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•Original article•

Hepatic metabolomics combined with network pharmacology to reveal the correlation between the anti-depression effect and nourishing blood effect of Angelicae Sinensis Radix

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[ABSTRACT] Angelicae Sinensis Radix (AS) is reproted to exert anti-depression effect (ADE) and nourishing blood effect (NBE) in a rat model of depression. The correlation between the two therapeutic effects and its underlying mechanisms deserves further study. The current study is designed to explore the underlying mechanisms of correlation between the ADE and NBE of AS based on hepatic metabonomics, network pharmacology and molecular docking. According to metabolomics analysis, 30 metabolites involved in 11 metabolic pathways were identified as the potential metabolites for depression. Furthermore, principal component analysis and correlation analysis showed that glutathione, sphinganine, and ornithine were related to pharmacodynamics indicators including behavioral indicators and hematological indicators, indicating that metabolic pathways such as sphingolipid metabolism were involved in the ADE and NBE of AS. Then, a target-pathway network of depression and blood deficiency syndrome was constructed by network pharmacology analysis, where a total of 107 pathways were collected. Moreover, 37 active components obtained from Ultra Performance Liquid Chromatography-Triple-Time of Flight Mass Spectrometer (UPLC-Triple-TOF/MS) in AS extract that passed the filtering criteria were used for network pharmacology, where 46 targets were associated with the ADE and NBE of AS. Pathway enrichment analysis further indicated the involvement of sphingolipid metabolism in the ADE and NBE of AS. Molecular docking analysis indciated that *E*-ligustilide in AS extract exhibited strong binding activity with target proteins (PIK3CA and PIK3CD) in sphingolipid metabolism. Further analysis by Western blot verified that AS regulated the expression of PIK3CA and PIK3CD on sphingolipid metabolism. Our results demonstrated that sphingolipid metabolic pathway was the core mechanism of the correlation between the ADE and NBE of AS.

[KEY WORDS] Angelicae Sinensis Radix; Blood deficiency syndrome; Depression; Metabonomics; Network pharmacology; Sphingolipid metabolism

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Introduction

Depression, a complex prevalent mental disorder, poses a huge burden on the society due to its high morbidity, high disability rate and high suicide rate [1]. According to a report issued by the World Health Organization (WHO), 350 million people around the world suffer from depression, and this disorder will become the leading cause of death by 2030 [2, 3]. In the modern Western system of medicine, some possible hypotheses concerning the mechanisms of depression were put forward, but the precise mechanisms of action have not been completely understood. Traditional Chinese medicine (TCM) is a holistic medical system for diagnosis, prevention and treatment of diseases. Understanding the pathogenesis of depression from TCM theory can provide new strategies for the clinical treatment of depression.

Blood deficiency, a common TCM syndrome, is a pathological state of blood dysfunction and organ dystrophy [4, 5]. Blood deficiency is accompanied with the change of blood cells and declined immune function, which is similar to anemia [4]. Epidemiological data show that blood deficiency syndrome is a common and serious problem in depression

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that should be drawn great attention [6, 7]. Individuals with depression usually suffer from physical symptoms related to blood deficiency, including palpitation, amenorrhea, and sleep disturbance [8, 9]. Scientific evidence comfirmed that depressive symptoms were related with the physical symptoms of palpitation, breath shortness [10] and menstrual problems such as amenorrhea, hypomenorrhea, and changes of blood clot [11]. It was speculated that the comorbidity mechanisms of depression and blood deficiency syndrome were related to the changes in neuroendocrine hormones (such as corticosterone) [12], neurotransmitters [13], immune function [14], and oxidative stress metabolism [15]. Depression and blood deficiency syndrome are inextricably correlated according to clinical manifestations and treatment experience. However, the experimental evidence and underlying mechanisms of action are less discussed.

Angelicae Sinensis Radix (AS), the root of *Angelica sinensis* (Oliv.) Diels, is an important herb used in TCM to enrich the blood, prevent and treat blood deficiency syndrome for thousands of years [16]. Recent studies have reported that AS exhibits pharmacological effect on depression [17]. Our previous study demonstrated that AS significantly improved the chronic unpredictable mild stress (CUMS) induced depressive symptoms, hematological anomalies, and hypoxia symptoms, indicating that AS can exert both anti-depression effect and nourishing blood effect on an animal model of depression [18]. The correlation and underlying mechanisms deserve further investigation.

Metabolomics is a mature technique of systems biology and attempts to identify and quantify the global metabolic profile of endogenous substances in biological systems [19]. Integrated with large-scale data extraction and multivariate variable processing, metabolomics can comprehensively reflect biochemical changes in biological fluids or tissues, and has been widely applied to investigate the prevention, diagnosis and prognosis of diseases and to explore the therapeutic mechanisms of medicines [20]. Network pharmacology can generate multi-level networks based on bioactive compounds, target molecules, and biological function, which contributes to the discovery of active ingredients and the explanation of underlying mechanisms involved [21, 22]. The potential mechanisms of Chinese herbal medicines are difficult to elucidate because of their multi-compound and multi-target characteristics. The holistic view of metabolomics and network pharmacology brings new opportunities. For example, Ren *et al.* combined metabolomics and network pharmacology to reveal the toxicity and the mechanism of detoxification of Yunnan Baiyao formulation [23]. Li *et al.* integrated network pharmacology and lipidomics to uncover the protective mechanisms of Astragali Radix (AR) against nephrotic syndrome (NS) [24].

In the current study, hepatic metabolomics and network pharmacology were combined to explore the underlying mechanisms of correlation between depression and blood deficiency syndrome, as well as relationship between the anti-depression effect and nourishing blood effect of AS. First, an UPLC-MS/MS metabolomics approach was utilized on the liver samples of rats to identify metabolic profiles, potential metabolites, and metabolic pathways. Principal component analysis and correlation analysis were performed to screen the key metabolites related to pharmacodynamics indicators. Second, network pharmacology was used to predict the common targets and metabolic pathways of depression and blood deficiency syndrome. The chemical constituents of AS extract were identified by UPLC-Triple-TOF/MS, and the targets of AS on both depression and blood deficiency syndrome were further predicted. Third, KEGG pathway analysis was used to predicted the pathways, while molecular docking was conducted to explore the interactions between key compounds and key target proteins. Finally, the expression of proteins (PIK3CA and PIK3CD) on sphingolipid metabolism was determined by Western blot. These findings will provide theoretical support for deeply understanding the correlation between depression and blood deficiency syndrome and the underlying mechanisms, and facilitate the clinical application of AS for the treatment of depression.

Materials and Methods

Animal experimental design

Briefly, 75% ethanol extract of AS was obtained, while a CUMS-induced depression-like behavior model of rats was employed for metabolomics analysis. A total of 50 male Sprague Dawley rats were randomly divided into five groups: a control group, a CUMS group, a high-dose AS treatment (HAS) group, a low-dose AS treatment (LAS) group, and a venlafaxine treatment (VLF) group. Rats in the CUMS, HAS, LAS and VLF groups were subjected to CUMS as previously described, with minor modifications. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The detailed methods of animal experiments are described in the Supporting Information.

Metabolomics analysis

After treatment for 28 days, all the rats were sacrificed and their livers were collected and stored at −80 °C. Prior to analysis, liver samples were thawed at 4 °C, before extraction of metabolites through the organic protein precipitation method. A quality control (QC) sample was prepared from 10 μL of each test sample, and injected in every six sample runs in the sequence to monitor the stability of the LC/MS platform and the reproducibility of the data. UPLC-MS analysis was performed on a Dionex UltiMate 3000 UHPLC system combined with a Q Exactive Orbitrap-MS spectrometer (Thermo Fisher Scientific Inc.,Waltham, Ma, USA). The obtained raw data from LC–MS were introduced to Compound Discoverer 2.0 (Thermo Fisher, USA) for further analysis. The obtained data was imported into SIMCA-P V13.0 (Umetrics, Sweden) for multivariate statistical analysis. The detailed methods of metabolomics analysis are described in the Supporting Information.

Network pharmacology analysis

Selecting the potential active components of AS

The powder of AS obtained in section 2.2 was dissolved in 50% methanol at a concentration of 25 mg·mL^{-1}. UPLC-MS analysis was performed on a "QTOF" 5600 time of flight mass spectrometer, equipped with electrospray ionization source (AB SCIEX company, USA). According to the fragment ions of known components from AS published in the literature and local Chinese medicine resource libraries, 66 components of AS were identified. Their Pubchem ID and 2D chemical structures were obtained on PubChem (https://pubchem.ncbi.nlm.nih.gov/), followed by screening of druggable compounds according to Lipinski's rule combined with human intestinal cell line Caco-2. The detailed methods of LC-MS and selection of the potential active components of AS are described in the Supporting Information.

Screening of targets and construction of network

All gene associated with depression and blood deficiency syndrome were collected from DisGeNET (http://www.disgenet.org/), Comparative Toxicogenomics Database (CTD, https://ctdbase.org/) and GeneCards (https://www.genecards.org) database. The information of the identified components of AS were obtained from the TCMSP database (https://old.tcmsp-e.com/tcmsp.php), while Lipinski's rule was used to screen the components of AS. The TCMSP database was employed to predict the relevant targets of ingredients in AS. The common targets of depression, blood deficiency syndrome and AS components were served as the targets related to the anti-depression effect (ADE) and nourishing blood effect (NBE) of AS. These targets were imported into Cytoscape 3.8.2 software to construct a target-pathway network of disease, a ingredient-target-disase network, and a target-pathway network of AS.

Construction of a protein-protein interaction (PPI) network

The predicted targets related to the ADE and NBE of AS were imported into the String 10.0 software (https://string-db.org/) to evaluate the protein-protein interaction. Then Cytoscape software was employed to screen core targets and construct the protein-protein interaction network.

KEGG pathway enrichment and Gene ontology enrichment analysis

KEGG terms of the putative proteins with P values < 0.01 and Overlap > 3 were employed and the data were collected by the Metascape (A gene annotation & analysis resource database, https://metascape.org/) prediction. Gene Ontology (GO) is a framework for the model of biology. Gene Ontology (GO) enrichment analysis was performed on Database for Annotation, Visualization and Integrated Discovery database (DAVID, https://david.ncifcrf.gov/). GO enrichment analysis included biological process, molecular function, and cellular components.

