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Original Article

Effects of Xiaoyao San on exercise capacity and liver mitochondrial metabolomics in rat depression model

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ABSTRACT

Objective: This study aimed to investigate the therapeutic effects of Xiaoyao San (XYS), a herbal medicine formula, on exercise capacity and liver mitochondrial metabolomics in a rat model of depression induced by chronic unpredictable mild stress (CUMS).

Methods: A total of 24 male SD rats were randomly divided into four groups: control group (C), CUMS control group (M), Venlafaxine positive treatment group (V), and XYS treatment group (X). Depressive behaviour and exercise capacity of rats were assessed by body weight, sugar-water preference test, open field test, pole test, and rotarod test. The liver mitochondria metabolomics were analyzed by using liquid chromatography-mass spectrometry (LC-MS) method. TCMSP database and GeneCards database were used to screen XYS for potential targets for depression, and GO and KEGG enrichment analyses were performed.

Results: Compared with C group, rats in M group showed significantly lower body weight, sugar water preference rate, number of crossing and rearing in the open field test, climbing down time in the pole test, and retention time on the rotarod test ($P < 0.01$). The above behaviors and exercise capacity indices were significantly modulated in rats in V and X groups compared with M group ($P < 0.05, 0.01$). Compared with C group, a total of 18 different metabolites were changed in the liver mitochondria of rats in M group. Nine different metabolites and six metabolic pathways were regulated in the liver mitochondria of rats in X group compared with M group. The results of network pharmacology showed that 88 intersecting targets for depression and XYS were obtained, among which 15 key targets such as IL-1β, IL-6, and TNF were predicted to be the main differential targets for the treatment of depression. Additionally, a total of 1 553 GO signaling pathways and 181 KEGG signaling pathways were identified, and the main biological pathways were AGE-RAGE signaling pathway, HIF-1 signaling pathway, and calcium signaling pathway.

Conclusion: XYS treatment could improve depressive symptoms, enhance exercise capacity, positively regulate the changes of mitochondrial metabolites and improve energy metabolism in the liver of depressed rats. These findings suggest that XYS exerts antidepressant effects through multi-target and multi-pathway.

1. Introduction

Depression is a common psychiatric disorder with hypokinetic symptoms such as reduced activity and increased tiredness and fatiguability (Wang et al., 2022). Depression is predicted to become the leading cause of the global burden of disease by 2030, and its prevention and treatment are of great importance (Dwyer et al., 2020). The pathogenesis of depression is complex due to a variety of factors, and several hypotheses have been proposed in the literature, including the monoamine transmitter hypothesis, the neuroendocrine hypothesis, and the neuroinflammatory hypothesis (Gu et al., 2021). Recently, the hypothesis of mitochondrial energy metabolism dysfunction has received increasing attention from researchers (Xie et al., 2020). The liver is the center of the body's substrates and energy metabolism, however, the relationship

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between the development of depression and the liver energy metabolism disorder, and the targets of treatments and their underlying mechanisms are still to be elucidated (Liu et al., 2021).

Exercise capacity is one of the key indicators of health-related fitness. A study found that individuals with depression spent more time on sedentary lifestyle and had lower exercise capacity (Kitagaki, Murata, Tsuboi, Isa, & Ono, 2020). There are several possible explanations for the association between exercise capacity and depression, and one of them is sedentary behavior (Santos et al., 2012). Daily sedentary time might be associated with depressive symptoms and exercise capacity. Another explanation is anxiety. One study suggested that a medium and high level of estimated exercise capacity during late middle-age was associated with lower odds of depression with anxiety (Shigdel, Stubbs, Sui, & Ernstsen, 2019).

Xiaoyao San (XYS) is a traditional Chinese herbal medicine formula that has been prescribed for treatment of depression (Wang et al., 2023). The formula consists of *Bupleuri Radix* (Chaihu in Chinese), *Angelicae Sinensis Radix* (Danggui in Chinese), *Paeoniae Radix Alba* (Baishao in Chinese), *Atractylodis Macrocephalae Rhizoma* (Baizhu in Chinese), *Poria* (Fuling in Chinese), *Glycyrrhizae Radix* et *Rhizoma* (Gancao in Chinese), *Menthae Haplocalycis Herba* (Bohe in Chinese), and *Zingiberis Rhizoma Recens* (Shengjiang in Chinese) (Wu et al., 2023). Both experimental studies and clinical applications have shown evidence that XYS is effective in ameliorating depression symptoms (Man, Li, Gong, Xu, & Fan, 2014; Chen et al., 2020; Zeng et al., 2022). It has been reported that treatment with XYS can result in an anti-depressant effect by regulating the expression of connexin 43 (Cx43), glucocorticoid receptor (GR), and brain-derived neurotrophic factor (BDNF) (Zhang et al., 2022). Venlafaxine (VLF) is a serotonin and norepinephrine reuptake inhibitor that is widely used in the treatment of psychiatric disorders, and can significantly ameliorate the clinical symptoms of depressed patients. The antidepressant effect of VLF has been extensively studied based on the "neurotransmitter hypothesis". Our group found that treatment with Venlafaxine improved mitochondrial morphology and respiratory chain complex activity in skeletal muscle of chronic unpredictable mild stress (CUMS) rats (Ji et al., 2022). However, studies on the effects of XYS on the regulation of liver mitochondrial energy metabolism in depressed states are scarce, and whether XYS can improve exercise capacity in depressed animals has not been reported. Therefore, in this experiment, VLF was used as a positive control intervention to investigate the efficacy and the underlying mechanism for an XYS intervention.

The mitochondrion is the organelle where the oxidative phosphorylation occurs. As one of the most abundant organs in terms of mitochondria, the liver plays an important role in regulating energy metabolism. It has been speculated that the hypokinetic symptoms of depression are likely to be closely related to mitochondrial energy metabolism dysfunction (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Karabatsiakis et al., 2014). Therefore, revealing the changes in mitochondrial metabolism in the liver of depressed animals is an important prerequisite for further elucidating the antidepressant efficacy of XYS.

Mitochondrial metabolomics is the latest technology in the search for elusive and desirable biomarkers or drug targets for mitochondrial diseases, and can be used to analyze the numerous downstream effects of mitochondrial dysfunction, including the metabolic effects of molecular events such as energy deficiency. Xu et al. used a mitochondrial metabolomics approach and found that mitochondrial energy metabolism probably played a crucial role in chronic atrophic gastritis development and *Astragali Radix* treatment.

