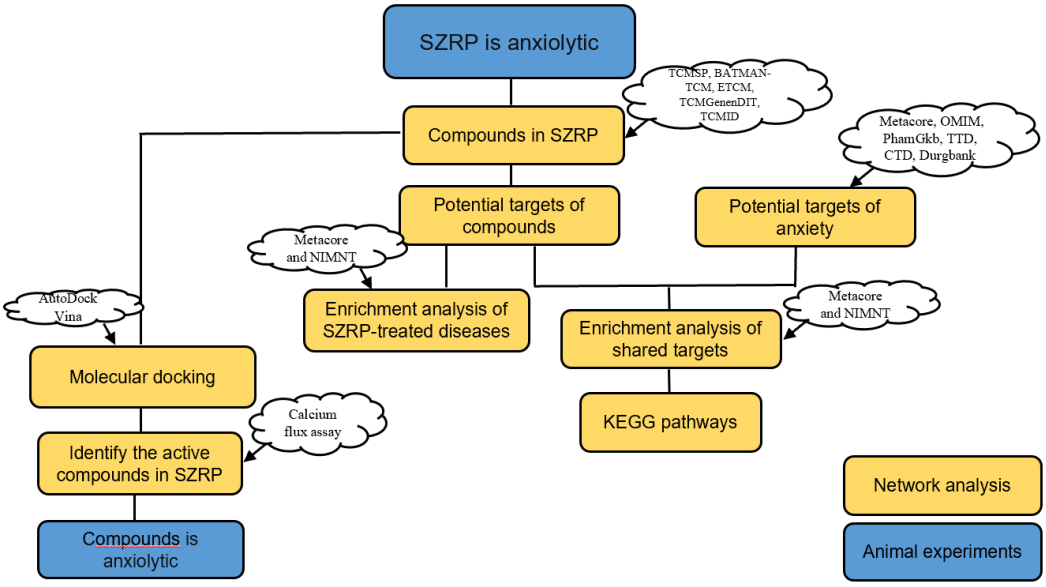

FIGURE 1: The workflow of this study.

2.1. Materials

2.1.1. Preparation of SZRP

Suanzaoren, Chuanxiong, Fuling, Zhimu and Zhigancao were purchased from Jinan Da Zhai Men Chinese Medicine Co. (Shandong, China) as raw material for the preparation of SZRP and fixed ratio of 14.3:2:2:2:1, respectively. The above drugs were macerated in eight volumes of distilled water for 60 min and decocted for 30 min. After filtration, the remaining drugs were added to six volumes of water and decocted for 20 min. The filtrates were mixed, concentrated to 2.17 g /mL.

2.1.2. Drugs

Diazepam (200703, Shandong Xinyi Pharmaceutical Co.) were 2.0 mg/mL (distilled in water). PTZ (1,5-Pentamethylenetetrazole, C11930987, Macklin) was prepared at 30 mg/kg (dissolved in saline) on the day of the behavioral experiment. Isomangiferin (B21543), Liquiritigenin (B20416), Kaempferol (B21126), Chrysophanol (B20238) and Emodin (B20240) (The above compounds were purchased from Shanghai yuanye Bio-Technology Co., Ltd, HPLC≥98%). Among them, Kaempferol Chrysophanol and Emodin were weighed and dissolved in of 0.5% carboxymethyl.

2.1.3. Experimental Animal

Male ICR mice (5-6 weeks) were purchased from Beijing charles river Laboratories (Beijing, China, SCXK (Jing) 2021-0006) and maintained in the SPF barrier environment in Experimental Animal Center of Shandong University of Traditional Chinese Medicine with a temperature of 22± 2°C, relative humidity of 40-60%, and a light/dark cycle of 12/12 h, and had free access to water and food. All mice received adaptive feeding for 1 week before drug administration. All the experimental protocols in this study were approved (NO. SDUTCM20210606002) by the Institute of Animal Care and Use Committee (IACUC) of Shandong University of Traditional Chinese Medicine.

2.2. Methods

2.2.1. Establishment of Anxiety Model

In the experimental study of anxiety-related behavior, it was found that PTZ can induce anxiety-like behavior [26-28].To establish the anxiety model, ICR mice were administered once with PTZ (30 mg/kg) at 30 min before behavioral experiment randomly.

2.2.2. Experimental Design and Drug Treatment Regimen

In SZRP improves anxiety-like behavior experiments, ICR mice were separated into 6 groups, each group had 15 mice. ICR mice in control group (Con) and model group (Mod) were given distilled water. Mice in positive group (Pos) were administrated with Diazepam (2.0mg/kg/d). Mice were administrated with SZRP at 14.5 g/kg/day (L), 28.9 g/kg/day (M), 43.4 g/kg/day (H) in SZRP groups by gavage (43.4 g/kg/day dose from "Jinkui Yaolue"). ICR mice were administered 90 min and injected PTZ (30 mg/kg) at 30 min before behavioral experiment.

In compound anxiogenic experiment, ICR mice were separated into 6 groups, each group had 14 mice. The mice were divided into four groups: control group, Kaempferol group (10 mg/kg/d) [29], emodin (20 mg/kg/d) [30], Chrysophanol group (20 mg/kg/d) by gavage. ICR mice were administered 60 min before behavioral experiment randomly. Turn on the red light for all behavioral experiments and test time is 5 min.

2.2.3. Behavioral Experimental Methods

2.2.3.1. Elevated Plus Maze test (EPM)

The elevated plus maze test was conducted using the XR-Super Maze (Shanghai Xinsoft Information Technology Co. Ltd.) tracking system and a polypropylene plastic cruciform apparatus that was elevated 76 cm above the floor. The cruciform box consisted of two open arms, two closed arms (30 cm × 5 cm for mice), and a central platform (the junction area, 5 cm × 5 cm for mice and 10 cm × 10 cm for rats). Each animal was placed on the central platform with the head towards the open arm, and the behavior was recorded within 5 min, including the open-arm entry times (OE), closed arm entry times (CE), time in the open arm (OT), time in the closed arm (CT). Then, the OE% and OT% were calculated as follows: OE%=OE/(OE+CE) ×100%, OT%=OT/(OT+CT) ×100%. The arena was cleaned with 75% ethanol after every trial. Anxiety-like behavior is to enter open arms and decrease the number of times and time.

2.2.3.2. Light Dark Box Test (LDB)

The light dark box consisted of two chambers of the same size (25 cm × 25 cm × 30 cm), with the dark and light compartments separated by a door (6.5 cm × 6.5 cm). The animals were placed in the region of the light box and allowed to move freely between the two chambers within 5 min. A video tracking system (XR Super Maze) was used to record the total distance, time spent in the light box, light box entry times, and the distance in the light box. As the rodents have an innate aversion to light areas and were allowed spontaneous exploration, the indexes previously mentioned could indicate their anxiety-like behavior.