Molecular docking

The 3D structures of the predicted proteins in sphingolipid signaling pathway (PIK3CA and PIK3CD) were obtained in the RCSB Protein Data Bank (http://www.rcsb.org). Then, the excess inactive ligands such as water molecules and phosphate radicals of the target proteins were removed by the PyMOL software. The proteins were hydrogenated and charged by the AutoDock software. The 3D structures of the candidate components was downloaded from ZINC (http://zinc.docking.org/substances/home/). Molecular docking was performed by Autodock Vina (1.5.6) and the results were processed by PyMOL (http://www.pymol.org).

Western blot analysis

The total proteins of the hippocampus were extracted, and the concentrations were measured by BCA assay. Samples containing 20 μg proteins were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane. The membrane was blocked with 5% bovine albumin (BSA) in Tris buffer saline-Tween 20 (TBST) at 37 °C for 2 h and then incubated at 4 °C overnight, before incubation with the corresponding primary antibodies diluted in 1 ∶ 1000. After washing with TBST, the membrane was then incubated with fluorescent secondary antibodies (1 ∶ 5000) at 37 °C for 2 h. After rewashing with TBST, the membrane was visualized by a fluorescent scanner (Odyssey CLX, Gene company limited, USA).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 software. Data are expressed as mean ± standard deviation (SD). Kolmogorov–Smirnov test was performed to assess if data were normally distributed. T-test and one-way ANOVA were used to compare the differences between two and more groups. $P < 0.05$ were considered as statistically significant. Principal component analysis and correlation analysis were conducted by SPSS 16.0 software.

Result

Metabolomics study

Metabolic profile analysis

The total ion chromatograms (TIC) of liver sample extracts from UPLC-MS/MS in the positive and negative modes are shown in Fig. 1S with major metabolites labeled. Variables were obtained from Compound Discoverer 2.0, and then imported into SIMCA-P V13.0 for multivariate statistical analysis. In the partial least squares-discriminant analysis (PLS-DA) score plot, obvious separation was observed between the control group and the CUMS group (Fig. 1A). The R^2Y and Q^2 of the PLS-DA model was 0.996 and 0.97, respectively, indicating that the model was valid without overfitting (Fig. 1B). Moreover, obvious separation was seen between the CUMS group and the treatment groups (Fig. 2S). Fig. 1E showed that all experimental groups were obviously separated in LC-MS metabolic profile. QC samples clustered together, reflecting the stability of the instrument and suggesting that the quality of all the LC-MS data for this study was satisfactory.

Potential biomarkers search

OPLS-DA models were utilized to discriminate differential metabolites (Fig. 1C). The S-plots of OPLS-DA revealed

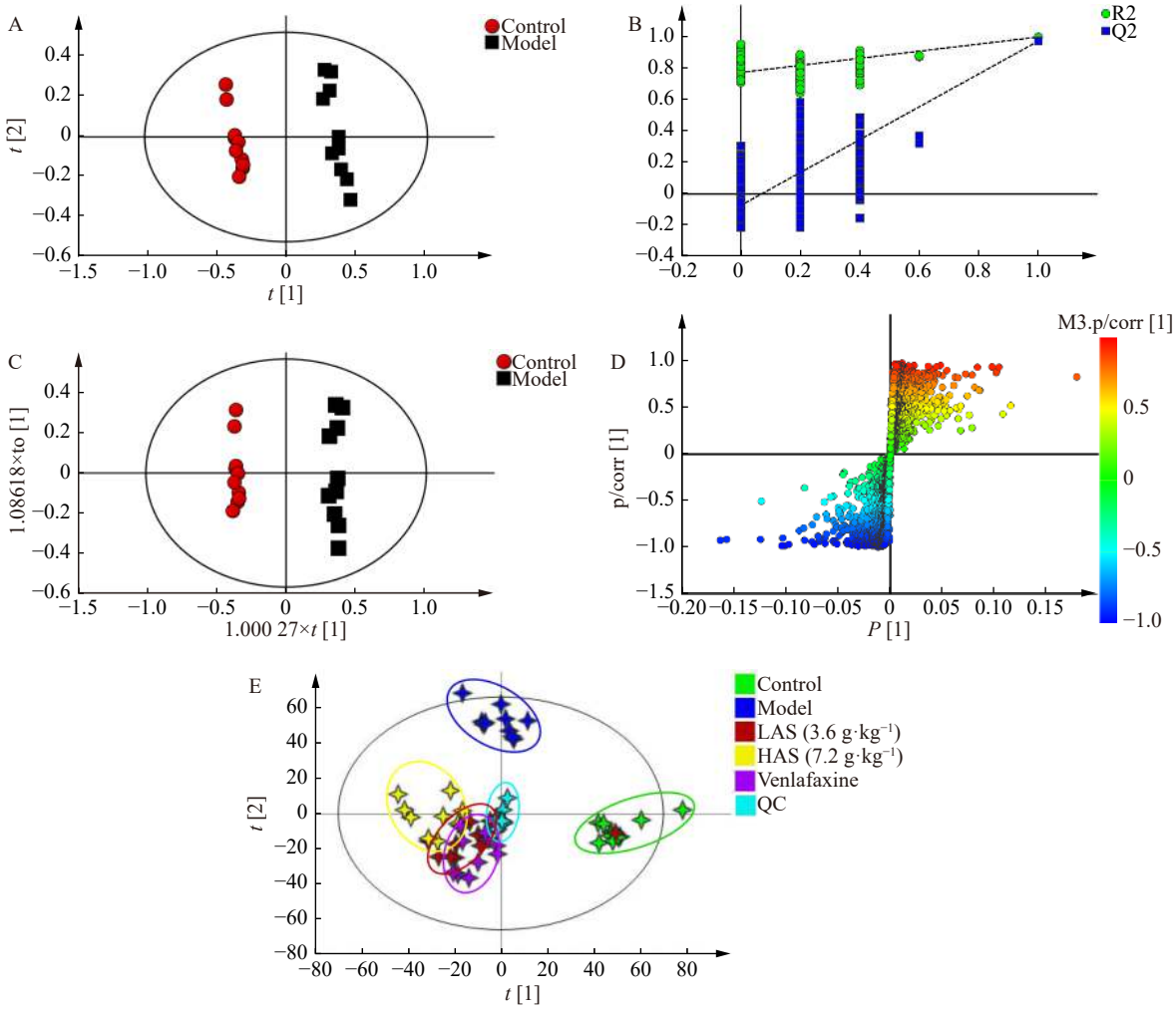


Fig. 1 Multivariate data analysis of liver samples from UPLC-MS/MS. (A) PLS-DA score plots; (B) Permutation test; (C) OPLS-DA score plots; (D) S-plot of OPLS-DA; (E) PCA score plots of serum samples collected from different groups

the variation of metabolites, as shown in Fig. 1D. Notably, 24 and 6 metabolites were selected as the differential metabolites with VIP > 1 and $P < 0.05$ (Table 1) in the ESI^+ and ESI^- ion modes, respectively, including increased levels of citric acid, nicotinamide, pyroglutamic acid, 4-oxoproline, oxidized glutathione, tryptophan, glycocholic acid, phytosphingosine, sphinganine, LysoPE(16:0/0:0), platelet-activating factor, palmitoyl sphingomyelin, PC(18:3(9*Z*,12*Z*,15*Z*)/18:2(9*Z*,12*Z*)), erucamide and PC(18:0/ 20:4(5*Z*,8*Z*,11*Z*,14*Z*)), as well as decreased levels of ornithine, lysine, taurine, gluconic acid, proline, ergothioneine, malic acid, glutamic acid, 2-hydroxyphenylalanine, adenosine, hypoxanthine, 5-methyltetrahydrofolic acid, taurochenodeoxycholic acid, LysoPC (18:2(9*Z*,12*Z*)), ceramide(d18:1/16:0).

AS significantly restored the levels of 18 metabolites to normal to different extents, including ornithine, lysine, taurine, gluconic acid, citric acid, ergothioneine, malic acid, glutamic acid, pyroglutamic acid, 4-oxoproline, tyrosine, hypoxanthine, 5-methyltetrahydrofolic acid, tryptophan, phytosphingosine, sphinganine, LysoPC(18:2(9*Z*,12*Z*)), LysoPE (16:0/0:0), platelet-activating factor, and palmitoyl sphingomyelin (Fig. 2 and Fig. 3S). The types of most restored metabolites were the same in both the LAS and HAS groups. In general, the degree of metabolite regulation increased with the increase of AS dosage. However, there were differences in several metabolites among different doses in this study. Tyrosine and LysoPC(18:2(9*Z*,12*Z*)) were significantly regulated in the HAS group, while no differences were observed between the CUMS group and the LAS group. In contrast, taurine and 4-oxoproline were significantly regulated in the LAS group, while no differences were observed between the HAS group and the CUMS group. The results indicated that there were still differences in pharmacological mechanisms among different doses of AS, which should be explored in further research.