Network pharmacology is a multidisciplinary approach based on the integration of biology, pharmacology, bioinformatics, and computer science (Dong et al., 2021). Unlike traditional single-component, single-target way of investigation, network pharmacology has great potential in elucidating the mechanism of multi-component drugs (Liu et al., 2021). For example, using the network pharmacology and molecular docking, Suanzaoren Decoction was found to play an important role in the treatment of Parkinson's disease with sleep disorders through a "multi-component, multi-target, multi-pathway" approach (Liu et al., 2021). It was reported that XYS exerted anti-depression effects through paeoniflorin, quercetin, licochalcone A, and other components acting on mitogen-activated protein kinase 1 (MAPK1), signal transducer and activator of transcription 3 (STAT3), IL-6, and transcription factor AP-1 (JUN) according to the metabolomic and network pharmacology results (Liu et al., 2021; Lin et al., 2021).

Therefore, in this study, we focused on the liver, an important metabolic organ, to explore the effect of XYS on exercise capacity and liver mitochondrial metabolomics in depressed rats at the subcellular organelle level. It was also combined with a network pharmacology approach to provide a new research strategy for the pharmacokinetic mechanism of XYS in the treatment of depression.

2. Materials and methods

2.1. Experimental animals

Male SPF-rated Sprague-Dawley rats (180–200 g, 8 weeks old), were obtained from Beijing Vital River Laboratory Animal Technology Co. [Animal license number: SCXK (Beijing) 2021–0006]. The animals were normally housed in a 12/12-h light–dark cycle in a room with 25 °C and 40 %–60 % of humidity. Experiments were carried out after one week of environmental adaptation. This experiment was approved by the Animal Experiment Ethics Committee of Shanxi University (SXULL2021039).

2.2. Medicines and main reagents

The XYS was made in the laboratory in proportion to the following quantity that consisted of *Bupleuri Radix* (30 g), *Paeoniae Radix Alba* (30 g), *Angelicae Sinensis Radix* (30 g), *Atractylodis Macrocephalae Rhizoma* (30 g), *Glycyrrhizae Radix* et *Rhizoma*, *Poria* (30 g), *Menthae Haplocalycis Herba* (10 g), and *Zingiberis Rhizoma Recens* (10 g). All the above herbs were purchased from Shanxi Huayang Pharmaceutical Company (Taiyuan, China) and authenticated as genuine. For positive control, Venlafaxine Hydrochloride Capsules (Batch No. 17103) were purchased from Chengdu Kanghong Pharmaceutical Group Co., Ltd. (Chengdu, China). Mitochondrial Isolation Kit (C3606) was purchased from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China). Acetonitrile and formic acid were purchased from Thermo Fisher Scientific (Shanghai, China).

2.3. Preparation of XYS extracts

The aqueous extract was prepared according to the method established by the research group (Zhao et al., 2023). All the herbs in the above-described XYS recipe were soaked for 40 min in 10-fold (volume to mass ratio) amount of distilled water, followed by decoction for 2 h. After collecting the filtrates, water was added to the residue (1:8, mass to volume ratio), and the mixture was extracted with boiling water for another 1.5 h. All filtrate was mixed and condensed to 2.12 g/mL (crude drug).

2.4. Grouping and CUMS model replication

SD male rats were adaptively fed for 7 d. The rats were randomly divided into four groups: control group (C, $n = 6$), CUMS control group (M, $n = 6$), Venlafaxine positive treatment group (V, $n = 6$), and XYS treatment group (X, $n = 6$). The dose of Venlafaxine was 35 mg/kg, and the doses of XYS was 2.12 g/mL (crude drug), which were based on the effective dose reported in previous research (Wu et al., 2023). The above drugs were dissolved in distilled water, and the rats were given intragastrically once a day at a dose of 10 mL/(kg·d) for 28 consecutive days.

The CUMS depression model was established based on a reported method (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). The CUMS procedure was described in the supplementary material. After one week of adaptation, the CUMS procedure was administered for 28 d. The experimental flow chart was shown in Fig. S1.

2.5. Behavior tests

2.5.1. Open field test

The rats were placed in a custom-made cage (100 cm × 100 cm × 40 cm) with black metal walls. The bottom of the cage was divided into 25 equal squares marked by white lines. The test was conducted in a quiet environment, with each rat gently placed in the central square of the cage. The rats were first allowed to acclimatize for 1 min and then the number of crossings to different squares and the number of rearing in the open field were recorded for the next 4 min (Wu et al, 2023).

2.5.2. Sugar water preference test

Two bottles of 1 % sugar solution were made available in the normal housing cage during the acclimatization week. In the formal experiment, each rat was placed individually in the cage and two bottles are prepared, one containing 1% sugar solution and the other containing purified water. The sugar solution bottle was placed on one side of the cage, and the purified water bottle on the opposite side. Sugar and water consumption (g) were measured after 12 h. The sugar preference was calculated using the following equation: sugar water preference (%) = sugar water consumption (mL)/total water consumption (mL) × 100. The total water consumption was the sum of the consumption from both bottles.

2.5.3. Pole test

The pole was 100 cm high and had a diameter of 2.5 cm. The tip of the pole was fitted with a small ball, which was wrapped with adhesive tape to ensure friction. The rat was placed head up on the top of the pole and allowed to climb downwards. The time of the rats' crawling down each time was recorded. Before the formal test, each rat was trained to climb down the pole (Ji et al, 2023). Three trials were performed by each rat and the average time was used in the statistical analysis.

2.5.4. Rotarod test

The speed and time were set for the rod rotator. All experimental rats were first adapted at a lower speed (5 r/min). For the formal test, the rat was placed on the spinning rod at 13 r/min and the time that the rat could hold on the rod at this speed was recorded (Gao, Cao, Du, & Qin, 2022). Three trials were performed by each rat and the average time was used in the statistical analysis.

2.6. Sample collection

All groups of rats were anaesthetized by intraperitoneal injection using 10 % chloral hydrate. Liver tissue samples were obtained by dissecting on ice and placed in a 1.5 mL vial in liquid nitrogen and stored at −80 °C before analysis. Liver mitochondria were extracted according to the instructions of the Tissue Mitochondrial Extraction Kit (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) and kept in −80 ℃ before analysis.

2.7. LC-MS analysis

2.7.1. Sample preparation

The extraction method for mitochondrial metabolomics analysis was according to a previous method, as described below. The mitochondrial precipitate was added to 1 mL of pre-cooled methanol, blended well, vortexed for 60 s, placed on ice, sonicated for 5 min, and centrifuged at 4 °C and 15 000*g* for 10 min. The supernatant was collected, and concentrated, dried, then re-dissolved in 80 μL of methanol before analysis.

2.7.2. LC-MS test conditions

The column used in UPLC was a SeQuant ZIC-cHILIC column (2.1 mm × 150 mm, 3 μm, Merck, USA) with mobile phase A was an aqueous solution containing 0.1% formic acid; and B was acetonitrile. The elution program was set as 0–8 min, 95%–85% B; 8–22 min, 85%–60% B; 22–25 min, 60%–95% B; 25–35 min, 95% B; with the column temperature 30 °C, injection volume 10 μL, and flow rate 0.25 mL/min.