2.2.3.3. Open-Field Test (OFT)

Test the animals were placed in a square apparatus (50 cm × 50 cm), with the arena divided into 16 equal squares for 5 min. Each mouse was placed individually into the centre and permitted free exploration. With the XR-Super Maze tracking system, the total distance, central area distance, time at central area were recorded during the test time. The arena was cleaned with 75% ethanol after every trial. Anxiety-like behavior was defined when the animal spent more time near the edges of the box than at the centre.

2.2.4. Network Pharmacology

Compounds of SZRP were collected from TCMSP (Link 1), BATMAN-TCM (Link 2), ETCM (Link 3), TCMGeneDIT (Link 4), TCMID (Link 5). Potential targets are from TCMSP, ETCM and BATMAN-TCM. The networks were herb-compound network (H-C network) by Cytoscape 3.7.2 (Isolated nodes do not appear in the external diagram).

Targets related to anxiety were derived from TTD (Link 6), OMIM (Link 7), PharmGKB (Link 8), CTD (Link 9), Drugbank (Link 10) and MetaCore (Link 11). And the protein targets were transformed into genes by STRING version 11.0 (Link 12). Disease and KEGG pathway enrichment analysis by MetaCore and NIMNT (Link 13). Disease enrichment analysis mapping was performed by excel. KEGG pathway analysis were conducted for the common potential targets of SZRP and anxiety. Selection of enrichment results was statistically significant (*P* value＜0.05) and rank top 30 terms for mapping by oebiotech (Link 14).

2.2.5. Molecular Docking

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. According to the results of KEGG pathways, the mean absolute value of the combined score of the three subtypes of CB1 (5tgz, 5xra, 5xr8) with compounds. The three-dimensional (3D) structures were retrieved from PDB database (Link 15). Water and ligands of the structure were removed using the PyMOL software, hydrogen bonds were added on the structure by AutoDock Tools software, and the structure was saved as a PDBQT file.

The compounds with molecular weight < 500 Da in SZRP for docking using AutoDock Vina. The 3D structures were downloaded from TCMSP and Pubchem database (Link 16). In AutoDock Tools, we input the structure of the component as a ligand and set the structure as follows: delete root, show root expansion, and choose torsions. Then, exported the structure to a ligand file in pdbqt format.

Use AutoDock Tools to select the docking region of the protein to be docked, the docking size on the X, Y, and Z axes. Save the information about the ligand receptor and the interworking region Settings in conf.txt. Use the cmd command to interconnect with AutoDock Vina. The results of docking can be shown by using Pymol to open both the receptor pdb file and the ligand output pdbqt file.

2.2.6. *In Vitro* Experiment

The positive compound was CP-55940 (Sigma, C1112). Compound was dissolved in HBSS (Gibco, 14175-095) buffer and diluted with DMSO (SIGMA-ALDRICH, D2650). The cell line CHO-K1/CB1/Gα15 expressing CB1 receptor (GenScript, M00299, R10011904-2) was used to investigate the activity of compound on CB1 receptor. The cell CHO-K1/CB1/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%CO_2. The CHO-K1/CB1/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% CO_2. 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 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 5x 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).

% 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=Bottom + (Top-Bottom)/ (1+10^ ((LogEC_{50}-X) ×HillSlope)).

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

2.3. Statistical Analysis

The Statistical Package for GraphPad Prism 8.0.1 was used for statistical analysis. The data are expressed as the Means ± SD. Statistical significance was determined by Student's t-test or One-way ANOVA followed by Dunnett's multiple comparisons test. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. The Effects of SZRP on Anxiety-Like Behavior

After 24 days of SZRP administration, the effect of SZRP on anxiety-like behavior was detected by LDB test. As shown in (Figure 2), number of light side entries in model group was significantly reduced compared with control group, time in light side, number of light side entries/total of entries, and time in light side/ total time was significantly reduced ((Figure 2A-2D), $P < 0.001$), suggesting that 30mg/Kg PTZ induced anxiety-like behavior of ICR mice. Compared to the model group, time in light side, number of light side entries/total of entries and time in light side/ total time in SZRP (14.5, 28.9, 43.4 g/kg/day) ((Figure 2B-2D), $P < 0.001$) significantly increased; The number of light side entries increased significantly in SZRP (14.5 g/kg/day) ((Figure 2A), $P < 0.01$) and the SZRP (43.4 g/kg/day) ((Figure 2A), $P < 0.05$).

After 30 days of administration, EPM test showed that (Figure 2), compared with the control group, the number of open arm entries/total of arm entries in the model group ((Figure 2G), $P < 0.05$), in the number of open arm entries, time in open arm and time in open arm/ total time were significantly reduced (Figure 2E, 2F & 2H), $P < 0.01$), which suggested that, PTZ (30 mg/kg) induced anxiety-like behavior. Compared with the model group, the number of open arm entries, time in open arm and time in open arm/ total time in in SZRP (43.4 g/kg/day) were significantly increased ((Figure 2E, 2F & 2H), $P < 0.01$),

suggesting that in SZRP (43.4 g/kg/day) could improve the anxiety-like behavior of mice induced by PTZ.

After 37 days of administration, OFT was used to test the improvement effect of SZRP on anxiety-like behavior. Compared with the control group (Figure 2), the center distance in the model group was reduced ((Figure 2C), $P < 0.05$), suggesting that PTZ (30 mg/kg) established anxiety-like behavior in ICR mice. Compared with model group, the number of enter entries and center distance in in SZRP (43.4 g/kg/day) were significantly increased ((Figure 2A & 2C), $P < 0.05$).

These results suggested that in SZRP (43.4 g/kg/day) could improve the anxiety-like behavior of PTZ after 37 days of administration.