These metabolites were involved in the metabolic pathways of phenylalanine and tryptophan biosynthesis, tyrosine metabolism, D-glutamine and D-glutamate metabolism, taurine and hypotaurine metabolism, and sphingolipid metabolism, etc. In contrast, venlafaxine regulated the levels of 14 metabolites. To further visualize the difference among the five groups, a heat map with 30 differential metabolites us-

Table 1 Differential metabolites detected in the liver of rats between the CUMS group and the control group

No.	Metabolites	t_R/(min)	*m/z*	Formula	VIP	*P*	Fold change	Trend	HMDB ID	Scan mode
1	Ornithine	0.819	133.0975	$C_5H_{12}N_2O_2$	2.88	***	0.30	↓	00214	+
2	Lysine	0.832	147.1131	$C_6H_{14}N_2O_2$	3.70	***	0.52	↓	00182	+
3	Taurine	0.923	124.0066	$C_2H_7NO_3S$	3.20	***	0.40	↓	00251	–
4	Gluconic acid	0.956	195.0506	$C_6H_{12}O_7$	3.18	**	0.79	↓	00625	–
5	Citric acid	1.007	193.0351	$C_6H_8O_7$	5.01	**	2.30	↑	00094	+
6	Proline	1.012	116.0708	$C_5H_9NO_2$	3.10	**	0.82	↓	00162	+
7	Ergothioneine	1.012	230.0962	$C_9H_{16}N_3O_2S$	2.90	***	0.66	↓	03045	+
8	Malic acid	1.026	135.0289	$C_4H_6O_5$	4.32	***	0.63	↓	00744	+
9	Glutamic acid[b]	1.045	148.0605	$C_5H_9NO_4$	3.34	**	0.63	↓	00148	+
10	Nicotinamide	1.131	123.0556	$C_6H_6N_2O$	8.71	***	2.53	↑	01406	+
11	Pyroglutamic acid	1.151	128.0346	$C_5H_7NO_3$	4.48	***	2.03	↑	00267	–
12	4-Oxoproline	2.047	128.0346	$C_5H_7NO_3$	2.90	**	2.17	↑	METPA0228	–
13	Tyrosine[b]	2.454	182.0815	$C_9H_{11}NO_3$	4.33	**	0.39	↓	06050	+
14	Oxidized glutathione	3.098	613.1603	$C_{20}H_{32}N_6O_{12}S_2$	10.97	***	3.30	↑	03337	+
15	Adenosine	3.186	268.1045	$C_{10}H_{13}N_5O_4$	1.95	***	0.53	↓	00050	+
16	Hypoxanthine	3.243	137.0462	$C_5H_4N_4O$	3.04	**	0.67	↓	00157	+
17	5-Methyltetrahydrofolic acid	3.472	458.1797	$C_{20}H_{25}N_7O_6$	2.02	***	0.38	↓	01396	–
18	Tryptophan[b]	3.832	205.0975	$C_{11}H_{12}N_2O_2$	2.05	*	1.15	↑	13609	+
19	Taurochenodeoxycholate	5.364	498.2897	$C_{26}H_{45}NO_6S$	4.80	***	0.40	↓	00951	–
20	Glycocholate	5.711	466.3168	$C_{26}H_{43}NO_6$	3.15	*	2.20	↑	00138	+
21	Phytosphingosine	6.189	318.3006	$C_{18}H_{39}NO_3$	5.22	***	2.13	↑	04610	+
22	Sphinganine	7.206	302.3058	$C_{18}H_{39}NO_2$	2.87	***	1.36	↑	00269	+
23	LysoPC(18:2(9*Z*,12*Z*))	8.497	520.3408	$C_{26}H_{50}NO_7P$	2.43	*	0.73	↓	10386	+
24	LysoPE (16:0/0:0)	8.999	454.2938	$C_{21}H_{44}NO_7P$	2.46	*	1.64	↑	11503	+
25	Platelet-activating factor	10.595	524.3718	$C_{26}H_{54}NO_7P$	5.32	**	2.18	↑	METPA0517	+
26	Palmitoyl sphingomyelin	10.626	703.5753	$C_{39}H_{79}N_2O_6P$	4.12	*	3.83	↑		+
27	Ceramide(d18:1/16:0)	11.039	538.5198	$C_{34}H_{67}NO_3$	1.80	**	0.46	↓	04949	+
28	PC(18:3(9*Z*,12*Z*,15*Z*)/18:2(9*Z*,12*Z*))	11.379	780.5519	$C_{44}H_{78}NO_8P$	2.76	*	2.96	↑	08204	+
29	Erucamide	11.801	338.3421	$C_{22}H_{43}NO$	3.66	*	1.32	↑		+
30	PC(18:0/20:4(5*Z*,8*Z*,11*Z*,14*Z*))	12.976	810.6008	$C_{46}H_{84}NO_8P$	4.63	*	3.38	↑	08048	+

"↓" or "↑" means the metabolite significantly decreased or increased in the CUMS group compared with the control group; $^*P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$ vs the control group; [b]Validated with standard reference samples.

ing hierarchical cluster analysis was plotted, as shown in Fig. 3. Each square represents the clustering value of each metabolite in an individual sample. Red or blue color represents the increase or decrease in the content of different metabolites.

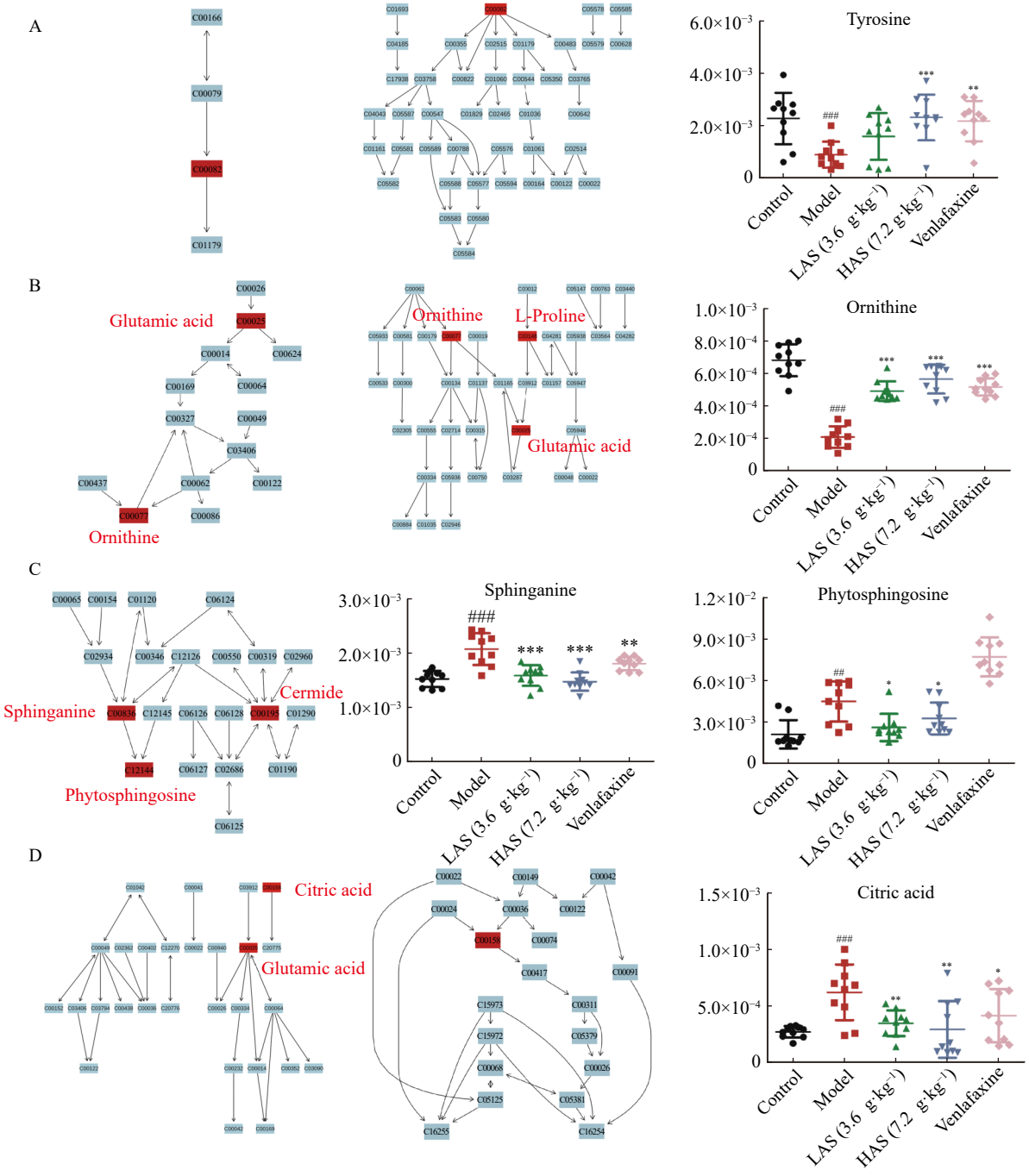
Screening of key metabolites

According to our previous study, AS significantly improved the CUMS-induced depressive symptom and anemia symptom. The key metabolites associated with both anti-depression effect and nourishing blood effect of AS were screened in the current study.

First, five behavioral indicators and nine hematological indicators of rats in the five groups (Table 1S) that were previously reported were selected for correlation analysis in the current study. As presented in Fig. 4S, a significant correlation was showed within blood gas indicators ($|r| \geq 0.8$ and $P < 0.01$), blood routine indicators ($|r| \geq 0.4$ and $P < 0.01$), as well as behavior indicators ($|r| \geq 0.4$ and $P < 0.05$). Besides, body weight was negatively correlated with PCO_2, but positively correlated with PO_2, SO_2, and pH ($|r| \geq 0.4$ and $P < 0.01$).

Platelet count (PLT) was significantly associated with three behavioral parameters, namely body weight, the number of crossings and rearings in the open-field test ($|r| \geq 0.6$ and $P < 0.01$), indicating that the anti-depression effect of AS was closely related to its nourishing blood effect.

Second, principal component analysis was used to comprehensively evaluate the pharmacodynamics indicators in current study. The results showed that two components accounted for 85.44% of the variance in the principal component analysis of five behavioral indicators (Table 2S), and three components accounted for 79.00% of the variance in the principal component analysis of nine hematological indicators (Table 3S). Correlation analysis was performed to yield a possible relationship between the metabolites and the comprehensive evaluation scores of pharmacodynamics indicators including behavioral indicator (F1) and hematological indicator (F2) in experimental rats. The results showed that 15 metabolites, including ornithine, taurine, pyroglutamic acid, lysine, malic acid, glutamic acid, 4-oxoproline, 2-hydroxyphenylalanine, 5-methyltetrahydrofolic acid, sphinganine, LysoPE(16:0/0:0), nicotinamide, oxidized glutathione, adenosine, and taurochenodeoxycholic acid were correlated