Mass spectrometry was set to ESI ionization mode, with detection in negative ion mode. The parameters were set to scanning mode: Full Scan /dd-MS2; the acquisition range was 80–1200 m/z; the spray voltage was 3.5 kV for positive and 2.5 kV for negative; the sheath gas flow rate was 35 arb; the auxiliary gas flow rate was 10 arb; the capillary temperature was 320 °C; and the resolution: MS Full Scan 35 000 FWHM, and MS/MS 17 500 FWHM.

2.7.3. LC-MS pattern analysis

The liver mitochondrial profiles were imported into the Compound Discoverer 3.1 software with the following parameters: mass range of 80–1200 Da, mass deviation of 15×10^{-6}, RT tolerance of 0.5, and S/N threshold of 1.5. The peak data obtained were normalized for the peak area and then the normalized data were imported into SIMCA-P 14.1 for PCA, PLS-DA, and OPLS-DA analysis. Finally, the S-plot plot was combined with *VIP* > 1.0 and an independent samples *t*-test was used to screen for significantly altered differential metabolites between the groups.

The identified biomarker data were imported into MetaboAnalyst 5.0 for pathway enrichment analysis. The metabolic pathways involved in the differential metabolites were then analyzed with reference to the online databases and relevant literature.

2.8. Network pharmacology analysis

2.8.1. Chemical composition and target screening of XYS

The eight herbs, namely *Bupleuri Radix*, *Paeoniae Radix Alba*, *Angelicae Sinensis Radix*, *Atractylodis Macrocephalae Rhizoma*, *Glycyrrhizae Radix* et *Rhizoma*, *Poria*, Menthae *Haplocalycis* Herba, and *Zingiberis Rhizoma Recens*, were entered into the TCMIP database. The screening conditions of oral bioavailability (OB) >30% and drug similarity (DL) >0.18 were used to explore the chemical composition and corresponding targets of the eight herbs in the XYS recipe.

2.8.2. Depression target collection

The potential targets associated with depression were identified through the GeneCards database. The database information was integrated by searching with the keyword "depression", and the human-derived (Homo Sapiens) target proteins were selected after removing duplicate targets.

2.8.3. Protein-protein interaction network (PPI) analysis

The targets corresponding to the chemical components in XYS were compared with those related to depression, and the intersecting targets were screened as potential targets of action of XYS in the treatment of depression. The potential targets were entered into the STRING platform for PPI analysis, with the species selected as "Homo sapiens", the interaction threshold was set to 0.7, and the remaining parameters left unchanged by default. The data was imported into Cytoscape 3.7.2 software to construct a PPI network map of the target sites. Core targets were also screened by network degree value (Degree $\geq$ 2 times was median), betweenness centrality (BC $\geq$ 1 times was median) and mediated centrality (CC $\geq$ 1 times was median).

2.8.4. GO and KEGG functional enrichment analysis

Metascape, an online analysis database, was used to perform GO gene functional analysis and KEGG pathway enrichment analysis on the depression targets of XYS. A threshold value of $P < 0.01$ was set to obtain the biological processes, molecular functions, cellular components, and related pathways of depression treated with XYS, and the top enrichment results were selected for visual analysis based on *P* values. This will reveal the molecular mechanism involved in its treatment.

2.9. Statistical analysis

Weight test using two-way repeated-measures ANOVA. Other data from behavioral tests were analyzed using one-way ANOVA. Differential metabolites were assessed by an independent *t*-test (two-tailed). Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA-P multivariate statistical analysis software. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant and highly significant, respectively.

3. Results

3.1. Changes in body weight and behavioral indicators

On day 0, there were no significant differences in body weight, sugar water preference, number of crossing, and rearing in the open field test between the groups. On day 28, all of the above behavioral indicators changed significantly in the M group. As shown in Fig. 1, the M group gained weight in significantly less amount compared to the C group ($P < 0.01$); The scores for the sugar water preference rate, and the number of crossing and rearing in the open field test were also significantly lower ($P < 0.01$). After the intervention, the rats in the V and X groups showed a significantly higher body weight and the number of crossing in the open field test compared with the M group ($P < 0.01$), as well as a higher score in the sugar water preference rate and the number of rearing in the open field test ($P < 0.05$).

3.2. Changes in exercise capacity of rats in each group

The exercise capacity of the rats was assessed by evaluating the score in the pole climbing test and the holding time in the rotarod test. After 28 d of modelling, the climbing down time in the pole test and the holding time in the rotarod test were significantly lower in the M group compared with that in the C group ($P < 0.01$). Compared with M group, the climbing down time in the pole test was higher in the V and X groups ($P < 0.01$, 0.05, respectively), and the holding time in the rotarod test was longer ($P < 0.05$), as shown in Fig. 2.

3.3. Changes in mitochondrial metabolites in liver

3.3.1. Mitochondrial metabolic profile analysis

The mitochondrial metabolite profiles were subjected to analysis, and PLS-DA was used to describe the major metabolic profiles of each group, and scatter plots and replacement test plots of PLS-DA score were obtained (Fig. 3A and B). As can be seen from the PLS-DA score plot, the C group was separated from M group, indicating that mitochondrial metabolism in rats was significantly altered after the four weeks of CUMS molding. The V and X groups were significantly separated from M group and tended towards C group, indicating that administration of VLF and XYS had a significant modulatory effect on the mitochondrial metabolic mechanism in the liver of the depressed rats. To test whether there is overfitting in the PLS-DA model, the PLS-DA permutation test was used and the intercept of the regression line of Q2 on the vertical coordinate was found to be less than 0, indicating that there was no overfitting and the PLS-DA model was reliable.

The model was further optimized using OPLS-DA. As can be seen from Fig. 3C, the M group was significantly separated from the C group. Potential biomarkers closely associated with depression were screened by *VIP* > 1.0 in the S-plot (Fig. 3D) and $P < 0.05$ in the One-way ANOVA analysis. Then, the OPLS-DA analysis was performed on the M Group and X group to screen for differential metabolites associated with improvement in depression with XYS. As shown in Fig. 3E and F, there was a separation of the mitochondrial metabolic profile between the M and X groups. It was also combined with *VIP* > 1.0 in the S-plot and $P < 0.05$ in the One-way ANOVA analysis to screen for metabolites that were regulated back by XYS.