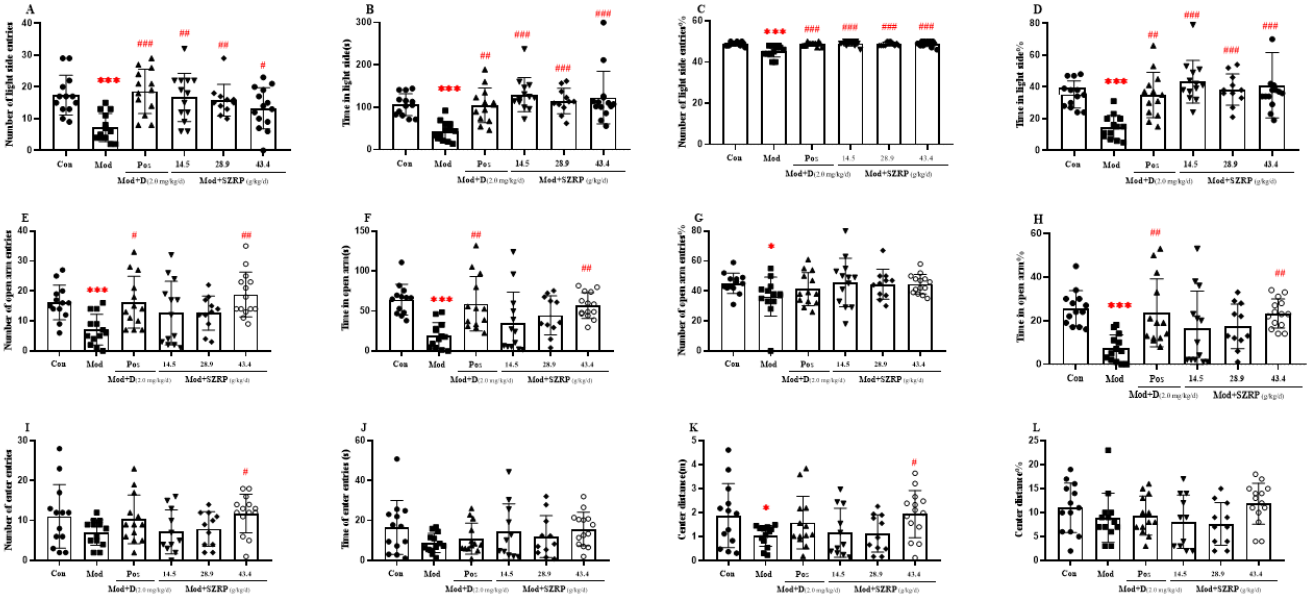

FIGURE 2: SZRP fights anxiety as a result of behavior. **A)** Number of light side entries in the LDB test. **B)** Time in light side in the LDB test. **C)** Number of light side entries% in the LDB test. **D)** Time in light side% in the LDB test. **E)** Number of open arm entries in the EPM test. **F)** Time in open arm in the EPM test. **G)** Number of open arm entries% in the EPM test. **H)** Time in open arm% in the EPM test. **I)** Number of enter entries in the OFT. **J)** Time of enter entries in the OFT. **K)** Center distance in the OFT. **L)** Center distance% in the OFT.
EPM: elevated plus maze; LDB: light dark box; OFT: open-field test; *P＜0.05, **P＜0.01, ***P＜0.001;#P<0.05, ##P<0.01, ###P<0.001. Mean±S.D., n=11-14. vs Con, Student's t-test; vs Mod, One-way ANOVA followed by Dunnett's multiple comparisons test by Graphpad 8.0.1.

3.2. Network Pharmacology

Ingredients in SZRP: 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 (Table 1). Based on TCMSP, BATMAN-TCM, ETCM, TCMGenenDIT and TCMID databases, we collected total of 545 compounds in the SZRP (Table 2). We established the herb-compound network for SZRP (Figure 3). 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.There were total 1050 potential targets in SZRP form TCMSP, BATMAN-TCM (gene score >20) and ETCM after eliminating duplicates (Table 3).

TABLE 1: Herbs in Suanzaoren prescription.

Chinese Name	Botanical Name	Common Name	Family Name
Suan Zao Ren	*Ziziphus Jujuba Var. Spinosa*	Semen Zizyphi	Rhamnus
Fu Ling	*Poria cocos Wolf*	Hoelen/Poria	Polyporaceae
Zhi Mu	*Anemarrhenae Rhizoma*	Anemarrhena	Liliaceae
Chuan Xiong	*Ligusticum chuanxiong Hort.*	Chuanxiong Rhizoma	Umbelliferae
Zhi Gan Zao	*Glycyrrhiza glabra L.*	Licorice	Licorice

TABLE 2: Number of chemical constituents in herbs of Suanzaoren prescription.

Herb	TCMSP	BATMAN-TCM	TCMGeneDIT	ETCM	TCMID	ALL	Union
Suan Zao Ren	33	37	15	36	51	172	64
Fu Ling	34	21	8	35	54	152	103
Zhi Mu	81	32	14	43	148	318	123
Chuan Xiong	189	28	81	83	181	562	292
Zhi Gan Zao	0	0	0	0	17	43*	28
Union	326	107	89	127	272		545

* These 43 compounds contained 17 compounds from TCMID, and 26 compounds from literature.

TABLE 3: The number of potential targets for herbs in Suanzaoren prescription.

Herbs	TCMSP		BATMAN-TCM	ETCM	Union
	Protein*	Gene			
Suan Zao Ren	129	119	369	537	360
Fu Ling	106	31	622	2428	637
Zhi Mu	414	292	484	757	468
Chuan Xiong	1203	923	603	452	647
Zhi Gan Zao	107	53	103	35	79
Union	305	111	847	408	1050

*There are protein targets from the TCMSP database are converted into gene targets by STRING.

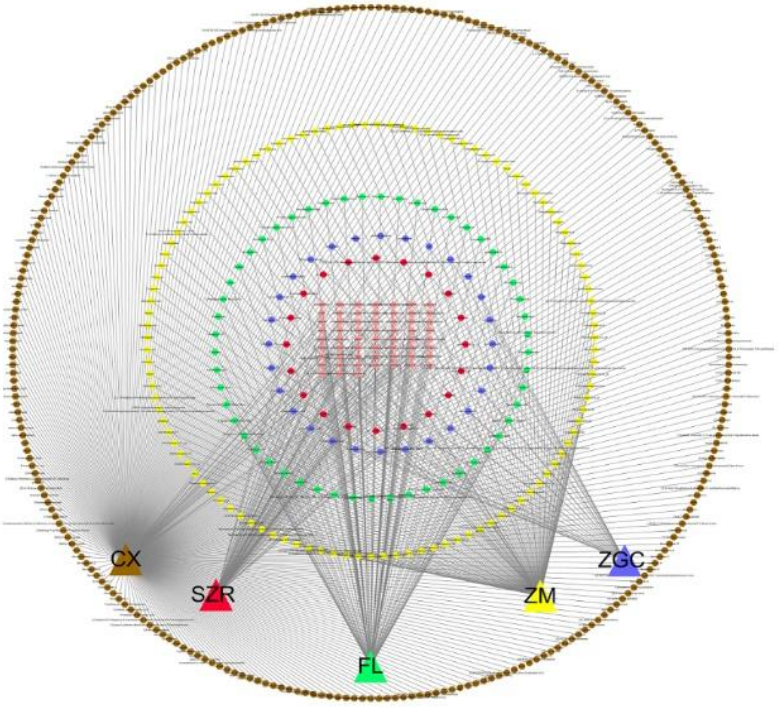

FIGURE 3: This network of compound and herb in Suanzaoren prescription. This network comprised 550 nodes and 610 edges (herb-compound). The triangle represents traditional chinese medicine and the circle represents compounds. Suanzaoren (SZR) and its compounds are shown in red, Fuling (FL) and its compounds 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.

