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Exploring of Antidepressant Components and Mechanisms of Zhizichi Decoction: Integration of Serum Pharmacochemistry, Network Pharmacology and Anti-inflammatory Analysis Verification

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ABSTRACT

Using LC–MS to screen and analyse the characteristics of components and biological systems is a new approach to study the pharmacological substance basis of traditional Chinese medicine, which has strong novelty in analytical science. This study analyses the antidepressant material basis of Zhizichi decoction through the integration strategy of serum pharmacochemistry, network pharmacology and immunoreactivity verification, which helps in overcoming the limitations of TCM research and provided a new perspective and approach for studying the components of ZZCD. First of all, blood from SD rats was collected before and after Zhizichi decoction administration initially. The migration constituents in the serum were then analysed using ultra-high performance liquid chromatography-Q-TOF-MS/MS. By integrating the TCMSP databases with the serum pharmacochemistry results, we constructed the 'ingredients–targets–pathways' network and the protein–protein interaction network for Zhizichi decoction's depression-relieving. Finally, the inhibitory effects of Zhizichi decoction and active ingredient groups comprised of pharmacodynamic components identified in prior network pharmacology study on IL-1β, IL-6 and TNF-α were measured through an inflammatory cytokines experiments. From the serum pharmacochemistry study, 146 migration constituents in serum and their attribution were hypothesized and characterized. They were identified as 18 prototype components and 128 metabolites, of which

Abbreviations: ADME, absorption, distribution, metabolism and excretion; AIGs, active ingredient groups; ANOVA, a one-way analysis of variance; AS, ankylosing spondylitis; BC, betweenness centrality; BDNF, brain-derived neurotrophic factor; BPCs, base peak chromatograms; CC, closeness centrality; CE, collision energy; CID, collision-induced dissociation; CUMS, chronic unpredictable mild stress; DAVID, Database for Annotation, Visualization and Integrated Discovery; DC, degree centrality; DL, drug-likeness; DMEM, Dulbecco's Modified Eagle Medium; DP, declustering potential; FBS, foetal bovine serum; GnRH, Gonadotropin-releasing hormone; GO, gene ontology; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IRS, insulin receptor substrate; LC-Q-TOF/MS, liquid chromatography quadrupole time-of-flight mass spectrometry; NGF, nerve growth factor; NT-3, neurotrophin 3; NT-4, neurotrophin 4; NTFs, neurotrophic factors; OB, oral bioavailability; p75NTR, p75 neurotrophin receptor; PPAR, peroxisome proliferator activated receptor; PPI, protein–protein interaction; TCM, traditional Chinese medicine; TCMSP, Traditional Chinese Medicine Systems Pharmacology; TIC, total ion chromatogram; TLRToll-like receptor, Toll-like receptor; TNF-α, tumour necrosis factor-alpha; VEGF, Vascular endothelial growth factor; XICs, extracted ion chromatograms; ZZCD, Zhizichi decoction.

Chuan Chai and Bo Jin contributed equally to this work.

121 were Phase I and 7 were Phase II metabolites. The Zhizichi decoction pharmacology network illustrated the relationships of the 20 definitive ingredients, 85 potential targets and 21 signalling pathways in connection with the depression. The targets predicted by pharmacology and protein–protein interaction network were reported to be associated with neuroinflammation, which suggested that further anti-inflammatory experiment was required. For the anti-inflammatory effect of AIGs 1 composed of 14 pharmacodynamic components was basically equivalent to that of whole ZZCD recipe, AIG 1 was hypothesized to be the critical pharmacodynamic components to inhibit inflammatory factors and defined as the antidepressant components' of Zhizichi decoction, providing a scientific foundation for the pursuit of potential new drugs for depression treatments.

1 | Introduction

Zhizichi decoction (ZZCD), a traditional Chinese medicine (TCM) formula originally recorded in 'Shanghan Lun' (*Treatise on Cold Pathogenic Diseases*) [1] dated thousands of years ago. It is composed of *Gardenia jasminoides* (*G. jasminoides*) Ellis and *Sojae semen praeparatum*, a product made from mature soybean seeds prepared via Chinese style fermentation [Glycine max (L.) Merr.] [2, 3]. ZZCD has the ability of clearing away heat, dispelling annoyance and dispersing gloomy heat [4]. It has been regarded as an effective recipe, used extensively for more than a century to treat depression in Chinese therapeutic practice.