3.3.2. Identification of differential metabolites in liver mitochondria

The differential metabolites related to depression and XYS intervention were identified based on the mass-to-charge ratio, retention time, molecular formula, and online databases. Compared with the C group, 18 biomarkers associated with depression were found in the M group, including lower levels of malic acid, taurine, glutamic acid, citric acid, succinic acid, glycerol 3-phosphate, adenine and glutamine, and higher levels of aspartic acid, leucine, lactic acid, xanthine, hypoxanthine, alanine, uracil, cytidine, phenylalanine and glutathione. After 28 d of intervention, nine differential metabolites were significantly back-regulated in the X group. Compared with the M group, the levels of aspartic acid, lactic acid, leucine, cytidine, alanine, phenylalanine, and glutathione were lower and malic acid and citric acid were higher in the X group, as shown in Table S1. The changes in the relative peak area levels of these differential metabolites in each group were shown in Fig. 4.

3.3.3. Metabolic pathway analysis of differential metabolites in liver mitochondria

Metabolic pathway enrichment analysis allows for a better understanding of the intrinsic linkages between metabolites. The 18 screened differential metabolites associated with depression, as well as those that were back-regulated after XYS administration, were imported into the Metaboanalyst 5.0 database for metabolic pathway analysis. A total of 15 were found to be metabolic pathways significantly associated with depression using the Impact > 0 as the criterion, as shown in Fig. 5a, including alanine, aspartate, and glutamate metabolism; *D*-glutamine and *D*-glutamate, aspartate, and glutamate metabolism; *D*-glutamine and *D*-glutamate metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; taurine and hypotaurine metabolism; phenylalanine metabolism; glutathione metabolism; citrate cycle (TCA cycle); arginine biosynthesis; arginine and proline metabolism;

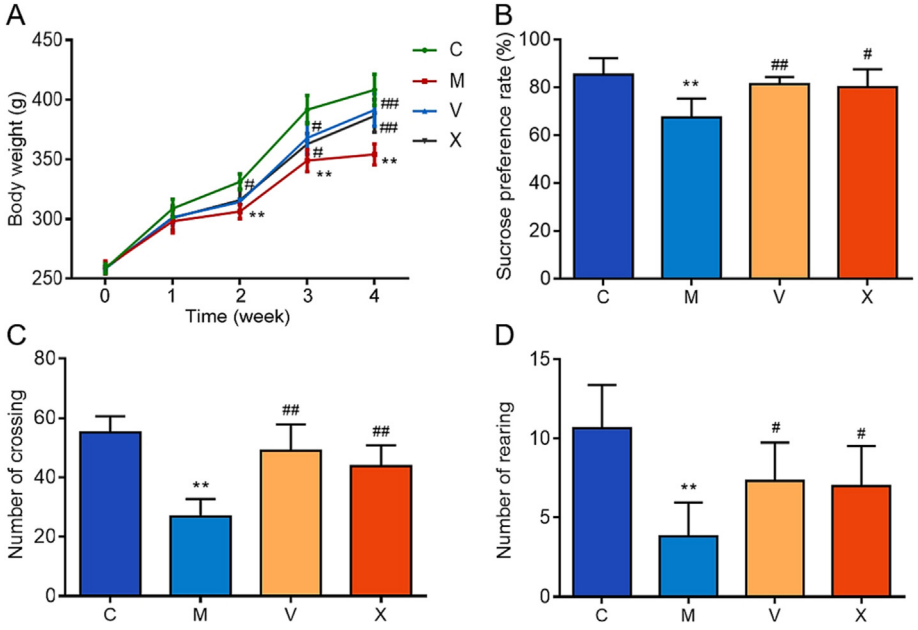

Fig. 1. Behavioral changes in response to 28-day intervention. (A) Body weight measured weekly; (B) Sugar water preference rate measured at end of intervention period; (C) Number of crossing in open field test measured at end of intervention period; (D) Number of rearing in open field test measured at end of intervention period. $^{**}P < 0.01$ *vs* C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* M group.

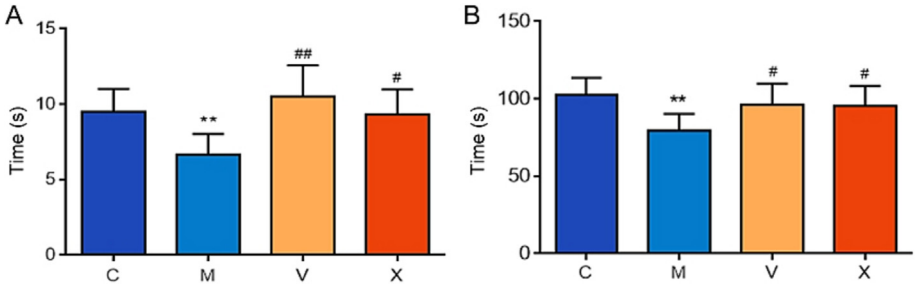

Fig. 2. Changes in exercise capacity assessed at the end of intervention period. (A) Climbing down time in pole test; (B) Holding time in rotarod test. $^{**}P < 0.01$ *vs* C group; $^{*}P < 0.05$, $^{\#\#}P < 0.01$ *vs* M group.