3.2.1. Disease Enrichment Analysis Potentially Treated by SZRP

The disease enrichment analysis of 1050 potential targets of SZRP by NIMNT and MetaCore databases. 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 (Figure 4). 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 (Figure 4).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.

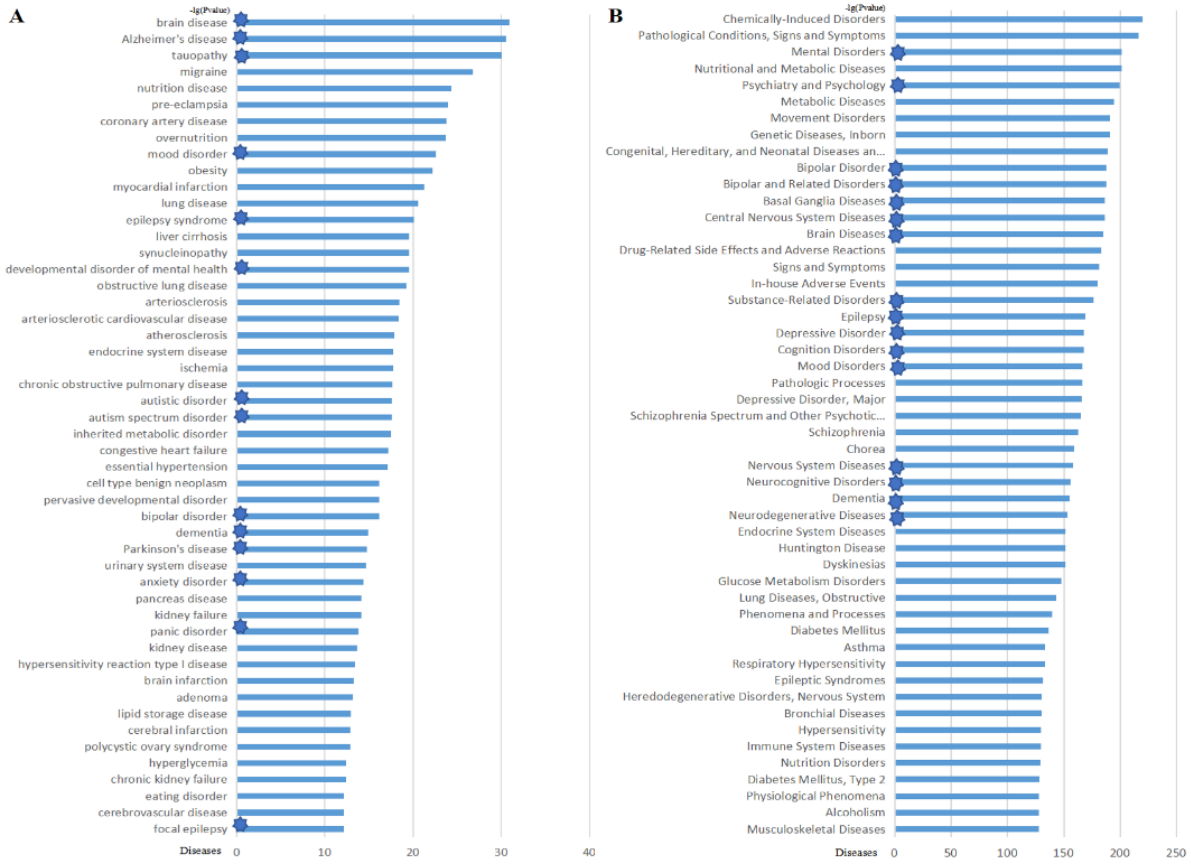

FIGURE 4: The top 50 diseases enriched for the potential targets of the Suanzaoren prescription. **A)** Diseases enrichment from NIMNT; **B)** Diseases enrichment from MetaCore; Select the top 50 for visualization. X-axis shows the -lg (P value) of the terms; Y-axis shows the different diseases (P<0.001). mean that the disease is related to nervous system or emotional disorder.

TABLE 4: The common diseases were enriched employing MetaCore and NIMNT based on potential targets of Suanzaoren prescription.

NIMNT	*P* value	MetaCore	*P* value
brain disease	1.21E-31	Brain Diseases	1.16E-185
Alzheimer's disease	3.02E-31	Dementia	6.76E-156
nutrition disease	5.03E-25	Nutritional and Metabolic Diseases	6.28E-202
mood disorder	2.36E-23	Mood Disorders	1.81E-167
lung disease	2.84E-21	Lung Diseases, Obstructive	2.03E-144
epilepsy syndrome	7.71E-21	Epileptic Syndromes	1.62E-132
obstructive lung disease	5.97E-20	Lung Diseases, Obstructive	2.03E-144
endocrine system disease	1.74E-18	Endocrine System Diseases	3.22E-152
inherited metabolic disorder	3.03E-18	Metabolic Diseases	1.91E-195
bipolar disorder	7.23E-17	Bipolar and Related Disorders	2.30E-188
		Bipolar Disorder	2.30E-188
dementia	1.18E-15	Dementia	6.76E-156

3.2.2. Targets Related to Anxiety

There were 249 targets related to anxiety (Table 5).We collected 1050 potential targets for compounds in SZRP and 249 targets associated with anxiety disorders. There were 117 share targets (Figure 5).

3.2.3. Enrichment Analysis of KEGG Pathway

MetaCore based pathway enrichment analysis results showed that the top 10 (*P* value < 0.05, arranged from small to large) (Figure 6) pathways included, tinnitus-associated changes in auditory pathway, protein folding and maturation_insulin processing, mediated direct regulation of xenobiotic metabolizing enzymes, nicotine signaling (general schema), retinol metabolism, estradiol metabolism, serotonin modulation of dopamine release in nicotine addiction. 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 (Figure 6). There were 6 pathways in total after taking the intersection of the top 30 (*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, Glutamatergic and cannabinoid signaling pathways. Notably, KEGG enriched pathways were closely related to GABAergic and cannabinoid systems.

TABLE 5: The number of potential targets related to anxiety.

Databases	Protein	Gene	Union
OMIM	67	67	67
PharmGkb		12	7
TTD	45*	45	41
CTD		53	50
Durgbank	73#	73	39
Metacore		1856	133
Union			249

The TTD and Durgbank databases being used to obtain potential protein targets for anxiety, while OMIM, PharmGkb, CTD, Metacore databases being used to obtain gene targets for anxiety.
*There were 45 therapeutic protein targets be collected for anxiety from TTD, these proteins were 41 genes mapped by STRING.
#There were 73 protein targets obtained from Durgbank for anxiety, it was 39 genes transformed using STRING.