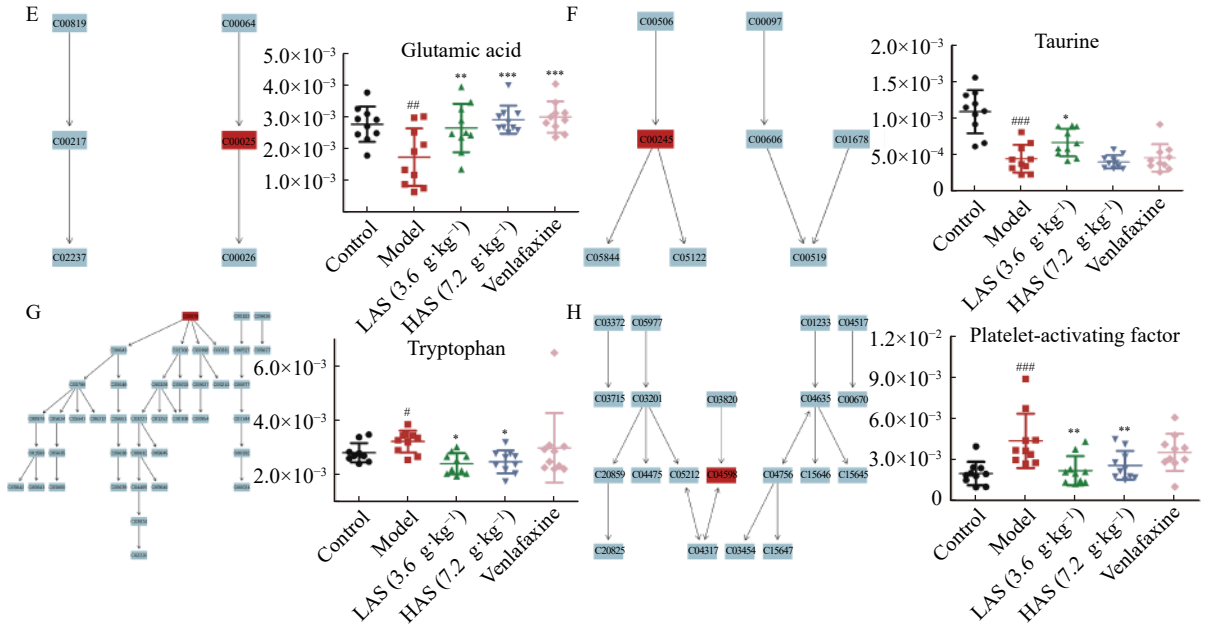


Fig. 2 Comparison of relative peak areas of the representative metabolites in UPLC-MS/MS associated with AS treatment. The content changes of tyrosine involved in phenylalanine, tyrosine and tryptophan biosynthesis, and tyrosine metabolism (A), ornithine in arginine biosynthesis, and arginine and proline metabolism (B), sphinganine and phytosphingosine in sphingolipid metabolism (C), citric acid in alanine, aspartate and glutamate metabolism, and citrate cycle (D), glutamic acid in D-glutamine and D-glutamate metabolism (E), taurine in taurine and hypotaurine metabolism (F), tryptophan in tryptophan metabolism (G), platelet-activating factor in ether lipid metabolism (H). Data are presented as means ± SD (n = 9). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the CUMS group

with F1 ($|r| \geq 0.3$ and $P < 0.05$), indicating that these metabolites were related to the anti-depression effect of AS. Moreover, eight metabolites, including ornithine, taurine, pyroglutamic acid, tryptophan, sphinganine, platelet-activating factor, oxidized glutathione, and ceramide(d18:1/16:0) were correlated with F2 ($|r| \geq 0.3$ and $P < 0.05$), indicating that these metabolites were associated with the nourishing blood effect of AS. Among these metabolites, oxidized glutathione, sphinganine, and pyroglutamic acid were negatively associated with both F1 and F2, while ornithine and taurine were positively associated with F1 and F2 (Fig. 4A), indicating that these five metabolites were the shared metabolites associated with the anti-depression effect and nourishing blood effect of AS. Besides, the correlations of these metabolites were also analyzed, and the results are showed in the Supporting Information (Fig. 5S).

Finally, receiver operating characteristic curve (ROC) analysis was performed to assess the clinical efficacy of the key metabolites as prognostic markers. The area under the curve (AUC) from ROC was calculated. The results revealed that the AUC value of three key metabolites were more than 0.75 (Fig. 4B), including oxidized glutathione (AUC = 0.78), sphinganine (AUC = 0.97), and ornithine (AUC = 1.00). This finding suggested that the model used has good prediction performance, and these three metabolites have better application value in evaluation of the therapeutic effect of Angelicae Sinensis Radix on depressive symptoms and blood deficiency symptoms induced by CUMS.

Metabolic pathways and function analysis

The differential metabolites of the liver were imported into MetaboAnalyst 5.0 for pathway enrichment. KEGG pathway analysis revealed that 11 metabolic pathways were involved in the development of depression (impact > 0.1, Fig. 5A), including sphingolipid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, etc. Among them, nine pathways were involved in the therapeutic effect of AS on CUMS-induced rats (Fig. 5C). Besides, three pathways were involved in the mechanisms of the anti-depression effect and nourishing blood effect of AS, including sphingolipid metabolism, arginine and proline metabolism and arginine biosynthesis (Fig. 5D). Enrichment analysis also found that other significant pathways (≥ 2 hits, fold enrichment ≥ 2.0, $P \leq 0.05$, Fig. 5B) were involved in the development of depression, including aminoacyl-tRNA biosynthesis, nitrogen metabolism and purine metabolism.

Network pharmacology study

Chemical Analysis

The typical TIC of extracts from Angelicae Sinensis Radix by UPLC-Triple-TOF/MS are presented in Fig. 6. Notably, 66 components were identified from literature and database (Table 4S). Meanwhile, 37 active components in AS passed the filtering criteria using Lipinski's rule and Caco-2 were obtained, including ferulic acid, ligustilide, vanillic acid, coniferyl ferulate, and senkyunolide I, etc. Detail information of these components is provided in Table 2.

Target prediction and network construction

The targets of depression and blood deficiency syn-

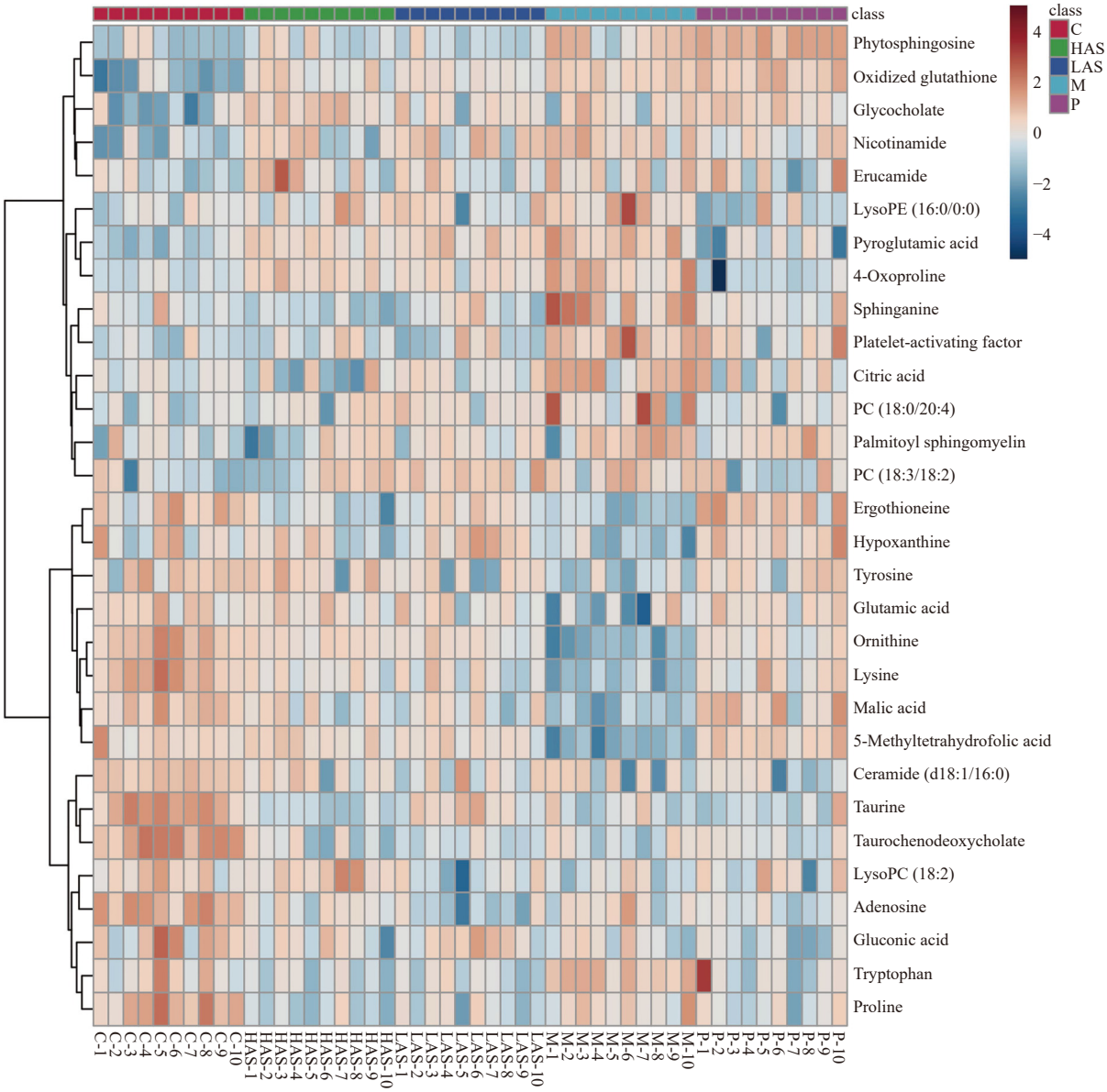


Fig. 3 Heat map of 30 differential metabolites. "C" represents the control group, "HAS" represents the high-dose AS treatment group, "LAS" represents the low-dose AS treatment group, "M" represents the CUMS group, and "P" represents the venlafaxine treatment group.

drome were obtained in Genecards database, Disgenet database, and CTD database. The top 10% targets were considered as important targets according to relevant scores. A total of 107 pathways were collected from the Metascape database. As shown in Fig. 7, the network consisted of 290 network nodes (including 183 targets, 9 unique pathways for depression, 11 unique pathways for BDS, and 87 common pathways) and 1469 edges.