Previous studied have focussed primarily on the chemical components and metabolic changes of ZZCD in vivo. Pharmacokinetic evaluations of five distinct combinations of ZZCD in rats were analysed by simultaneously determining the plasma concentrations of geniposide and genistein [5]. Several ZZCD ingredients and their metabolites in rat bile and urine were examined by using ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS) [6]. Other studies have focussed on the metabolomics of ZZCD related to depression. To look into ZZCD's neuroprotective effects, pharmacology and cell metabolomics were combined. [7]. Following oral administration, a total of 56 chemicals were detected in the full metabolic profile of ZZCD in the faeces of chronic unpredictable mild stress (CUMS)-induced depression and normal rats [8]. In our previous study, we applied serum metabolomics to identify potentially typical metabolites ZZCD metabolic pathways in mouse serum generated by CUMS. These findings were further verified by analysing the signalling pathway's target expression in mouse hippocampi [9]. There are also some discussions about the mechanism of ZZCD in network pharmacology-based depression treatment [10, 11]. However, the treatment of depression, a severe and intricate central nervous system illness, remains challenging as its mechanism has not yet been thoroughly elucidated [12, 13]. There are still some unfilled gaps in the global system biology research on the material and mechanism of ZZCD-based relieving depression.

Systems biology is consistent with the 'holistic theory' of TCM, limited to vague characterization of internal systems [14]. Chemomics is suitable for the material basis research of prescriptions with significant therapeutic effects, limited to the expression of information in external systems [15]. It can be a supplementary reference to systems biology but cannot be directly related to it. How to integrate the two and conduct a systematic, comprehensive and dynamic study on the material basis and mechanism of action of ZZCD for relieving depression? The research of network biology and the validation of pharmacological and pharmacodynamic indicators are the technical bridges that can connect the interactions between systems.

Network pharmacology can systematically, comprehensively and dynamically study the TCM material basis and its mechanism of action [16, 17]. Because TCM works through the combination of small molecules and the target protein of organism [18]; to explore the ZZCD material basis of relieving depression, it is necessary to build the action network of its effective components and targets. Biological processes at different levels could be represented by gene regulation network, protein interaction network and metabolic network [19]. To study the mechanism of ZZCD in relieving depression, it is necessary to build a network related to genes, proteins, metabolites and depression in its system biology research. Some studies have utilized multiple databases to establish a metabolic disease network, elucidating the relationship between diseases and genes from a global perspective [20, 21]. However, the conclusions drawn from these studies lack reliability as the information on chemical components used in network pharmacology analyses is not derived from blood or tissue samples, but rather from databases and literature references.

TCM is predominantly administered orally, and its active constituents must be transported via the bloodstream to exert their effects [22]. Consequently, post-administration serum is the primary focus, containing the direct active substances responsible for TCM's therapeutic effects [23]. The field of serum medicinal chemistry in TCM, a nascent area of research, combines pharmacological substances with scientific TCM studies. Integrating this with network pharmacology could more accurately depict TCM's actual effects in the body [24]. To date, there have been no reports on the discovery of compound compositions in ZZCD based on the synergy between network pharmacology and serum pharmacochemistry.

Liquid chromatography-mass spectrometry (LC–MS) represents a sophisticated and comprehensive analytical technique. Unlike traditional methods that isolate single chemical components from complex matrices for subsequent identification by spectroscopy and mass spectrometry, LC–MS integrates chromatography and mass spectrometry to streamline sample separation, qualitative analysis, and quantification. Recently, the rapid advancement of LC–MS in analytical science has broadened its application in various TCM research areas, including the qualitative and

quantitative analysis of complex mixtures, in vivo metabolism, pharmacokinetic studies, pharmacological basis research, and quality control of Chinese medicinal materials, thus advancing the modernization of TCM. The complexity of studying TCM's chemical compositions and compound compatibilities underscores the significance and promising future of LC–MS in this research domain. Employing LC–MS to explore ingredient characteristics and biological systems offers a novel method to investigate the pharmacological bases of TCM, showcasing significant innovation in analytical science. LC-Q-TOF/MS is now deemed an indispensable tool for examining migration constituents in serum, investigating their reactions and metabolism, and determining the material basis of TCM [25–27].