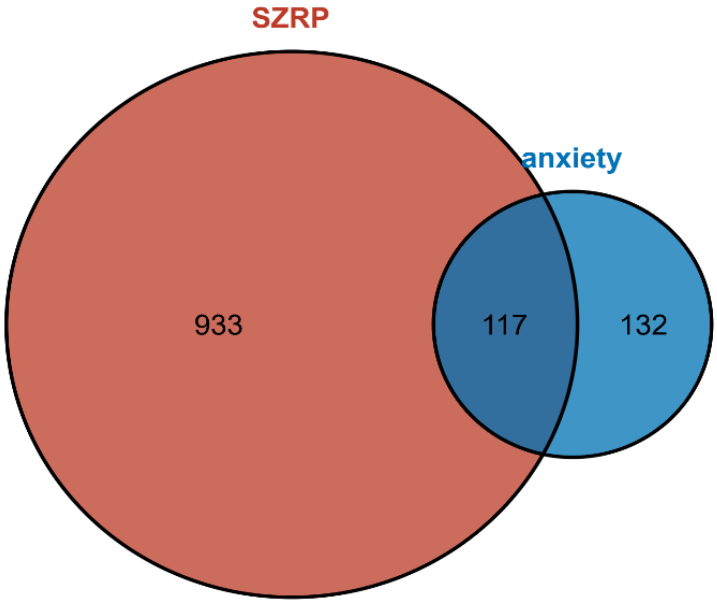

FIGURE 5: Venn diagram of chinese medicine (SZRP) and disease (anxiety) targets. The shaded areas are the intersection targets of chinese medicine and disease.

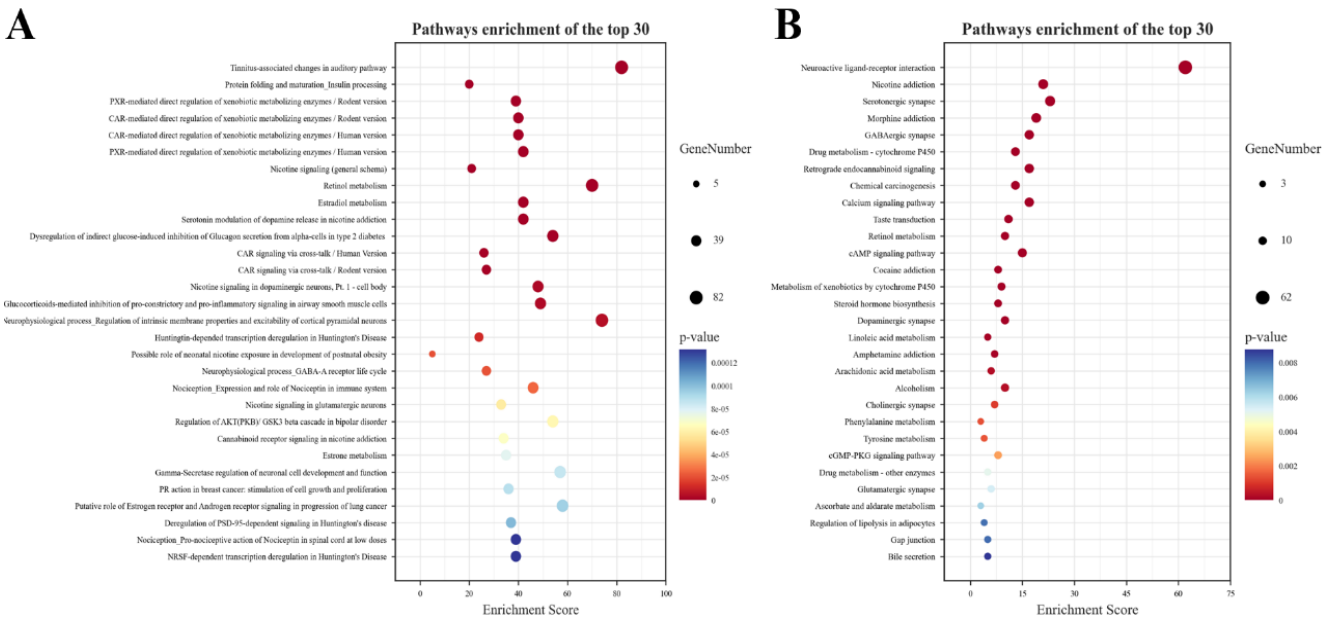

FIGURE 6: Top 30 pathways for gene target enrichment of potential target for shared target. **A)** Pathway enrichment come from Metacore, **B)** Pathway enrichment come from NIMNT. X-axis shows t Gene count, the left Y axis shows the different pathway (P<0.05).

3.3. Molecular Docking

Based on KEGG enrichment analysis results and literature, CB1 was found to be closely related to anxiety, so CB1 was selected as the receptor for molecular docking (Table 6).

There were many studies [31-33] indicated that cannabinoid receptor 1 (CB1) played the key role in the pathophysiological mechanism of anxiety. Therefore, we executed molecular docking of compounds with molecular weight < 500Da in SZRP employing AutoDock vina. Results showed there were the biggest affinity between CB1 and isomangiferin, asperglaucide (Table 6).

TABLE 6: The affinity between compounds in Suanzaoren prescription and targets with related to anxiety by molecular docking.

Target	PDB-ID	Ligand	Total energy
DRD2	6luq	Jujuboside B	-9.8
		ononin	-9.4
		Anemarsaponin B_qt	-8.8
		Anemarsaponin B	-8.7
		Jujuboside B_qt	-8.4
		Timosaponin BII	-8.3
		Timosaponin BII_qt	-8.2
		Timosaponin B III	-8
		Anemarsaponin E	-7.8
		Timosaponin B III_qt	-7.5
		Spinosin	-7.1
		Anemarsaponin E_qt	-6.7
BDNF	5mo9	Jujuboside B	-9.9
		Timosaponin B III_qt	-9.5
		Timosaponin BII_qt	-9
		Anemarsaponin B_qt	-9
		Timosaponin B III	-8.5
		ononin	-8.5
		Anemarsaponin B	-8.4
		Anemarsaponin E	-8.3
		Jujuboside B_qt	-8.3