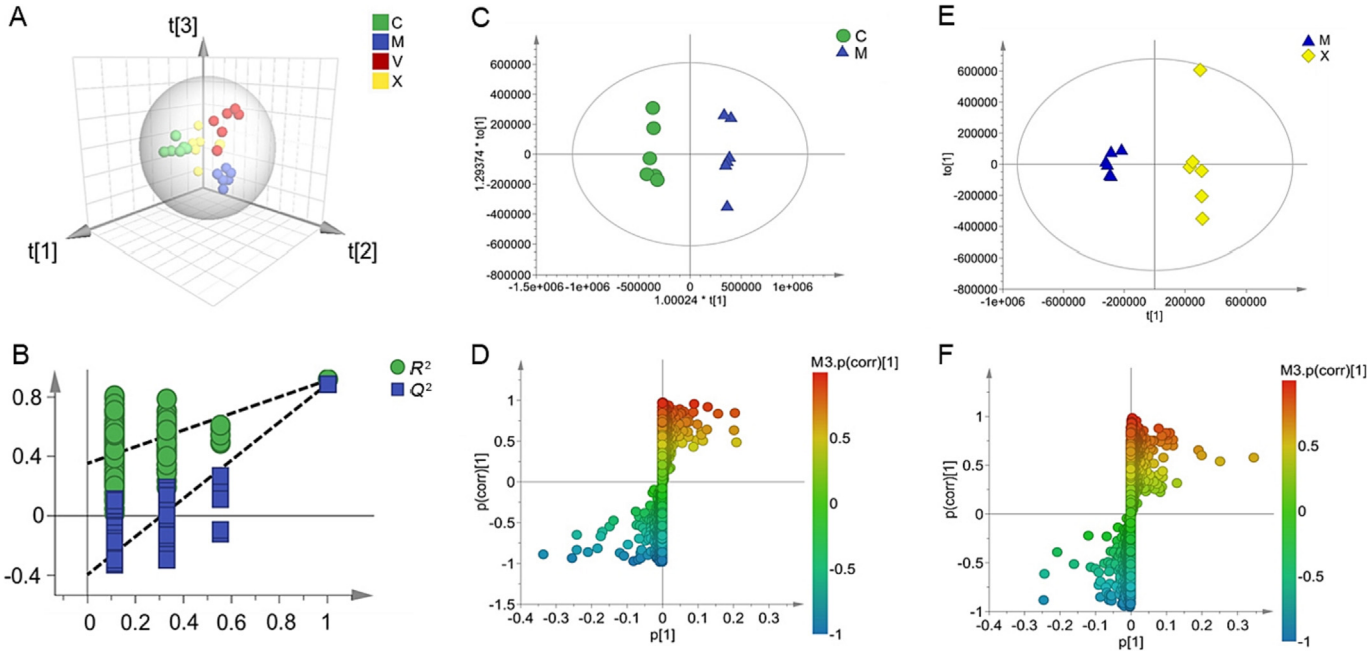

Fig. 3. Multivariate statistical analysis of mitochondrial metabolomics data. (A) Plots of PLS-DA score for C, M, V, and X groups; (B) Plots of PLS-DA replacement tests for C, M, V, and X groups; (C) Plots of OPLS-DA score for C and M groups; (D) S-plot for C and M groups; (E) OPLS-DA score plots for M and X groups; (F) S-plot for M and X groups.

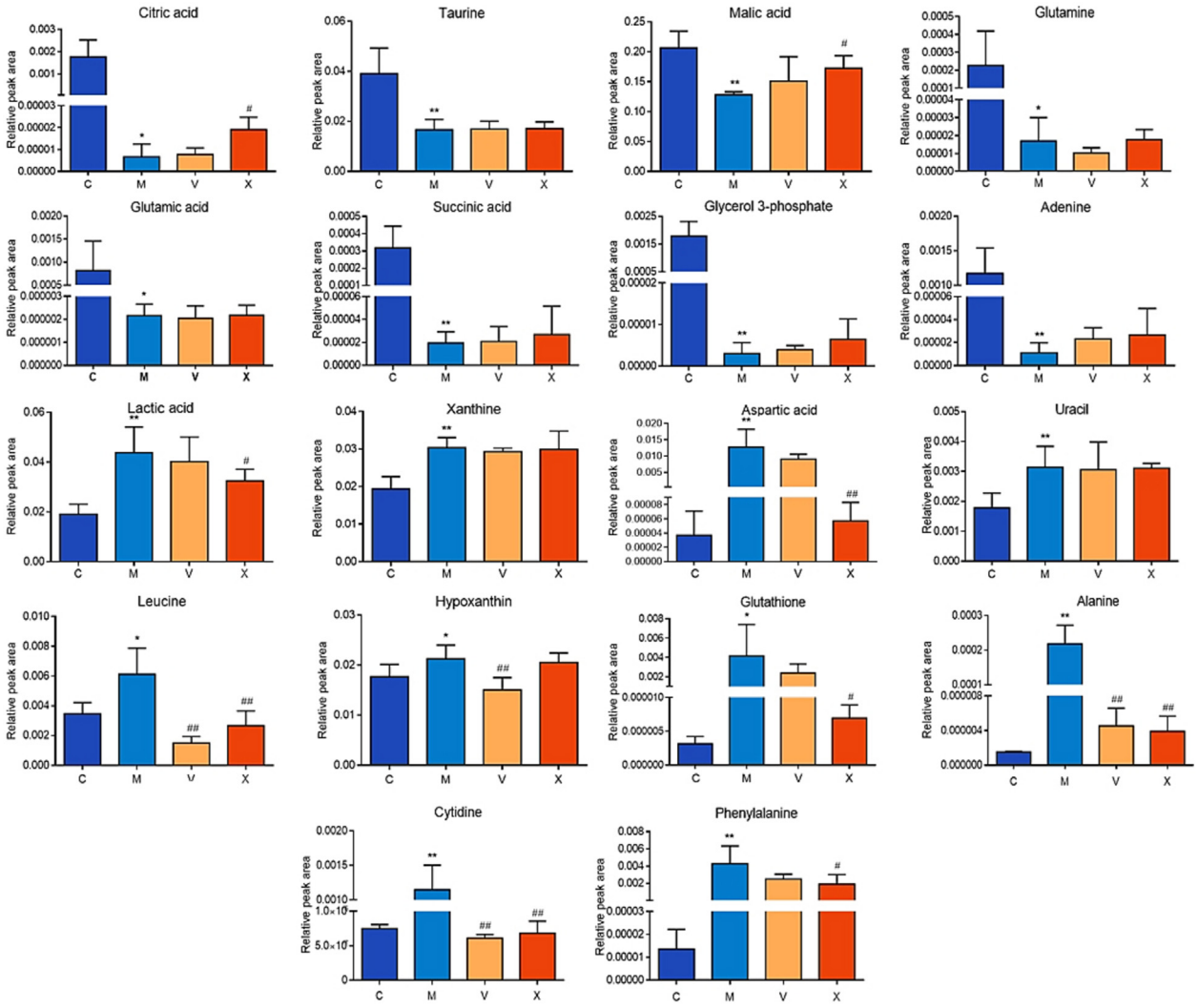

Fig. 4. Relative peak areas of differential metabolites in rat liver mitochondria. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* C group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* M group.