Pharmacodynamic analysis can be used to validate and supplement the results of network pharmacology [28–30]. Depression is often accompanied by pain symptoms [31, 32]. The TCM posits that when liver constraint transform into fire, the body enters an inflammatory state, AND exhibit neurohumoral metabolism disorder and sympathetic nervous system overexcitement [33]. Furthermore, a number of investigations have revealed antidepressant effects of anti-inflammatory treatment [34–38].

Therefore, we hypothesized that there is a correlation between the depression-relieving and anti-inflammatory effects of ZZCD, tailed with the network pharmacology results. Investigating the 'multi-component, multi-target, and multi-pathway' mechanism of ZZCD in relieving depression through network pharmacology combined with serum pharmacochemistry is the goal of the current study. On top of that, we aimed to identify the 'antidepressant components' via anti-inflammatory analysis verification.

2 | Materials and Methods

2.1 | Chemical and Materials

G. jasminoides Ellis was bought from KANGTAI TCM Co. (Jurong, China), and *Sojae semen praeparatum* were bought from YiFeng TCM shop (Nanjing, China).

The reference standards of geniposidic acid, deacetyl asperulosidic acid, deacetyl asperulosidic acid methyl, scandoside methyl ester, shanzhiside, shanzhiside methyl ester, genipin 1-β-gentiobioside, geniposide, gardenoside, crocin-I, genipin, crocin-II, crocetin, daidzin, daidzein, genistin, genistein, glycitin, glycitein, rutin, isoquercetin, quercetin, salicylic acid, vanillic acid, malic acid, cinnamic acid, ferulic acid, gallic acid, protocatechuic acid, protocatechuic aldehyde, caffeic acid, chlorogenic acid and p-coumaric acid were all bought from Sigma-Aldrich (St. Louis, MO, USA)

LC/MS-grade formic acid, methanol, acetonitrile and water were purchased from Merck Co. (Darmstadt, Germany).

BV2 microglia cells were acquired from China Center for Type Culture Collection (CCTCC, Wuhan, China). Dulbecco's Modified Eagle Medium (DMEM) high-glucose complete medium, foetal bovine serum, penicillin/streptomycin and all other required cell culture materials were bought from Gibco-BRL (New York, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) and Lipopolysaccharide (LPS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tumour necrosis factor-alpha (TNF-α), IL-6 and IL-1β ELISA kits were purchased from Thermo Fisher Scientific Inc. (Shanghai, China).

2.2 | Chromatographic-Mass Spectrometric Conditions

A triple time-of-flight mass spectrometer (TripleTOF 5600 system, AB Sciex, Foster City, CA, USA) with an electrospray ionization source was linked to an ultra-high performance liquid chromatography (UHPLC) system (Shimadzu Corporation UHPLC XR, Kyoto, Japan).

An injection volume of 10 μL, including ZZCD solution, standard solutions, control serum samples and drug-containing serum samples, were all separated by a XBridge C_{18} column (4.6 × 100 mm, 3.5 μm). A binary solvent gradient consists of Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile with 0.1% formic acid). The gradient was ran for a total of 30 min with the following profile: from 0 to 5 min, the gradient went from 10% to 50% B; from 5 to 22 min, the gradient went from 50% to 95% B; from 22 to 24 min, the gradient was held at 95% B; from 24 to 25 min, the gradient went from 95% to 10% B; and from 25 to 30 min, the gradient was held at 10% B for the final washing and column equilibration. The column temperature was maintained at 40°C.