As illustrated in Fig. 8A, after target prediction, 37 candidate compounds were docked with 184 target proteins. After intersecting the AS-related targets with diseases-related targets, 46 potential targets related to the mechanism of the ADE and NBE of AS were obtained. These targets were imported into 3.8.2 Cytoscape software to obtain a ingredient-target-disease network, which consisted of 85 network nodes (including 37 compounds, 46 predicted-targets and 2 diseases) and 157 edges. Two important parameters, betweenness centrality and degree, were used to evaluate the key therapeutic targets of AS on ADE and NBE. Notably, four targets, including MAPT (degree = 22, betweenness centrality = 0.3814), CA2 (degree = 15, betweenness centrality = 0.1256), DYRK1A (degree = 11, betweenness centrality = 0.1125), and PTPN1 (degree = 8, betweenness centrality = 0.0898) were probably the crucial targets in the mechanism due to their important positions in the network.

PPI network analysis

A total of 46 predicted targets screened above were imported into the String database to obtain a protein-protein interaction (PPI) relationship. The results were imported into Cytoscape software to construct a PPI network, as shown in

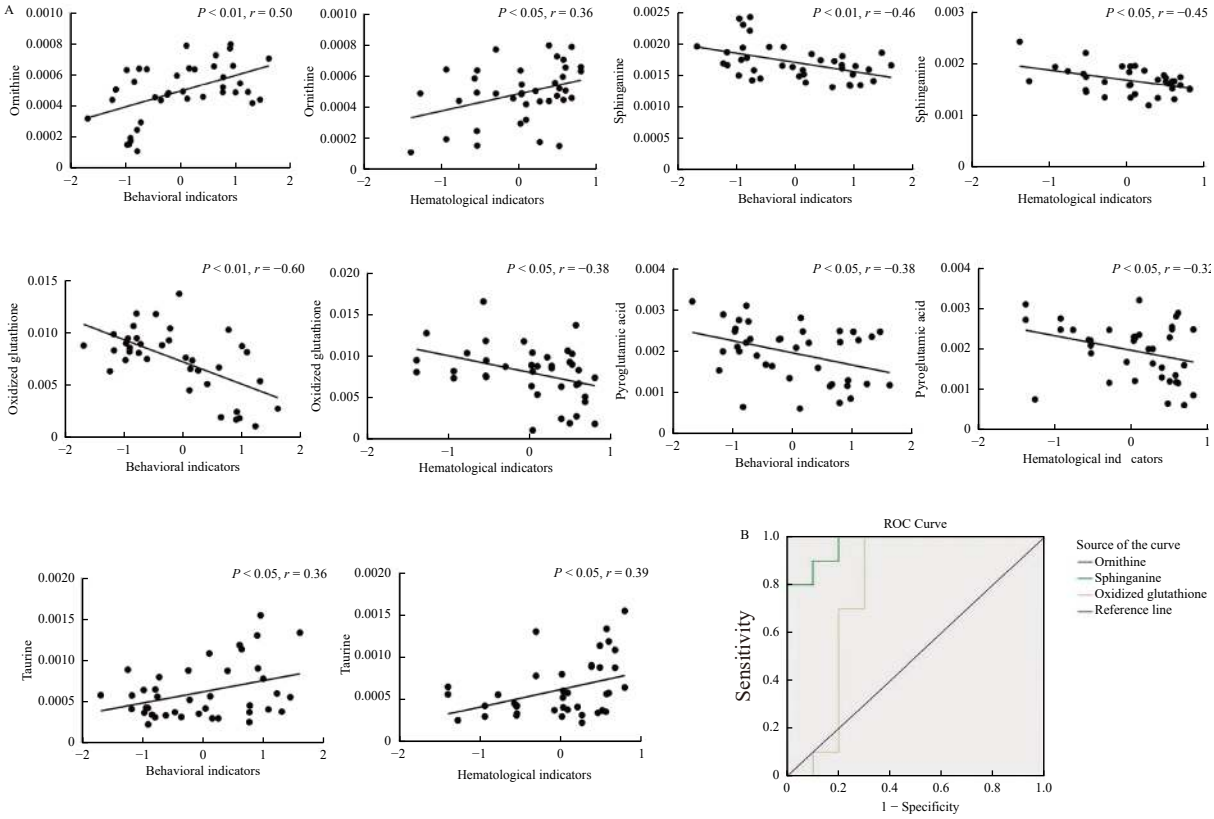


Fig. 4 (A) Correlation analysis between key metabolites and pharmacodynamics indicators; (B) ROC curve analysis of oxidized glutathione, sphinganine, and ornithine for therapeutic evaluation

Fig. 8B. Herein, 38 nodes and 214 edges were involved in the network. JUN (degree = 26), SRC (degree = 25), STAT3 (degree = 24), PIK3CA (degree = 22), PTGS2 (degree = 22), EGFR (degree = 22), STAT1 (degree = 19), NFKB1 (degree = 15), TLR4 (degree = 19) and ESR1 (degree = 19) were the top 10 targets in the network.

Pathway enrichment analysis and GO analysis

Pathway analysis was performed and the pathway terms directly related to other diseases (such as pathways in cancer) were removed. As a result, the major targets were enriched in ten important related pathways, including prolactin signaling pathway (hsa04917), PI3K-Akt signaling pathway (hsa04151), estrogen signaling pathway (ko04915), HIF-1 signaling pathway (hsa04066), neurotrophin signaling pathway (hsa04722), TNF signaling pathway (ko04668), sphingolipid signaling pathway (ko04071), and tyrosine metabolism (hsa00350), etc. (Fig. 8C). Among these pathways, HIF-1 signaling pathway invovling seven targets is regarded as one of the most important pathways for the comorbidity mechanisms of depression and blood deficiency syndrome, as well as the mechanisms of the anti-depression effect and nourishing blood effect by AS. The result is in accordance with our previous data [18] that AS can regulate the protein expression of the target genes of HIF-1α, such as lactate dehydrogenase-A (LDHA) and pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK-1). In the current study, we found that AS also regulated seven targets in HIF-1 signaling pathway, including EGFR, ERBB2, NFKB1, PIK3CA, PIK3CD, STAT3, and TLR4. By KEGG pathway analysis, we speculated that AS may regulate HIF-1α protein *via* a series of upstream regulators like TLR4, NFKB1, PIK3CA, PIK3CD, and STAT3, resulting in the influence of EGFR, ERBB2 LDHA and PDK-1 cascade.

A total of 214 GO terms were closely related to the metabolisms of the ADE and NBE of AS. Briefly, 150 Go-terms were obtained in the Biology Process, mainly involving inflammatory response (GO:0006954), neurotransmitter catabolic process (GO:0042135), response to oxidative stress (GO:0006979), and lipid metabolic process (GO:0006629). Furthermore, 44 Go-terms were obtained in the Molecular Function, which are associated with enzyme binding (GO:0019899), identical protein binding (GO:0042802), and heme binding (GO:0020037). Also, 20 Go-terms were obtained in the Cellular Location, primarily concerning cytosol (GO:0005829), axon (GO:0030424), and perinuclear region of cytoplasm (GO:0048471). The top 10% terms were selected for the diagram according to relevant scores (Fig. 8D).

Molecular docking

According to pathway enrichment in metabolomics and network pharmacology, two target proteins in sphingolipid signaling pathway (PIK3CA and PIK3CD) and the active component of AS (*E*-ligustilide) were selected for molecular docking. The results indicated high affinities between *E*-ligustilide and the two targets (Figs. 9A, 9B). *E*-ligustilide

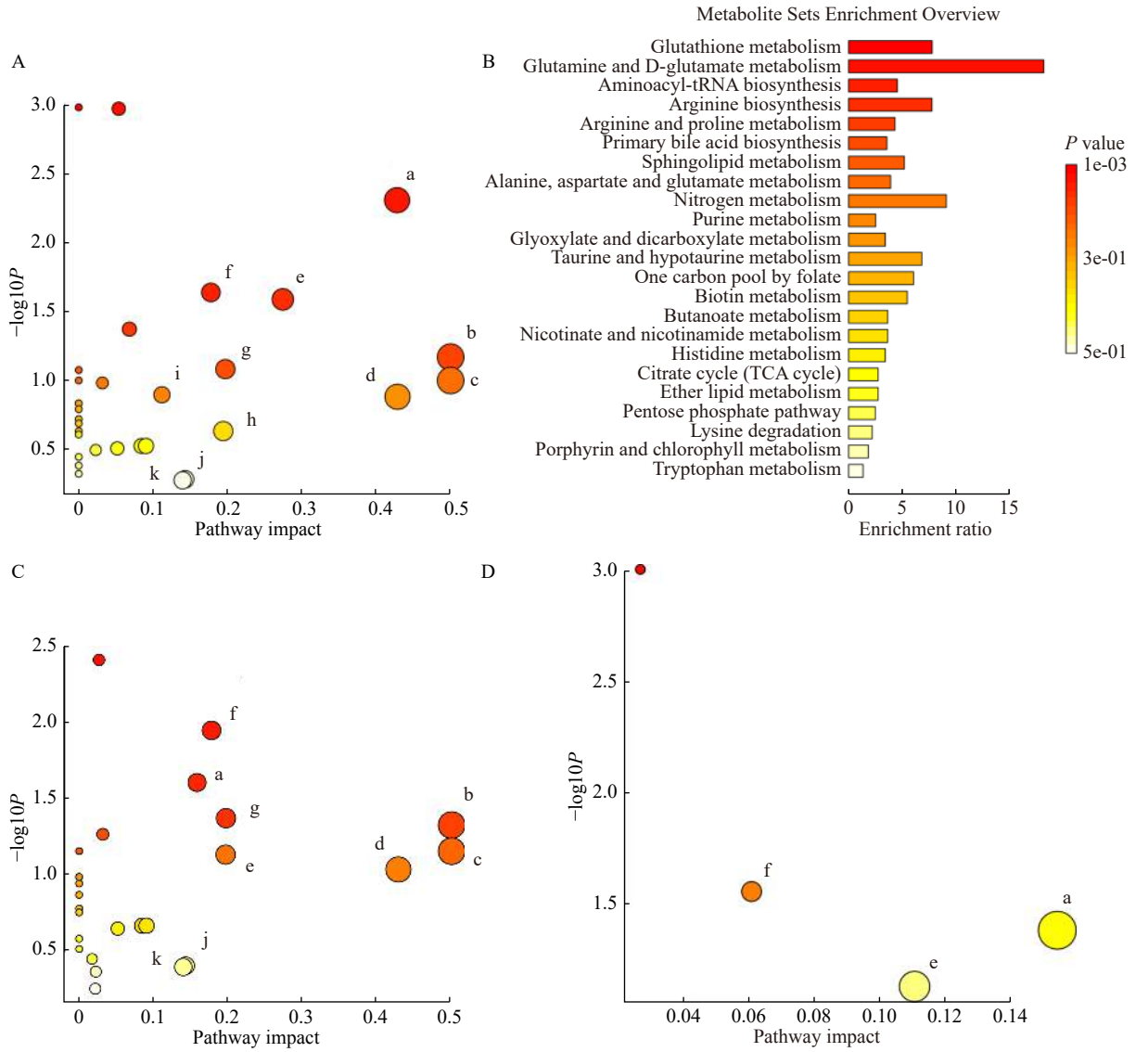


Fig. 5 Overview of metabolic pathway analysis (A) and enrichment analysis of the differential metabolites between the CUMS group and the control group (B). Pathways related to the therapeutic effect of AS on CUMS-induced rats (C). Shared pathways associated with the anti-depression effect and nourishing blood effect of AS (D). Sphingolipid metabolism (a), phenylalanine, tyrosine and tryptophan biosynthesis (b), D-glutamine and D-glutamate metabolism (c), taurine and hypotaurine metabolism (d), arginine and proline metabolism (e), arginine biosynthesis (f), alanine, aspartate and glutamate metabolism (g), nicotinate and nicotinamide metabolism (h), glycerophospholipid metabolism (i), tryptophan metabolism (j), and tyrosine metabolism (k).