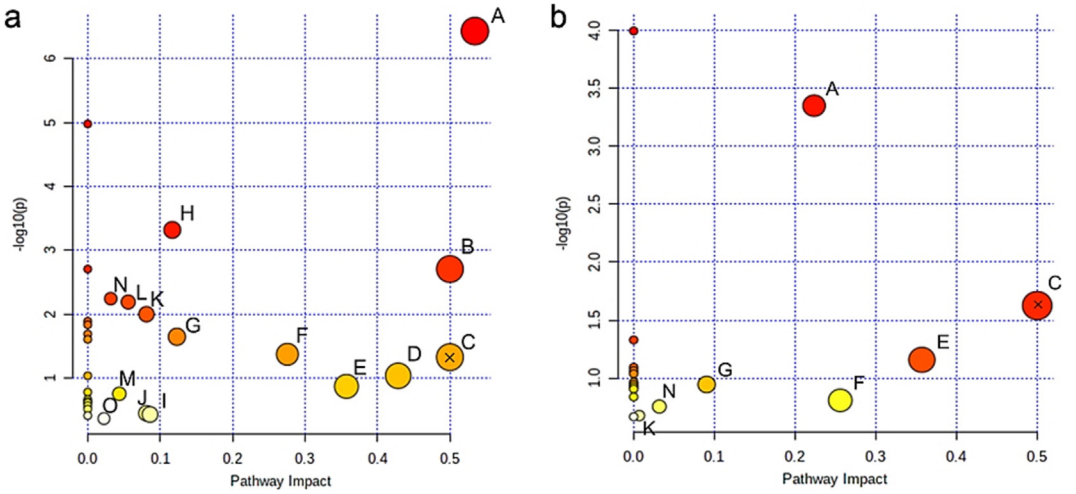

Fig. 5. Metabolic pathway analysis. (a) Depression-associated metabolic pathways; (b) XYS affected metabolic pathways associated with depression. A. alanine, aspartate, and glutamate metabolism; B. *D*-glutamine and *D*-glutamate metabolism; C. phenylalanine, tyrosine, and tryptophan biosynthesis; D. taurine and hypotaurine metabolism; E. phenylalanine metabolism; F. glutathione metabolism; G. citrate cycle (TCA cycle); H. arginine biosynthesis; I. arginine and proline metabolism; J. glycerophospholipid metabolism; K. pyrimidine metabolism; L. purine metabolism; M. glycerolipid metabolism; N. glyoxylate and dicarboxylate metabolism; and O. primary bile acid biosynthesis.

glycerophospholipid metabolism; pyrimidine metabolism; purine metabolism; glycerolipid metabolism; glyoxylate and dicarboxylate metabolism; and primary bile acid biosynthesis. Subsequent analysis revealed that tyrosine and tryptophan biosynthesis occur only in plants and bacteria, not in animals, so this pathway was ruled out (Parthasarathy et al., 2018). Thus, this study identified 14 metabolic pathways that were associated with depression. As shown in Fig. 5b, after XYS administration, six of these metabolic pathways can be significantly modulated, namely phenylalanine metabolism; glutathione metabolism; alanine, aspartate, and glutamate metabolism; citrate cycle (TCA cycle); glyoxylate and dicarboxylate metabolism; and pyrimidine metabolism.

3.4. Potential mechanism of action for XYS to alleviate depression based on network pharmacology

3.4.1. Potential target prediction

The TCMSP platform was used to search for the active compounds and their protein targets of action in XYS, and a total of 223 targets were obtained. In addition, 1 262 depression-related targets were searched through the GeneCards database. A total of 88 targets were obtained from the intersection of the targets of XYS and the targets of depression, which are the potential targets of XYS for the treatment of depression, and the Venn diagram was shown in Fig. S2.

3.4.2. PPI network construction

Eighty-eight intersecting targets were analyzed for protein interactions based on the STRING database, and the interaction network of potential target proteins for the treatment of depression with XYS was constructed in Cytoscape 3.7.2 software (Fig. 6). To some extent, the PPI network can reflect the complex and intertwined interrelationships between potential targets, which do not work in isolation. The darker the node and larger the diameter, the larger the degree of the interaction. Thicker lines indicate a close interaction between the two target proteins. Topological analysis was conducted on 15 key targets screened by Degree (≥2 times median), CC (≥1 times median), and BC (≥1 times median), including IL-1β, IL-6, TNF, EGFR, AKT1, IL-2, MAPK3, STAT3, TP53, CXCL8, CASP3, PTGS2, MMP-9, VEGFA, IL-10. These targets are the pivotal proteins in the entire network and play a more critical role, and can be seen as a key target in the treatment of depression with XYS.

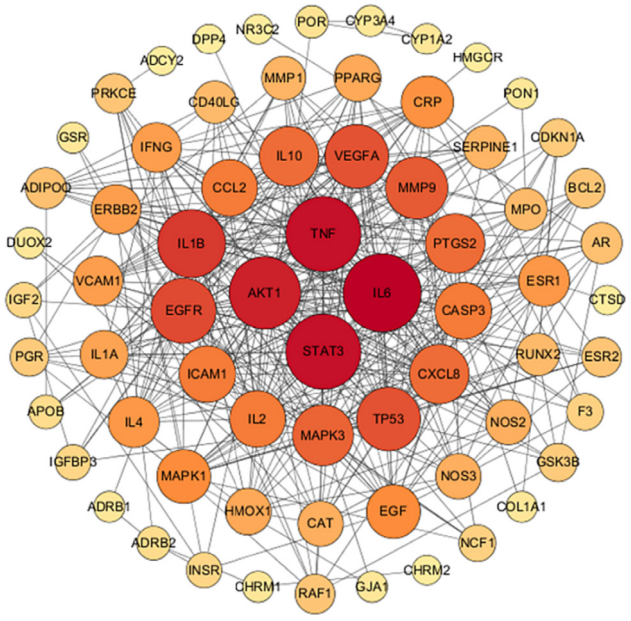

Fig. 6. PPI diagram of potential targets of XYS intervention for depression.

3.4.3. GO function and KEGG pathway enrichment analysis

GO enrichment includes biological processes (BP), cellular components (CC), and molecular functions (MF), and GO functional enrichment analysis can be used to obtain the biological functions of genes. The GO functional enrichment analysis yielded a total of 1 553 GO gene functional annotation entries ($P < 0.01$), including 1 320 biological processes, 90 cellular components, and 143 molecular functions. The BP enrichment results are mainly related to positive regulation of protein phosphorylation, cellular response to nitrogen compound, regulation of MAPK cascade, response to hormone, positive regulation of cell motility, positive regulation of locomotion, negative regulation of cell population proliferation, cellular response to organonitrogen compound, cellular response to lipid, and positive regulation of cell migration. The CC enrichment results are mainly related to membrane raft, membrane microdomain, postsynapse, synaptic membrane, postsynaptic membrane, receptor complex, plasma membrane protein complex, neuronal cell body, cell body, and dendrite. The MF enrichment results are mainly related to signaling receptor regulator activity, signaling receptor activator activity, receptor ligand activity, oxidoreductase activity, protein homodimerization activity, cytokine activity, cytokine receptor binding, inorganic molecular entity transmembrane transporter activity, neurotransmitter receptor activity, and channel activity (Fig. 7).

The KEGG pathway enrichment results of 181 entries were obtained by Metascape platform analysis, and the top 20 entries with $P < 0.01$ were selected for graphing, as shown in Fig. 8. The effect of XYS on depression may be related to AGE-RAGE signaling pathway in diabetic complications, pathways in cancer, HIF-1 signaling pathway, IL-17 signaling pathway, calcium signaling pathway, diabetic cardiomyopathy, retrograde endocannabinoid signaling, insulin resistance, allograft rejection, type II diabetes mellitus, transcriptional misregulation in cancer, longevity regulating pathway, small cell lung cancer, AMPK signaling pathway, salivary secretion, amyotrophic lateral sclerosis, inflammatory mediator regulation of TRP channels, leukocyte transendothelial migration, dopaminergic synapse, and viral myocarditis.