		Timosaponin BII	-8
		Anemarsaponin E_qt	-7.9
		Spinosin	-7.7
COMT	4pyi	Jujuboside B	-10.5
		Timosaponin B III	-9.7
		Jujuboside B_qt	-9.7
		Anemarsaponin B	-8.5
		Timosaponin BII	-8.3
		ononin	-8.3
		Timosaponin B III_qt	-8.3
		Anemarsaponin E	-8.2
		Anemarsaponin B_qt	-8.2
		Anemarsaponin E_qt	-8.1
		Timosaponin BII_qt	-7.9
		Spinosin	-7.7
SLC6A4	6w2c	Jujuboside B	-10.1
		Anemarsaponin B	-9.6
		Timosaponin BII	-9.4
		Timosaponin B III	-9.2
		Jujuboside B_qt	-9.1
		Anemarsaponin E	-8.9
		ononin	-8.5
		Spinosin	-8.1
		Anemarsaponin B_qt	-7.8
		Timosaponin BII_qt	-7.6
		Timosaponin B III_qt	-6.9
		Anemarsaponin E_qt	-6.7
HTR1A	4iar	Anemarsaponin B_qt	-10.4
		ononin	-9.7
		Jujuboside B_qt	-8.7
		Jujuboside B	-8.5
		Anemarsaponin B	-8
		Timosaponin BII	-8
		Timosaponin B III	-7.9
		Spinosin	-7.1
		Anemarsaponin E_qt	-6.8
		Anemarsaponin E	-6.7
		Timosaponin BII_qt	-6.7
		Timosaponin B III_qt	-5.9
CNR1	5xr8	Isomangiferin	-9
		Liquiritigenin	-8.8
		Triterpene	-8.7
		Kaempferol	-8.5
		Anemarcoumarin A	-8.4
		Saponin	-8.4
		1-methoxy-2-methylanthraquinone	-8.3
		Liquiritin	-8.3
		5-Hydroxymethyl-6-Endo-(3'-Methoxy-4'-Hydroxyphenyl)-8-	-8.3
		Polystachoside	-8.3
	5xra	Asperglaucide	-9.3

		Isomangiferin	-9.3
		Chrysophanol	-9.2
		Triterpene	-9
		Aurantiamide acetate	-8.9
		Folic Acid	-8.8
		Folinic acid	-8.8
		Triterpene	-8.8
	5tgz	Perlolyrine	-9.1
		Anemarcoumarin A	-9.1
		(?)-caaverine	-9
		Triterpene	-9
		Isomangiferin	-8.9
		Saponin	-8.9
		Dehydroabietic Acid Methyl Ester	-8.7
		1-methoxy-2-methylanthraquinone	-8.7
		Kaempferol	-8.7
		Vallesiachotamine	-8.7
MAPK3	3she	n-methylasimilobine	-9.4
		Lysimachoside	-9.2
		(?)-caaverine	-9.2
		Saponin	-9.2
		dl-Nuciferine	-9
		Emodin	-9
		Polystachoside	-9
		4H-Benzopyran-4-one, 6-beta-D-glucopyranosyl-5-hydroxy-2-	-8.9
		5,6-dihydroergosterol	-8.9
		Ergosta-7,22E-dien-3beta-ol	-8.9

3.4. Molecular and *In Vitro* Experiments

According to the results of molecular docking, the mean absolute value of the combined score of the three subtypes of CB1 (5tgz, 5xra, 5xr8) with compounds was greater. And based on compounds reported, we selected five compounds to verified the activity of targeting on CB1 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 CB1 (CHO-K1/CB1/Gα15 cell). The positive compound was CP-55940, with a maximum concentration of 200 μM and 3-fold dilution, divided into 8 concentration points and results showed the EC_{50} of positive compound CP-55940 targeting on CB1 was 49.44 μM in this study.

The four compounds, liquiritigenin, isommangiferin, kaempferol, emodin were diluted twice, and 8 concentrations were detected, ranging from 100μM to 0.781μM. The compound chrysophanol was detected at 9 concentrations in a two-fold dilution, ranging from 200μM to 0.781μM. The EC_{50} of isomangiferin, liquiritigenin, kaempferol, chrysophanol and emodin activating on CB1 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 (Figure 7). Chrysophanol and kaempferol had the maximum effective rate of 81% and 68%.

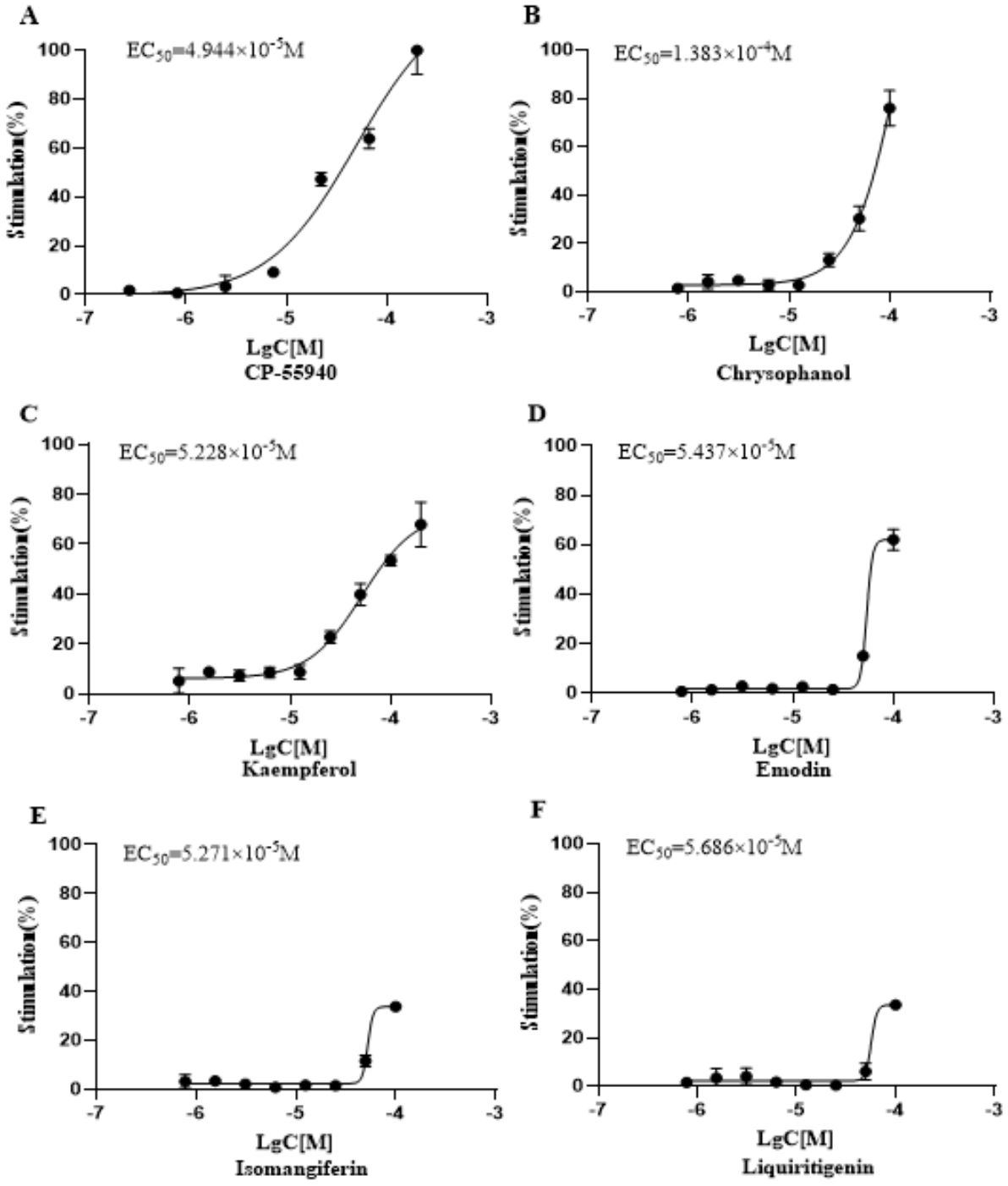

FIGURE 7: The EC50 of compound in Suanzaoren prescription against the target of Cannabinoid receptor 1. **A)** CP-55940 - CB1; **B)** Chrysophanol - CB1; **C)** Kaempferol - CB1; **D)** Emodin - CB1; **E)** Isomangiferin - CB1; **F)** Liquiritigenin - CB1. Mean±D., N=3.