made a hydrogen-bonding interaction with PIK3CA at GLN-522, and made hydrogen-bonding interactions with PIK3CD at VAL-445 and SER-444.

Experimental validation

To verify the results obtained by network pharmacology, the levels of PIK3CA and PIK3CD in the hippocampus were determined by Western blot. In the current study, we observed markedly decreased expression of PIK3CA and PIK3CD in the hippocampus of rats in the CUMS group compared with that in the control group ($P < 0.01$, Fig. 10), suggesting that sphingolipid metabolism was disrupted after CUMS procedures. By contrast, the expression of the two proteins obviously increased after treatment with AS ($P < 0.01$). The results suggested that sphingolipid metabolism is involved in the underlying mechanisms of the ADE and NBE of AS.

Discussion

Depression and blood deficiency syndrome are closely interrelated. However, the experimental support and underlying mechanisms are less discussed. As a well-known Chinese herb used for replenishing the blood and promoting the circulation for thousands of years, AS has been proved to exert anti-depression effect and nourishing blood effect in a rat model of depression. The correlation of the two therapeutic effects and its underlying mechanisms deserves further

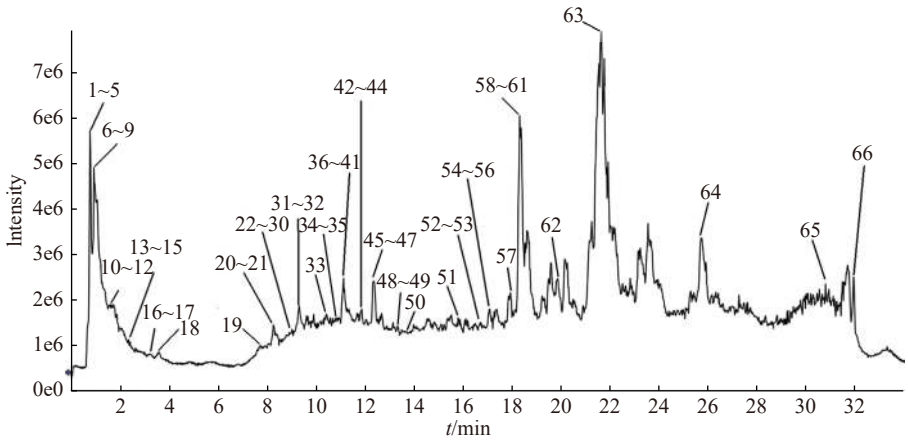


Fig. 6 Total ion chromatogram of extracts from Angelicae Sinensis Radix

study. In this study, an UPLC-MS/MS based metabolomics approach was adopted to analyze the correlation between depression and blood deficiency syndrome, as well as the ADE and NBE of AS, in the metabolites and metabolic pathways. In addition, network pharmacology was performed to screen the related targets and metabolic pathways. The results suggested the presence of many common features shared between the ADE and NBE of AS, including metabolites, targets and metabolic pathways (Fig. 11). According to the results of two techniques, common pathways (such as sphingolipid metabolism and tyrosine metabolism) and differential pathways (such as arginine and proline metabolism, and HIF-1 signaling pathway) related to the ADE and NBE of AS were discovered, indicating the intercommunity and complementarity of two techniques in systems biology. These findings provide experimental evidence for understanding the correlation between depression and blood deficiency syndrome and the underlying mechanisms. In the future work, other omics technologiwa with high sensitivity and strong characterization ability such as proteomics and transcriptome will be applied to reveal the correlations between depression and blood deficiency syndrome at protein and gene levels.

Depression is a metabolic disease closely related to hepatic function. In TCM theory, “stagnation of qi in the liver” is believed to be the core symptom of depression.The liver stores and regulates blood function, which is related to the pathogenesis of blood deficiency. The liver is believed to reflect the onset and cure of depression and blood deficiency syndrome. For instance, Liu *et al.* employed a hepatic metabolomics approach to explore the scientific connotations and compatibility effect of XiaoyaoSan [25]. In the current study, the liver was selected for metabolomics analysis to explore the comorbidity mechanisms of depression and blood deficiency syndrome, as well as the therapeutic mechanisms of AS.

Technology integration The active constituents, target proteins and pathway networks related to the ADE and NBE of AS were obtained through network pharmacology analysis, which reflect the characteristics of multi-target and multi-pathway of TCM. Based on the results of metabolomics and molecular docking, the core metabolites and proteins can be mapped to the pathway predicted by network pharmacology. For example, tyrosine identified by metabolomics and tyrosinase (TYR) predicted by network pharmacology suggested the importance of tyrosine metabolism (hsa00350) in the therapeutic mechanisms of the ADE and NBE of AS. Furthermore, metabolites including phytosphingosine, sphinganine, palmitoyl sphingomyelin and ceramide (d18:1/16:0) in sphingolipid metabolic pathway detected by metabolomics can be mapped to the sphingolipid signaling pathway (ko04071) predicted by network pharmacology. The result was further proved by Western blot analysis.

Sphingolipid metabolism Sphingolipids are important structural components of membranes involved in various cellular processes such as membrane tracking, cell morphology, cell-cell interactions, as well as cell proliferation, differentiation, apoptosis, and migration [26]. Sphingolipid metabolism was proved to be linked with multiple pathophysiological mechanisms of depression, such as activation of the HPA axis, transport and transmission of neurotransmitters, neurodegeneration and inflammation [27]. It was demonstrated that sphingolipid and phospholipid metabolism in the hippocampus of chronic unpredictable stress rats were affected, and the level of lysophosphatidylcholine was correlated with blood corticosterone level [28]. Gulbins *et al.* summarized the importance of acid sphingomyelinase/ceramide system in the pathogenesis of major depression and put forward that sphingolipids may serve as a novel target for anti-depressants [29]. Besides, abnormal sphingolipid metabolism was related to the pathophysiology of blood deficiency syndrome [30]. For example, sphingolipids inhibited the convertion of erythropoiesis to myelopoiesis by mediating inflammatory signaling pathway [31], and played an important role during megakaryopoiesis and platelet formation as the regulators of cytoskeletal organization [32]. The current study found that sphinganine was the key metabolite related to behavioral indicators and hematological indicators based on metabolomics analysis, indicating that sphingolipid metabolism was in-

Table 2 Components in AS for further analysis after ADME screening

ID	Components	Mr	Log*P*	Caco	HDON	HACC	RBN
AS1	Spermidine	145.29	−1.17	−0.12	5	3	7
AS2	Pipecolinic acid	129.18	0.4	0.32	2	3	1
AS3	Adenine	135.15	−0.58	−0.3	3	4	0
AS4	Nicotinic acid	123.12	0.28	0.34	1	3	1
AS5	Nicotinamide	122.14	−0.32	0.44	2	3	1
AS6	6-Hydroxypurine	136.13	−0.03	0.09	2	4	0
AS7	*p*-Coumaric acid	164.17	1.64	0.46	2	3	2
AS8	Uracil	112.1	−1.01	0.05	2	4	0
AS9	3-Formylindole	145.17	1.88	1.25	1	1	1
AS10	Caffeic acid	180.17	1.37	0.27	3	4	2
AS11	4-Hydroxycoumarin	162.15	1.34	0.77	1	3	0
AS12	7-Hydroxycoumarin	162.15	1.63	0.74	1	3	0
AS13	Phthalic acid	166.14	1.04	−0.05	2	4	2
AS14	Chlorogenic acid	354.34	−0.42	−1.03	6	9	5
AS15	Vanillic acid	168.16	1.15	0.43	2	4	2
AS16	Coumarin	146.15	1.9	1.2	0	2	0
AS17	Hymecromone	176.18	2.08	0.78	1	3	0
AS18	7-Methoxycoumarin	176.18	1.88	0.97	0	3	1
AS19	Isoferulic acid	194.2	1.62	0.49	2	4	3
AS20	Ferulic Acid	194.2	1.62	0.47	2	4	3
AS21	8-Hydroxy-6,7-dimethoxycoumarin	222.19	1.46	0.89	1	4	2
AS22	Senkyunolide G	208.28	2.54	0.63	1	3	3
AS23	Senkyunolide D	222.26	1.8	0.12	1	4	3
AS24	Senkyunolide I	224.25	2.74	0.87	1	3	2
AS25	Coniferyl ferulate	356.4	3.64	0.71	2	6	8
AS26	Scoparone	206.21	1.87	0.85	0	4	2
AS27	Calycosin	284.28	2.32	0.52	2	5	2
AS28	Dibutyl phthalate	278.38	4.2	0.8	0	4	10
AS29	Senkyunolide A	192.28	3.19	1.3	0	2	3
AS30	4'-Hydroxyacetophenone	136.16	1.3	0.87	1	2	1
AS31	Osthole	244.31	3.74	1.15	0	3	3
AS32	Sedanolide	194.3	3.37	1.24	0	2	3
AS33	Cinnamic acid	148.17	1.9	0.91	1	2	2
AS34	*Z*-Ligustilide	190.24	3	1.3	0	2	2
AS35	*E*-ligustilide	190.24	2.94	1.28	0	2	2
AS36	Senkyunolide O	380.52	4.97	0.94	0	4	4
AS37	Psoralen	186.17	2.2	1.05	0	3	0

volved in the comorbidity mechanisms of depression and blood deficiency syndrome, as well as the therapeutic mechanisms of AS. Consistent with the results of metabolomics analysis, sphingolipid metabolism was also predicted to be involvled in the anti-depression effect and nourishing blood effect of AS in network pharmacology analysis.