4. Discussion

With an understanding that the original prescription of XYS is based on the theoretical framework of traditional Chinese medicine, this study aimed to investigate the effect of an XYS intervention on behavioral changes, exercise capacity, and the liver mitochondrial metabolomic profiles to advance our understanding of the potential mechanism underlying the effect. The results of this study demonstrated that the rats under CUMS exhibited a significantly reduced body weight, performance in the open field test, exercise capacity, and sugar water preference rate, and these depressive symptoms can be ameliorated by a 28-day intervention of XYS and Venlafaxine, which are consistent with previous reports (Liu, Zheng, Du, Li, & Qin, 2019; Jiao et al., 2019), and re-confirmed the antidepressant effect of XYS in the CUMS model.

The major new contributions from this study are the identifications of potential target substrates, proteins, and metabolic pathways, for the effect of XYS, with evidence collected using the metabolomic and network pharmacological analyses. The mitochondrion is the major site of cellular energy transformation, where many key metabolic reactions occur, such as the TCA cycle, adenosine triphosphate (ATP) and reactive oxygen species (ROS) production, β oxidation, and amino acid synthesis (Liu & Xu,

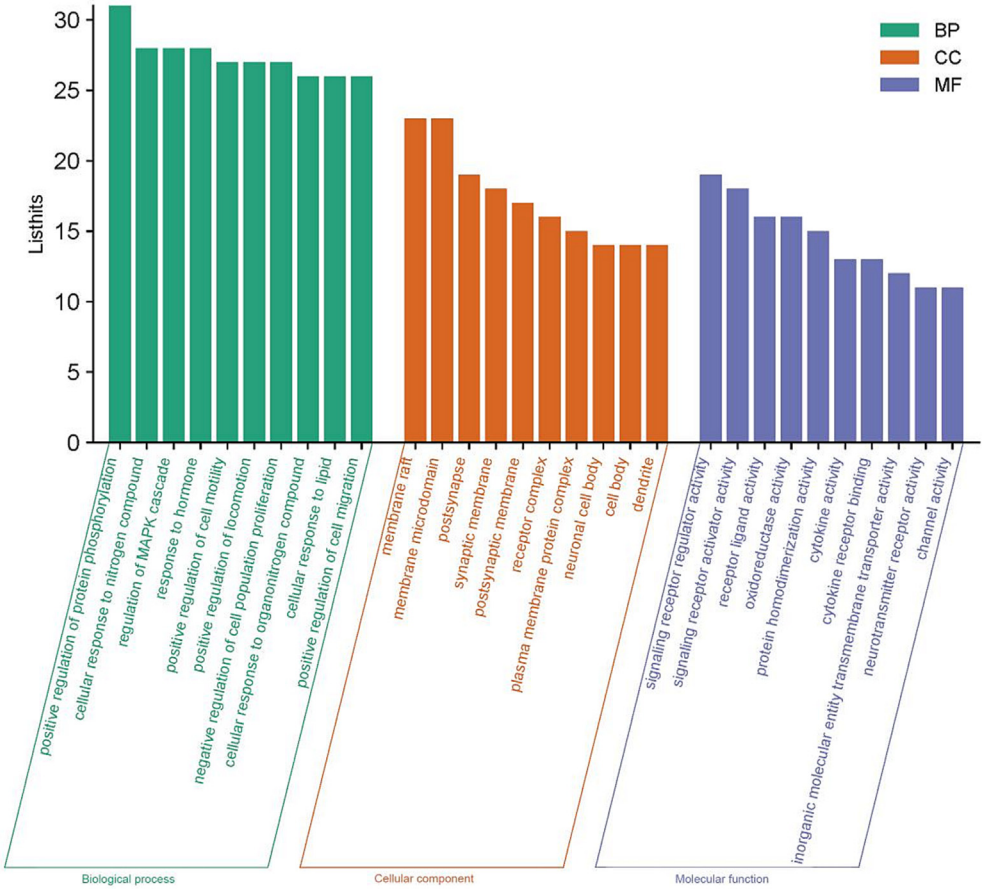

Fig. 7. Histogram of GO enrichment analysis.

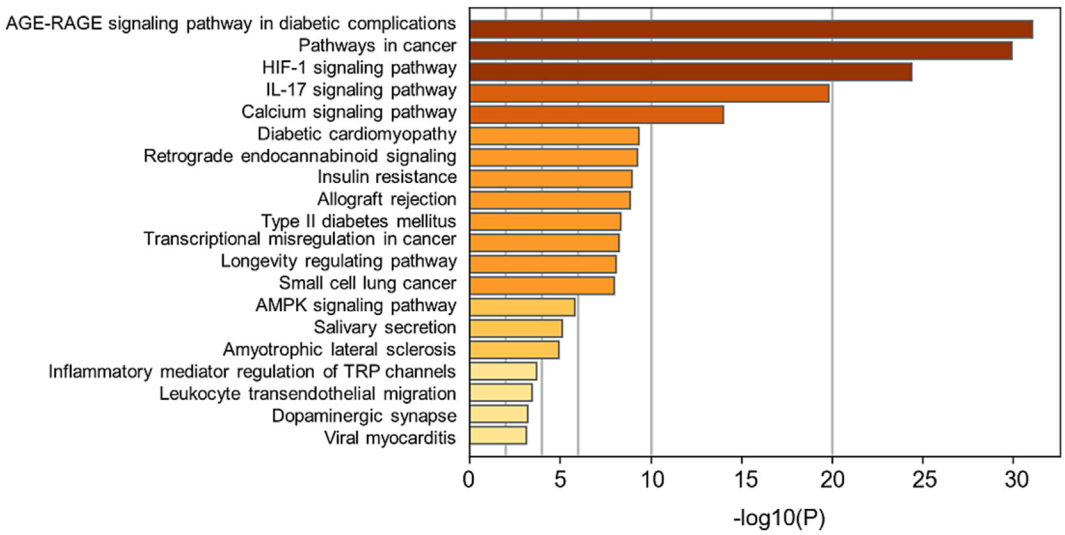

Fig. 8. Histogram of KEGG analysis.

2018). In this study, the screening of the liver mitochondrial metabolomic identification identified 18 potential biomarkers associated with depression, such as malate, taurine, and glutamate, etc., of which XYS was able to back-regulate nine differential metabolites, such as aspartate, lactate, and leucine, etc. KEGG analysis revealed that XYS intervention had an effect on six metabolic pathways including phenylalanine, glutathione, alanine, aspartate, and glutamate metabolic pathways, which were mainly involved in amino acid metabolism and energy metabolism.