3.5. The Effect of Compounds on Anxiety-Like Behavior

After one dose of administration, OFT result showed that (Figure 8), compared with the control group, total distance in chrysophanol group were significantly increased ((Figure 8), $P < 0.01$). Compared with the control group, the number of enter entries in kaempferol group were significantly increased ((Figure 8E), $P < 0.01$). These results suggest that chrysophanol and kaempferol can improve anxiety-like behavior induced by one dose of administration.

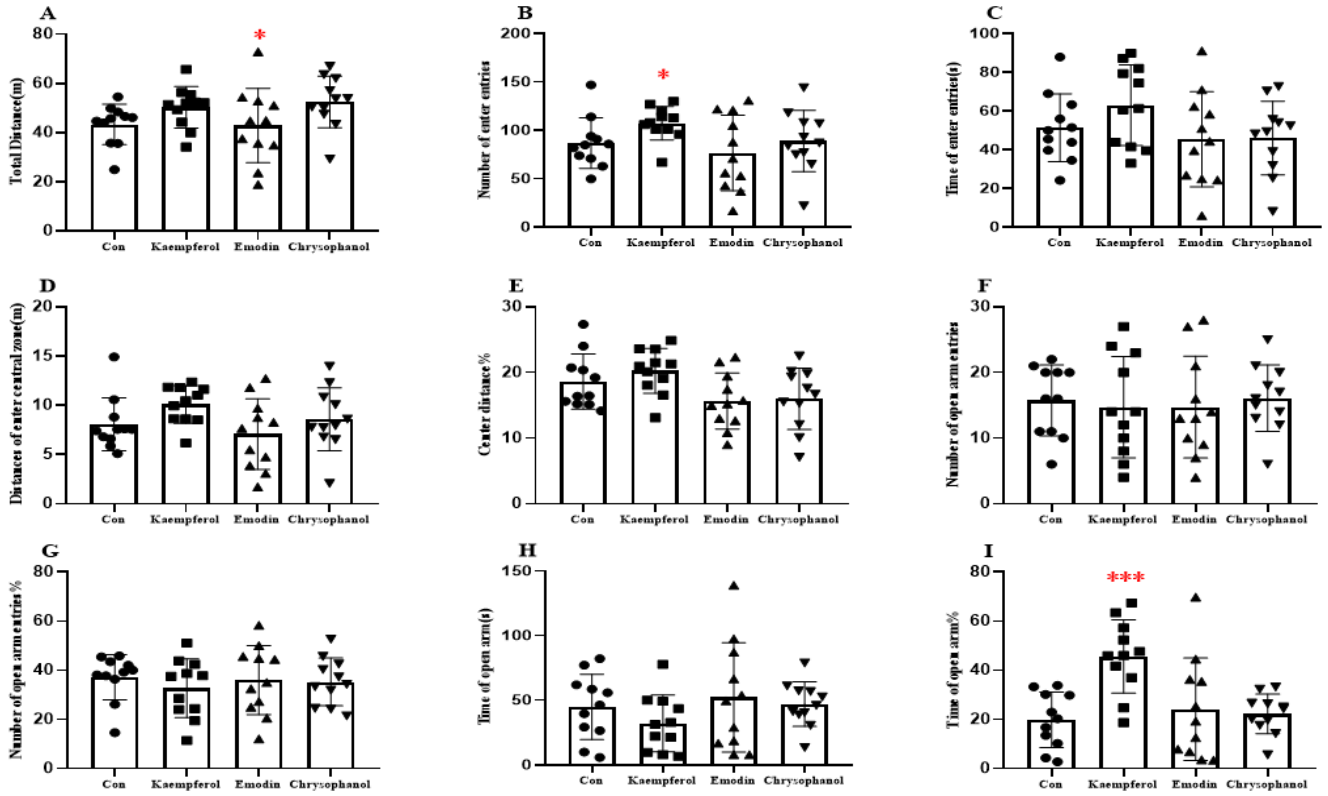

FIGURE 8: Results of the kaempferol, emodin and chrysophanol against anxiety in behavior. **A)** Total distance in the OFT. **B)** Number of enter entries in the OFT. **C)** Time of enter entries in the OFT. **D)** Center distance in the OFT. **E)** Center distance% in the OFT. **F)** Number of open arm entries in the EPM. **G)** Number of open arm entries% in the EPM. **H)** Time in open arm% in the EPM test. **I)** Time in open arm% in the EPM test.
EPM: elevated plus maze; LDB: light dark box; OFT: open-field test; *P＜0.05, ***P＜0.001. Mean±S.D., n=11-14. vs Con, Student's t-test by Graphpad 8.0.1.

After four dose of administration, EPM test was used to detect the improvement effect of compounds on anxiety-like behavior. Compared with the control group (Figure 8), time in open arm% in the kaempferol group was increased ((Figure 8C), $P < 0.001$), These results suggested that the kaempferol group could improve the anxiety-like behavior of administration.

4. Discussion

In this study, EPM test, LDB test and OFT results showed that PTZ (30 mg/kg) could stably establish the anxiety-like behavior model of ICR male mice. The SZRP (43.4 g/kg) group improved the anxiety-like behavior established by PTZ more significantly. 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 anxiogenic may be via regulating membrane potential and synaptic transmission, etc., which involve in 117 biomolecules. Especially, we found that Chrysophanol, Kaempferol and Emodin activated CB1 with EC_{50} 1.383×10^{-4} M, 5.228×10^{-5} M and 5.437×10^{-5} M respectively. And Chrysophanol and Kaempferol has excellent anti-anxiety effect.

In this study, 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.

In addition, the study showed that exploration was increased when CB1 receptor was deleted in cortical and striatal GABAergic neurons, however, deletion of CB1 receptor from cortical glutamatergic neurons caused a decreased exploration in these task [34]. And in mice lacking the CB1 receptor either in cortical glutamatergic or in GABAergic neurons, CB1 receptors expressed in cortical glutamatergic neurons favors novelty seeking, whereas CB1-dependent control of inhibitory GABAergic neurons favors behavioral inhibition by novelty-induced behavioral inhibition test [35].