HIF-1 signaling pathway HIF-1, a heterodimeric transcription factor composed of alpha (α) and beta (β) subunits, has been discovered to regulate hundreds of genes, including vascular endothelial growth factor (VEGF), lactate dehydrogenase-A (LDHA), glucose transporter-1, 3 (GLUT1, 3), insulin-like growth factor-2 (IGF-2), erythropoietin (EPO),

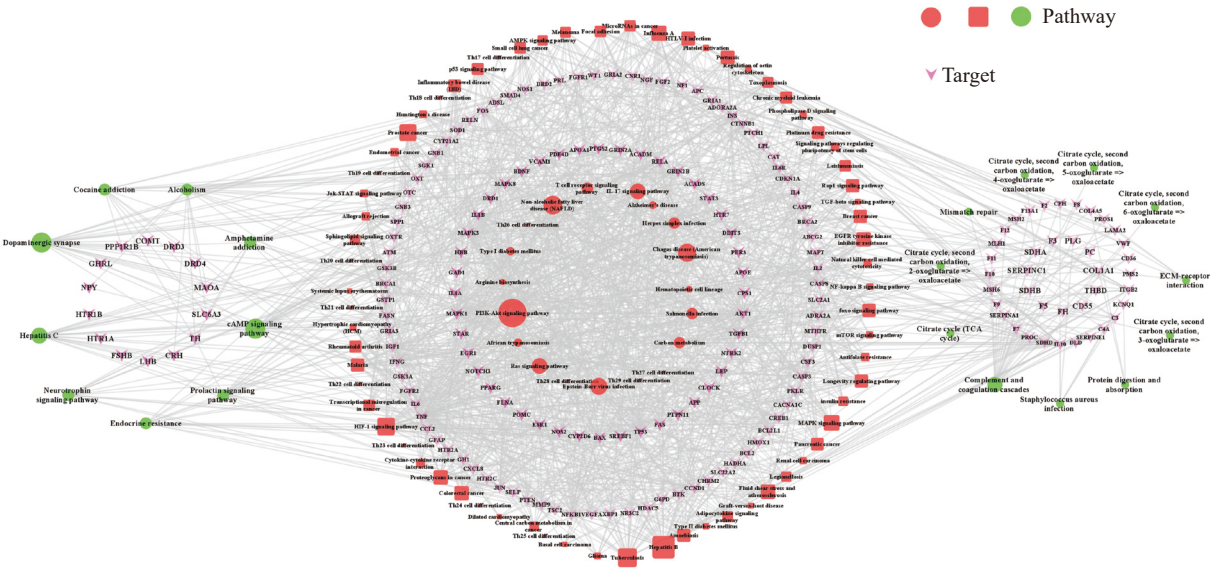


Fig. 7 Target-pathway network of depression and blood deficiency syndrome

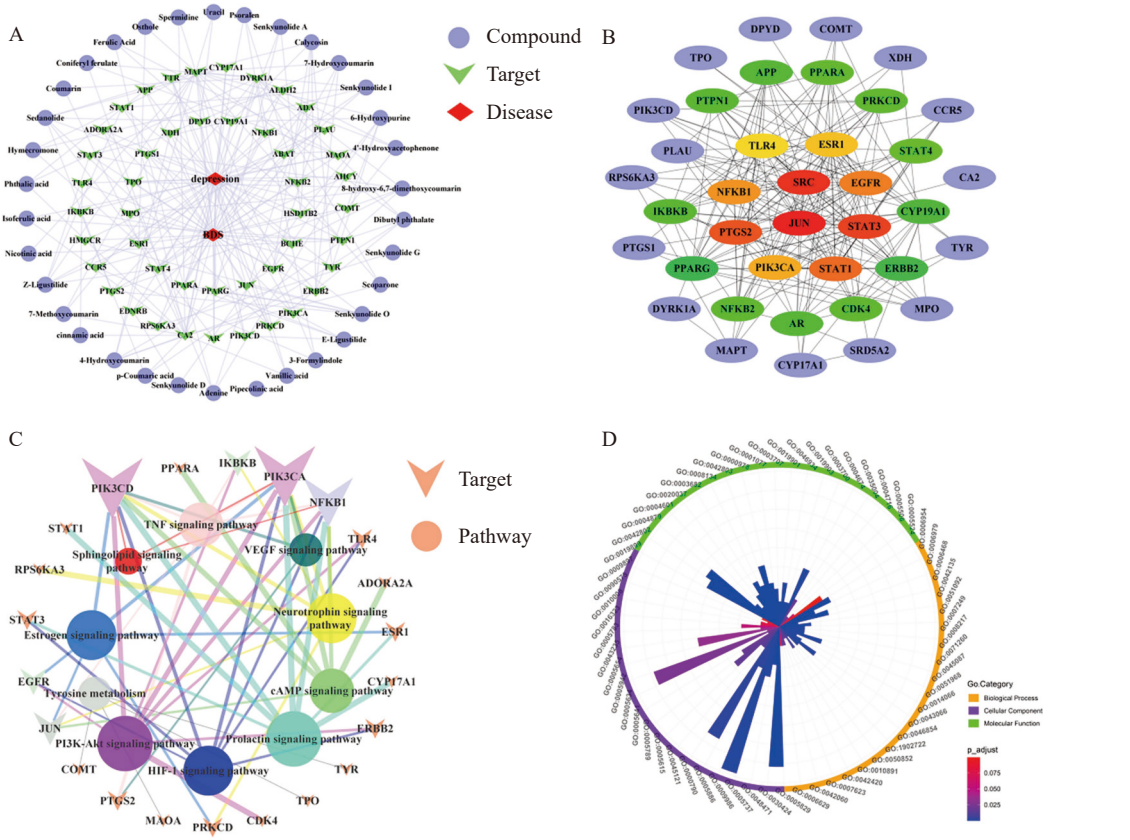


Fig. 8 (A) Ingredient-target-disease network. (B) PPI network of the key targets. (C) Target-pathway network related to the ADE and NBE of AS. (D) GO functional enrichment analysis

phosphoglycerate kinase1 (PGK1), pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK-1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), and BCL2 [33, 34]. Under the pathological conditions, the expression of these genes is activated by HIF-1, which then influences glucose metabolism, cell proliferation/survival, and apoptosis [35]. Evidence has demonstrated that HIF-1 is involved under the pathological conditions such as inflammation, hypoxia,

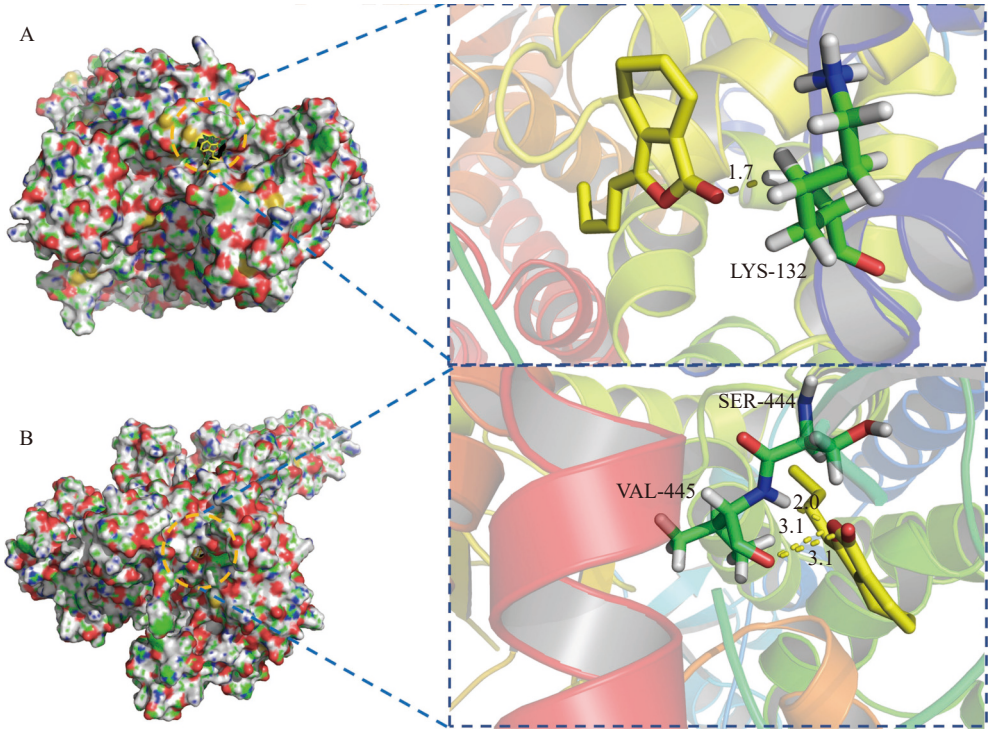


Fig. 9 3D interaction diagrams between *E*-Ligustilide and the targets of PIK3CA (A) and PIK3CD (B) in sphingolipid signaling pathway. Active site amino acid residues are represented as stick model with green colored, while the inhibitor is shown as stick model with yellow colored.