Amino acids play a vital role in the life activities of living organisms. Phenylalanine acts as a precursor to tyrosine and can be further converted into dopamine (DA), norepinephrine (NE), and other neurotransmitters. It is well known that deficiencies in DA and NE contribute to the development of depression. Therefore,

reduced phenylalanine levels may lead to a decrease in catecholamine biosynthesis, which may further have an impact on depression (Gu et al., 2021). Glutathione (GSH) is a small compound containing a sulfhydryl group, consisting of three amino acids: cysteine, glutamate, and glycine. It has been demonstrated that both oxidative stress and mitochondrial damage are associated with disturbed GSH homeostasis (Von Bohlen Und Halbach, 2022). Alanine can be interconverted with pyruvate to produce acetyl coenzyme A, which enters the tricarboxylic acid cycle and participates in energy metabolism (Tian et al., 2016; Li et al., 2020). Aspartic acid is involved in the ornithine cycle, enhancing liver function and reducing fatigue. Glutamate, an important component of excitatory neurotransmitters, is closely associated with depression (Moriguchi et al., 2019). Reduced levels of glutamate negatively affect the energy supply which plays a key role in the development of depression (Villa et al., 2017). A study has shown that the antidepressant effect of XYS is related to the regulation of glutamine and glutamate metabolism to maintain ammonia homeostasis in the body and promote energy metabolism (Chen et al., 2020). In the present study, we found significant changes in the levels of metabolites such as phenylalanine, alanine, and glutamate in the liver mitochondria of depressed rats, suggesting that depression is associated with dysregulation of amino acid metabolic pathways.

Disturbed energy metabolism is one of the main manifestations of depression, and the TCA cycle is the common pivot pathway linking the metabolism of the three major energy-supplying substances, carbohydrates, lipids, and amino acids, and the mitochondrial electron respiratory chain. Experimental studies in proteomics combined with metabolomics have revealed that the TCA cycle is disrupted in the cerebellum of depressed rats (Shao et al., 2015). Citric acid and malic acid are intermediates in the TCA cycle that affect the body's energy metabolism. Liu et al. found that plasma citric acid levels were significantly lower in patients with depression, suggesting that depression was associated with an impaired TCA cycle, which subsequently caused disturbances in energy metabolism (Liu et al., 2015). In this study, we found that the metabolite content in the liver mitochondria of CUMS rats was altered, and the levels of lactic acid, malic acid, aspartic acid, and other metabolites were reversed towards normal in response to the XYS treatment, suggesting that XYS can improve the energy metabolic disorders in mitochondria that would contribute to the antidepressant effect.

In the network pharmacology study, 88 possible targets for the treatment of depression were obtained, including 15 core targets such as TNF, AKT1, and STAT3, etc., suggesting the multi-component and multi-target characteristics of the XYS treatment in treating depression. It has been suggested that the vicious cycle of "inflammation-mitochondrial damage" causes functional damage to nerve cells and is one of the important pathophysiological mechanisms of depression (Islam, 2017). Mitochondria are closely associated with inflammatory processes, and the most directly regulated inflammatory factor is TNF-α (Jeong & Seol, 2008). TNF-α is a classical cytokine that causes interference between mitochondrial respiratory chain complexes I and III, and can induce mitochondrial dysfunction and lead to mitochondrial membrane rupture and apoptosis. Akt is a serine/threonine protein kinase that is an important target downstream of PI3K. Research has confirmed that XYS ethyl acetate fraction can alleviate depression-like behaviors in CUMS mice possibly by promoting hippocampal neurogenesis and inhibiting the over-activation of the IGF-1Rβ/PI3K/Akt pathway (Zeng et al., 2022). STAT3 is a regulator of respiration within the mitochondria and plays an important role in regulating the electron respiratory chain and is involved in maintaining cellular homeostasis. STAT3 is able to participate in energy metabolism via acetylation (Xu et al., 2016).

In this study, the enrichment analysis of GO function and KEGG pathway predicted that XYS might work through AGE-RAGE signaling pathway in diabetic complications, pathways in cancer, HIF-1 signaling pathway, and other pathways to induce a treatment effect on depression, which has a multi-target and multi-pathway feature. Although the results of the KEGG pathway enrichment analysis did not directly suggest that the antidepressant effect of XYS was associated with mitochondria, the analysis revealed a correlation between mitochondrial function and the pathway involved in the enrichment results. The mitochondrial calcium uptake regulated by the calcium signaling pathway plays an important role in normal cellular physiological functions (Zhang et al., 2021), including stimulation of ATP production, inhibition of autophagy, rectification of intracytoplasmic calcium signaling, and regulation of cell death (Rossi, Pizzo, & Filadi, 2019). In fact, Ca^{2+} is a key intracellular second messenger that reaches the mitochondrial membrane space and then the matrix, responsible for regulating the proteins, enzymes, and transport proteins of ATP synthesis (Contreras, Drago, Zampese, & Pozzan, 2010). It was found that depression is associated with a dysregulation of Ca^{2+}, which acts mainly through the calcium signaling cascade (Paul, 2001). The above studies provide theoretical support for the possible antidepressant effect of XYS through the regulation of mitochondrial energy metabolism.

Intracellular life activity is a combination of genes, proteins, and small metabolites, with functional changes in the upstream macromolecules ultimately manifested at the metabolic level. Lei et al. systematically investigated the antidepressant effects of paeoniflorin on CUMS rats based on a combination of LC-MS metabolomics and network pharmacology analysis, and identified the citrate cycle as an important metabolic pathway, and SLC6A4, TNF, IL6, and SLC6A3 as key targets (Lei et al., 2022). Han et al. analyzed the urine of a mouse model of Alzheimer's disease for metabolomics and combined it with network pharmacology, and found that Dihuang-Yinzi improved four energy-related metabolic pathways to alleviate cognitive deficits in AD disease, including glycerophospholipid metabolism, niacin/nicotinamide metabolism, glycolysis and the tricarboxylic acid cycle (Han et al., 2022). Therefore, our study integrated network pharmacology and metabolomics and provided a new research strategy for the antidepressant effect of XYS. However, there are still limitations in this study. Core targets such as TNF, AKT1, and STAT3 can be selected for follow up experiments to confirm the predictions of network pharmacology and to investigate the effect of XYS on mitochondrial energy metabolism in depressed rats.

5. Conclusion

This study produced new evidence on the effects of a 28-day XYS intervention on the symptoms of depression, exercise capacity, and the mitochondrial metabolites profile and metabolic pathways in the liver of the rats under CUMS. The antidepressant effects of XYS appeared to be manifested through multiple pathways such as AGE-RAGE signaling pathway, cancer pathway, and HIF-1 signaling pathway, with multi-target and multi-pathway characteristics.

CRediT authorship contribution statement

Weidi Zhao: Writing-original draft, Data curation, Methodology. **Cui Ji:** Data curation, Methodology. **Jie Zheng:** Data curation, Methodology. **Shi Zhou:** Writing-review & editing. **Junsheng Tian:** Writing-review & editing. **Yumei Han:** Writing-review & editing, Funding acquisition. **Xuemei Qin:** Writing-review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2023.09.004.

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