They were then analysed on the hybrid system with scanning mass range of 100–1500 m/z in both positive and negative ion modes. A calibrated delivery system was used to ensure the accuracy of mass less than 1 ppm. The ion spray voltage was set to 5500 V in positive ion mode and −4500 V in negative ion mode, together with the following ambient parameters: temperature of 500°C, curtain gas pressure of 35 psi, heater gas pressure of 55 psi and nitrogen gas for nebulization at 55 psi. High resolution settings were used for the TripleTOF MS spectrum acquisition survey. For the positive ion mode, the optimal declustering potential (DP) and collision energy (CE) were chosen at 80 and 15 eV, and for the negative ion mode, at −80 and −15 eV. A sweeping CE setting at 35/−35 eV ± 15 eV was applied for collision-induced dissociation (CID). Data acquisition and processing analysis were carried out using the Analyst TF 1.6 software (AB Sciex, Foster City, CA, USA).

2.3 | Animal Handling

A total of 16 healthy male SD rats (weighing 200 ± 20 g, animal license no. SCXK2017-0011, qualified no. 311620400001018) were purchased from Qinglongshan Animal Resources Centre (Nanjing, China). All animal experiments were approved by the animal experiment ethics committee of Nanjing University of Chinese Medicine, under ethics application number 201905A031, which complied with the Ministry of Health's Detailed Rules for the Implementation of the Administration of Medical Laboratory

Animals, State Science and Technology Commission's Regulations on the Administration of Laboratory Animals, ARRIVE guidelines and the AVMA euthanasia guidelines 2020. Before the experiment, the rats were acclimated for at least a week in a climate-controlled room with a 12-h light/dark cycle, 23–27°C and 30–70% humidity. The rats was then randomly divided into a ZZCD group ($n = 8$) and a control group ($n = 8$). According to the previous research [39], rats in the ZZCD group received ZZCD at a dose of 2 g/mL/100 g body weight in a single administration. Rats in the control group were given the same amount of ordinary water orally.

2.4 | Cell Culture

BV2 cell lines were maintained in DMEM high-glucose complete medium supplemented with 1% (v/v) penicillin/streptomycin and 10% (v/v) foetal bovine serum (FBS). Cells were plated on a 75 cm^2 flask in a 5% CO_2 incubator at 37°C. For the next few trials, the culture medium was swapped out every two to three days.

2.5 | Standard Solutions and ZZCD Sample Preparation

By precisely weighing the standard ingredients and combining them with 70% methanol, the mix standard solution was created. This standard mixture was filtered by a filter membrane (0.22 μm).

ZZCD is made up of *Sojae semen praeparatum* and *G. jasminoides* Ellis at the ratio of 105:248. The decoction was prepared in the laboratory following the procedures given in full by Shanghan Lun [1].

The procedures and parameters were listed as follows: *G. jasminoides* Ellis (1470 g) was cooked with water (56 L) until the combination was reduced to 35 L. *Sojae semen praeparatum* (3472 g) was then added and decocted until the mixture was reduced to 21 L. The filtered decoction was concentrated by freeze drying. The measurement of extract yield was around 35.52% (w/w). For subsequent oral administration, water was added to the extract to dissolve it to a relative concentration of 2 g/mL. For the follow-up UHPLC-Q-TOF-MS/MS analysis, it was 10 times diluted with water via vortex mixing for 1 min. The mixture was then rediluted with 50% methanol to a relative concentration of 1.75 mg/mL and then centrifuged at 12000 rpm for 10 min. After being moved to a different tube, the supernatant was filtered by a filter membrane (0.22 μm). For subsequent cell treatment, ZZCD whole prescription extracts were dissolved with 1% methanol to a relative density listed in Table S1.

Active ingredient group (AIG) 1 was composed of the p-coumaric acid, quercetin, shanzhiside, geniposide, deacetyl asperulosidic acid, genipin 1-β-gentiobioside, gardenoside, geniposidic acid, scandoside methyl ester, daidzein, daidzin, genistein, genistin and glycitin. The 14 active ingredients belonged to the 20 definitive ingredients derived from the previous network pharmacology study, which was mixed according to their original proportion in ZZCD. Ethyl oleate, mandenol and stigmasterol were removed for their nondetection, whereas kaempferol, 3-methylkaempferol and isoimperatorin were not included due to their low quantity in ZZCD.