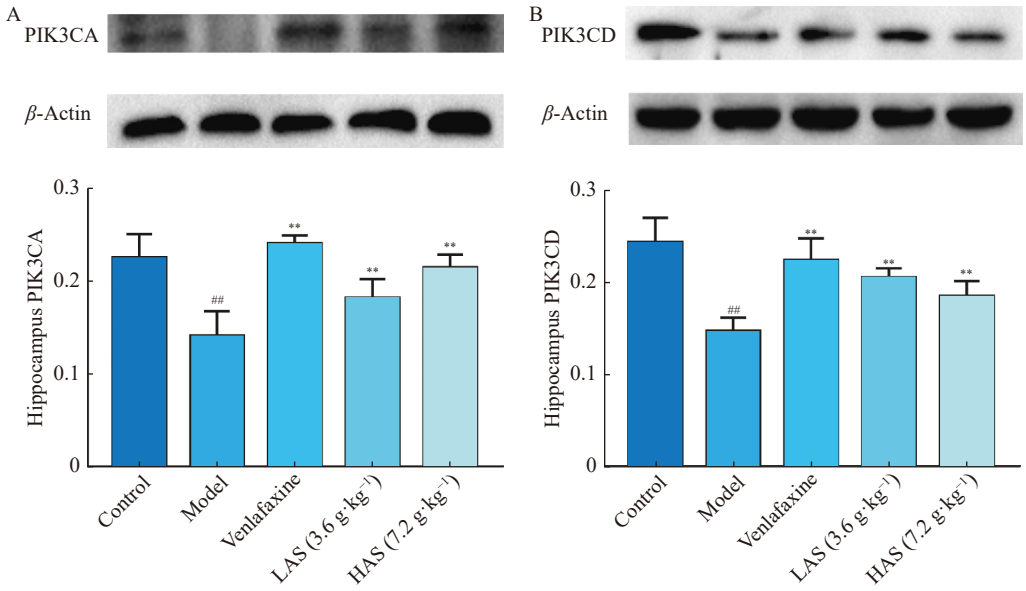


Fig. 10 Effect of Angelicae Sinensis Radix on the expression of PIK3CA (A) and PIK3CD (B) in the hippocampus of rats. Data are presented as means ± SD (n = 5). $^{\#\#}P < 0.01$ *vs* the control group; $^{*}P < 0.01$ *vs* the CUMS group

ischemic disease, and cancer [36-38]. Recently, it has been proved that HIF-1 signaling pathway is associated with the pathophysiology of depression. For instance, Shibata *et al.* proved that the mRNA expression of HIF-1 and its target genes (LDHA, VEGF, PGK1, GLUT1, and PFKFB3) in the peripheral white blood cells of patients with major depressive disorder (MDD) was upregulated [39]. Li *et al.* demonstrated that HIF-1 signaling pathway might be involved in the pathogenesis of depression based on metabolomics study [34]. In the present study, we demonstrated that seven targets including EGFR, ERBB2, NFKB1, PIK3CA, PIK3CD, STAT3 and TLR4 in HIF-1 signaling pathway were involved in the comorbidity mechanisms of depression and blood deficiency syndrome based on network pharmacology analysis, as well

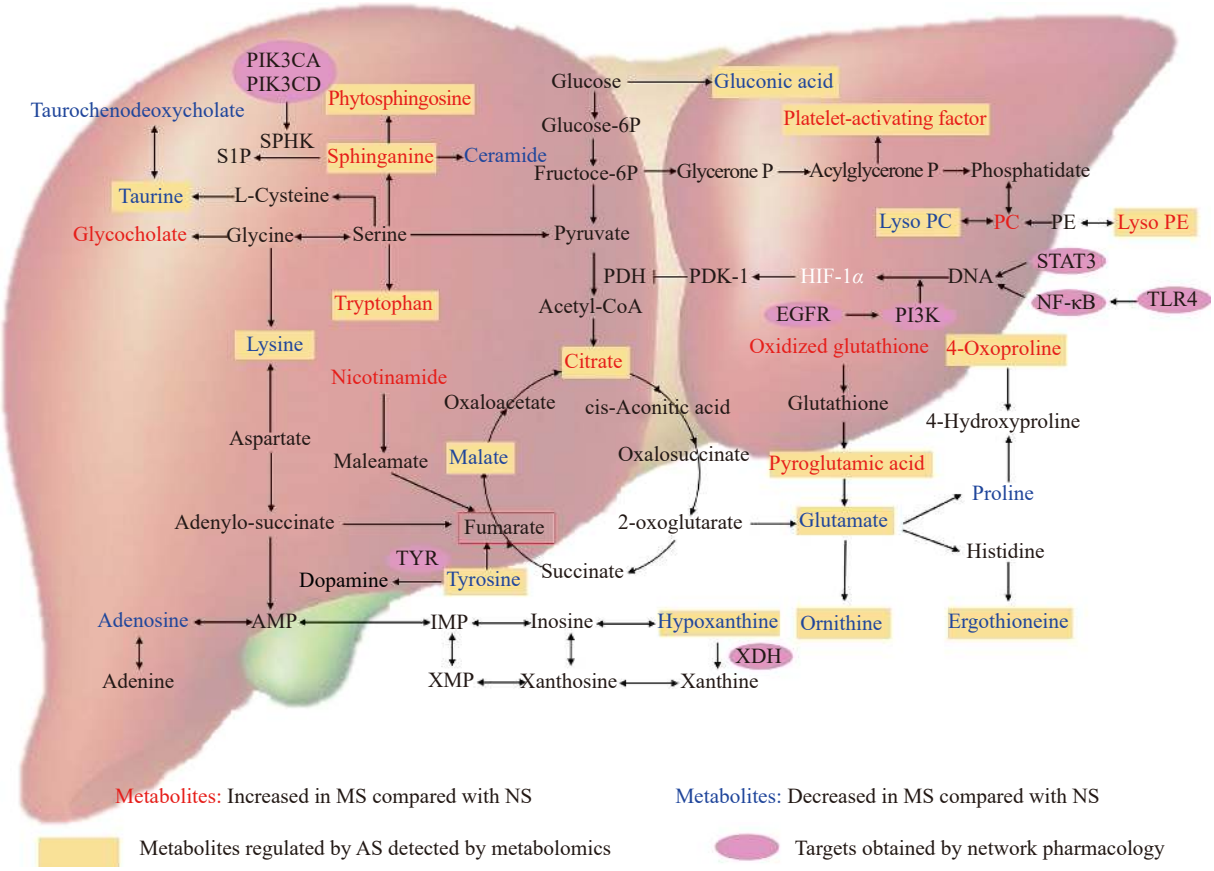


Fig. 11 Representation of pathways involved in the correlation of the anti-depression effect and nourishing blood effect of AS

as the mechanisms of the anti-depression effect and nourishing blood effect of AS. The protein expression of the target genes (LDHA and PDK-1) of HIF-1α has been detected by Western blot in our previous study [18], which supported the results of this study.

Liver-brain axis Liver-brain axis plays an important role in the pathophysiological function of hypohemia-related depression. The liver is an organ resposible for metabolic function. Recently, the liver has been valued by researchers for its important function in the liver-brain axis. Dysfunctional and structural abnormalities of the liver may cause dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis [40], reducing hippocampal neurogenesis [41], and affecting neuroplasticity [42] and neurosteroidogenesis [43]. These pathophysiological processes may exacerbate depression by acting on related brain areas. Conversely, neural signals from the central nervous system (CNS) affect glucose, lipid and protein metabolism in the liver [44]. Besides, the dysfunctional abnormalities of the liver induced by the change of neural signals from the CNS may influence the production and secretion of hepcidin, a crucial peptide hormone responsible for regulating both iron absorption and erythrocyte recycling, followed by exacerbation of blood deficiency symptom [45, 46]. Our findings primarily quantify an overall liver metabolic profile in the rat model of hypohemia-related depression and elucidate novel insight (liver-brain axis) into the multifaceted mechanisms of correlation of the anti-depression effect and nourishing blood effect of AS. In the future, in-depth studies will be performed to explore the invovlement of liver-brain axis in the mechanisms of hypohemia-related depression, and correlation between the ADE and NBE of AS.

Predicted correlation mechanism Integrating the results of metabolomics, network pharmacology and molecular docking in the current study, we found that sphingolipid metabolism is the core pathway involved in the mechanisms of correlation between the ADE and NBE of AS. In the depressed rats, the abnormalities of neural signals from the CNS may affect sphingolipid metabolism in the liver, such as accumulation of ceramide (Cer) and sphinganine (Sph). Cer accumulation influence the expression of hepcidin, with a consequent change in intracellular iron content [47]. We speculated that AS could less inhibited ferroportin (Fpn) through regulating sphingolipid metabolism and hepcidin expression, promote iron release to the blood stream and binding to the trasporter fransferrin (Tf) in its ferric form (Fe^{3+}), then reach the bone marrow to contribute to the hematopoietic response, and finally resulted in improvement in the blood deficiency symptom of the CUMS-rats. On the other hand, AS may act on a special lipid region formed by cholesterol, polyunsaturated fatty acids, and sphingolipids on the membrane that mediates neurotransmitter signaling *via* G-protein coupled receptors and ion channels [48], in order to exert anti-depressant

effect. Furthermore, AS may affect the key enzymes in sphingolipid metabolism and the production of sphingosine-1-phosphate (S1P) [49], an oxygen-independent regulator of HIFs that regulates the disorder of downstream metabolic pathways. In the future, molecular biology experiments will be conducted to verify the predicted correlation mechanisms.

Limitations The results of the current study are of great significance to reveal the correlation between the anti-depression effect and nourishing blood effect of AS and elucidate the underlying mechanisms. However, this study still has several limitations. First, there are only two dose groups of AS, with respect to the effective doses reported in previous studies. It is necessary, in the future, to explore the dose-response relationship of AS on depressed rats based on multipe dose groups. Second, although that all human genes known to be associated with diseases have orthologues in rat genome [50], different species used in metabolomics analysis and network pharmacology analysis still influence the following correlation analysis. In the future, more attention should be drawn to clinical samples for exploring the metabolic mechanisms in a more rational manner.

Conclusion

In the current study, hepatic metabolomics, network pharmacology and molecular docking are used to explore the underlying mechanisms of correlation between depression and blood deficiency syndrome, as well as relationship between the ADE and NBE of AS. The results demonstrate that three metabolites including glutathione, sphinganine, and ornithine, as well as 46 relevant targets are related to the ADE and NBE of AS. Integrating the results of metabolomics, network pharmacology and molecular docking, sphingolipid metabolism is the core pathway involved in the mechanisms of correlation between the ADE and NBE of AS. These findings suggest that metabolomics combined with network pharmacology and molecular docking can serve as a potent approach for exploring the mechanisms of diseases, as well as the therapeutic mechanisms of TCM.

Supporting Information

Supporting information can be requested by sending E-mail to the corresponding author.

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