Thus, CB1 on cortical glutamatergic and GABAergic neurons exert opposing on anxiety-like behavior, but this function seems to operate within specific limits of neuronal activity [36]. Numerous pharmacological studies support the view of bidirectional regulation of anxiety-like behavior by CB1 [37, 38]. Interestingly, experimental studies have shown that anti-anxiolytic-like effects in the low dose of cannabinoids via the CB1 receptor on cortical glutamatergic terminals, on the contrary, the CB1 receptor on the GABAergic terminals induce an anxiogenic-like effect under a high-dose treatment [39]. Experimental studies have also found, while a mild activation of CB1 receptors in the prefrontal cortex ventral hippocampus attenuates anxiety and a slight CB1 receptor stimulation in the amygdala results in an anxiogenic-like response [40]. The results of enrichment of potential pathways were consistent with the experimental verification.

Chrysophanol belonged to anthraquinones and was yellow powder and soluble in methanol and ethanol. It has anti-inflammation, anti-cancer and offers neuroprotection [41-43]. In a study, 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-1β and reactive oxygen species (ROS), increasing neurotransmitter (NE, DA, 5-HT) concentrations and BDNF expression [44].

Flavonoids Kaempferol was yellow powder and slightly soluble in water, soluble in hot ethanol, ether or alkali solution. It has anti-inflammation and anti-oxidative [45, 46]. Some studies indicated Kaempferol had effects on anxiety. Kaempferol can enhanced number of entries and time spent in the open arm inEPM test, which demonstrated anxiolytic activity [47]. 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 [48]. 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-HT1A (WAY 1006355) [49]. Using light/dark model and hole-board experiment to evaluated whether Kaempferol has anxiogenic 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 [50].

Anthraquinones Emodin was yellow powder and soluble in ethanol, slightly soluble in ether, chloroform, benzene, insoluble in water. It has the role of anti-inflammatory [51-53], neuroprotective [54] and cardiovascular protective [55, 56]. No reports were found about Emodin effecting on anxiety. In this study, we found Chrysophanol, Kaempferol and Emodin in SZRP activated CB1 with EC_{50} 138.3, 52.28 and 54.37μM respectively.

Of course, in our previous literature study, we summarized SZRP anti-anxiety in clinical and experimental studies. We found that SZRP combined with other drugs or traditional Chinese medicine acupuncture or acupoint application and other methods for the treatment of anxiety and insomnia, cardiac intervention, myocardial infarction, cardiovascular neurosis, cancer, hypertension, type 2 diabetes and other diseases associated with anxiety, SZRP combined with other drugs or methods can significantly reduce patients with Hamilton Anxiety Scale (HAMA), patients with self-rating anxiety scale (SAS) and other anxiety scores, improve anxiety symptoms of significant efficacy and fewer side effects. And Experimental pharmacological studies showed that SZRP could regulate the levels of brain hormones and neurotransmitters such as DA, HVA, NE, GABA, 5-HT, 5-HIAA, Glu, β-EP and so on. By decreasing the levels of inflammatory cytokines such as IL-1β and TNF-α, enhancing the proliferation of B lymphocytes, the phagocytic function of peritoneal macrophages and down-regulating the proliferation of T lymphocytes to regulate immune function; By increasing the levels of ACTH and CORT and GR expression, endocrine level can be regulated, and then the homeostasis of nervous system, endocrine system and immune system can be regulated to prevent and treat anxiety [57]. In addition, our results showed that SZRP contained 145 compounds , including flavonoids, triterpenoids, steroids, coumarins, phthalides, and volatile oils, etc., while five herbs contain 1104 compounds. And there were 80 common compounds in SZRP and its five herbs, which accounted for 6.8% of total compounds in all 5 herbs and 55.2% of compounds in SZRP [58].

The anxiogenic effect of SZRP has been reported in literature [59]. In this paper, main chemical compounds and potential targets of SZRP were collected from TCMSP, and potential targets of disease were obtained from Gene Cards Database. DAVID database was used for pathway enrichment analysis, and active compounds for molecular docking. In our study, we obtained more comprehensive chemical composition from TCMSP, BATMAN-TCM, ETCM, TCMGeneDIT and TCMID databases, and obtained as many potential compound targets as possible from TCMSP, BATMAN-TCM and ETCM databases. More disease targets were obtained from TTD, OMIM, PharmGKB, CTD, DrugBank and Metacore databases. In addition, we use two databases to increase the reliability of the pathway enrichment analysis, especially the Metacore database with high reliability. In terms of molecular docking, we screened the active ingredients for docking through preliminary experimental studies, include Liquiritigenin, Isomangiferin, Kaempferol, Emodin and Chrysophanol. There were evaluated based on molecular docking results and literature studies. In addition, Kaempferol and Chrysophanol have been found to have anti-anxiety effects in experimental studies. Furthermore, I think our study is more comprehensive and depth.

5. Conclusion

In this study, the therapeutic compounds and mechanism of SZRP for anxiety disorders were investigated by using network pharmacology combined with experimental verification. The main findings were as follows: i) SZRP (43.4 g/kg) improved the anxiety-like behavior established by PTZ more significantly. ii) 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. iii) The 249 potential targets of anxiety were obtained, and 117 possible targets of SZRP against anxiety were found. iv) The 117 potential targets played roles in many pathways, such as GABAergic and cannabinoid system. v) Molecular docking and cell experiments for validating binding affinities of compounds and targets, Chrysophanol, Kaempferol and Emodin have activation effects on CB1 with EC_{50} 138.3μM, 52.28μM and 54.37μM respectively. More importantly, the anxiolytic effects of Kaempferol and Chrysophanol were found in the short term.

However, there are limitations to this study. The ingredients in SZRP and anxiety-related genes may not be global or full by network pharmacology. We have not yet verified the specific anxiogenic mechanism of SZRP, Kaempferol and Chrysophanol. At present, few reports have confirmed the anti-anxiety effects of Kaempferol and Chrysophanol. We will conduct further studies based on the available data in the future. In conclusion, this study provided theoretical basis and clues on the pharmacological mechanism of SZRP, Kaempferol and Chrysophanol against anxiety. It provides certain reference significance for the development of new drugs for anxiety

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article Supplementary Material.

Author Contributions

Xiaohong Bao and Tianyuan Ye provided research materials and statistics. Lu Han and Tongxing Wang provided the article design and data analysis. Xiaorui Cheng, Dongmei Qi and Xin Wang provided administrative support and article design. All the authors contributed to the manuscript writing and final review.

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Data Availability

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

None.

Ethics approval

All animal experimental operations were approved by Shandong University of Traditional Chinese Medicine.

Supplementary Materials

Supplementary material is available on the publisher's website along with the published article.

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