AIG 2 was composed of p-coumaric acid, deacetyl asperulosidic acid, genistin, daidzin and glycitin. The five active ingredients belonged to the seven main contributing pharmacodynamic components related to the top 10 associated targets with higher degree and betweenness derived from the previous network pharmacology study, which was mixed according to their original proportion in ZZCD. Kaempferol and 3-methykaempferol were also not included for their low quantity.

2.6 | Serum Pharmacochemistry Study

2.6.1 | Collection and Preparation of Rat Serum

The rat blood was drawn from the orbital vein before administration and at 0.5, 1, 1.5, 2 and 4 h after administration. After allowing to settle for 30 min, The blood was centrifuged at 4000 rpm for 10 min after it had settled for 30 min to extract the serum, which was then kept at −80°C until analysis. After the blood collection, all the rats were euthanized. Prior to analysis, the serum samples were thawed at room temperature. A 200 μL aliquot of each serum sample was mixed with 800 μL of methanol for protein precipitation and then vortexed for 1 min. Following a 10-min centrifugation at 12,000 rpm, the supernatant was vacuum-evaporated to dryness at 45°C. The dried residue was reconstituted with 100 μL of methanol. The solution was then vortexed for 1 min and centrifuged at 12,000 rpm for additional 10 min; the supernatant was transferred to another tube and filtered through a filter membrane (0.22 μm).

2.6.1.1 | Data Processing of Migration Constituents in Serum. Peakview 1.2 software (AB Sciex, Foster City, CA, USA) was used for post-acquisition analysis. It used a comprehensive list of fragmentation information to generate a number of extracted ion chromatograms (XICs). In comparison, the endogenous components present in both the control and drug-containing serums were subtracted. The ion peaks present in the drug-containing serum chromatogram, but not in the control serum chromatogram, were identified, These were hypothesized to be the blood transitional components of ZZCD. The hypothesized components were compared with the standard solution chromatogram, along with factors such as retention time, accurate molecular weight, mass charge ratio and secondary fragments. If a successful match occurs, they will be defined as the blood transitional prototype components of ZZCD.

Furthermore, the total ion chromatogram (TIC) of the prototype components standards was imported into MetabolitePilot 1.5 programs to establish a compound library database. Biotransformation parameters were subsequently set to Phase I and Phase II metabolic pathways. The drug-containing serum and control serum chromatograms were then introduced as samples and blank controls, respectively. Finally, the potential metabolites were identified based on the metabolite retention time, secondary fragments in accordance with both the software and relevant references.

2.7 | Network Pharmacology Study

2.7.1 | Prediction and Docking of Components and Targets

In order to determine the components of ZZCD, we enlisted a database by searching Traditional Chinese Medicine Systems Pharmacology (TCMSP) server using '*Gardenia jasminoides* Ellis' and '*Sojae semen praeparatu*' as the keywords. A literature search in PubMed Central of the NCBI database led to the manual supplementation of the TCMSP components. According to absorption, distribution, metabolism and excretion (ADME) properties, drug-likeness (DL) ≥ 0.18 and oral bioavailability (OB) $\geq 30\%$ were set as filtering criterion for candidate components. The results of serum pharmacochemistry were also referenced.

To reverse predict the targets of candidate molecules in ZZCD, we first converted the compound structure into the SDF (*.sdf) structure format by ChemBio3D Ultra 12.0 software. These files were then uploaded to the PharmaMapper server website, and targets with fit-score values above 4.5 or the top 10 targets (even if their values were below 4.5) for each compound, were selected. It was followed by adjusting all the ZZCD targets to their official symbols using gene tool from NCBI database for the additional analysis. The gene name and ID were subsequently obtained by mapping the ZZCD targets to the UniProt database.

To predict the target genes associated with depression, we searched GeneCards, DisGeNet, CTD and TTD databases using 'depression', ' depressive', ' depressed' and 'antidepressant' as the keywords.

The ZZCD targets and disease genes were matched, and then the overlapping targets' PDB-ID searching were performed using PDB website. The data were further docked to the candidate components using SYBYL X 2.1 software by generating a total-score for each connection. The higher the score, the more reliable the reliability in the interaction. Consequently, we chose the docked components and their top 10 total-score value targets as assigned components and targets for subsequent study.

2.7.2 | Gene Ontology Enrichment and KEGG Pathway Annotation Analyses

To understand the functions and processes that might be influenced by the identified targets, we submitted the targets to the Database for Annotation, Visualization and Integrated Discovery (DAVID) server for gene ontology (GO) enrichment and KEGG pathway annotation analyses. GO enrichments were categorized into cellular components analysis, molecular functions and biological processes, based on a significance threshold of $p < 0.05$.

2.7.3 | Establishment of Ingredients–Targets–Pathways Network and Protein–Protein Interaction Network

For initially exploring 'multi-ingredients, multi-targets and multi-pathways' mechanism of ZZCD in relieving depression, we constructed an ingredients–targets–pathways network with the screening pathways, along with their correlated ingredients and targets via merge function of Cystoscope 3.2.1 software. Nodes represented components, targets, and pathways in the drawn map, whereas the edges represented their association.

To screen potential ingredients and targets, the connection obtained was further used to analyse topological parameters involving betweenness and degree by Network analyzer, a plugin app of Cytoscape 3.2.1 software.

The protein–protein interaction (PPI) network was established by entering the gene ID of the potential targets into the String Version 10.5 server; Cytoscape 3.2.1 software was then used to view the results, which were saved in TSV format. Along with the possible core targets, the mid-value of the topological parameters including degree centrality (DC), closeness centrality (CC) and betweenness centrality (BC) were also acquired. They were then matched with the top pathway-associated targets in ingredients–targets–pathways network.

2.8 | Anti-Inflammatory Study

2.8.1 | Cell Treatment and MTT Assay

Anti-proliferative capacity was assessed using MTT colorimetric assay [40]. In brief, BV2 cell lines were isolated using 0.25% trypsin and then plated at a density of 5×10^4 cells/well in 96-well microplates for an overnight period. The wells were split up into 12 groups ($n = 5$) at random: a control group, a solvent containing group, a model group and three ZZCD samples treated groups, which were further divided into three subcategories of low-, medium- and high-dosage groups, following a 24-h incubation period to promote cell adhesion. Control and solvent containing groups did not received and treatments, whereas the other groups were treated with LPS (100 ng/mL) after 4 h of medium or different concentrations of ZZCD samples incubation. The supernatants were collected 24 h later for the measurement of pro-inflammatory cytokines. Then 100 μL of MTT solution were added to each well by incubating for an additional 4 h. After discarding the MTT solution, the insoluble formazan crystals in the live cells were dissolved in 150 μL DMSO and shaken for 15 min. An ELISA plate reader was used to measure the absorbance at 490 nm.

2.8.2 | Measurement of TNF-α, IL-6 and IL-1β Release

The levels of TNF-α, IL-6 and IL-1β in collected supernatants were detected by ELISA kits according to the manufacturer's instructions.

2.8.3 | Statistical Analysis

Using SPSS version 17.0 (IBM, Chicago, Illinois, USA) software, a one-way analysis of variance (ANOVA) and Tukey's test were conducted for multiple comparisons. Accepted statistical significance was $p < 0.05$. The data were presented as the means ± standard error of three separate experiments.

3 | Results and Discussion

3.1 | Serum Pharmacochemistry Analysis

3.1.1 | Method Optimization

Rats were administered ZZCD at concentrations of 10, 20 and 30 times the clinical adult dosage to optimize the administration dose. The chromatogram at the 20 times dosage exhibited a greater variety and quantity of serum components compared to the 10 times dosage. Moreover, the 30 times dosage was too viscous for oral administration and failed to produce the desired results. Consequently, 20 times the clinical adult dosage was established as the optimal administration dose.

Blood sampling times (0.5, 1, 1.5, 2, 4, 6 and 8 h after administration) were optimized to achieve stronger peak intensities for both common and unique components in the drug-containing serum. The data showed that the variety and quantity of serum components significantly decreased in the 4-h samples, indicating that the components were almost completely metabolized 4 h after administration. Thus 0.5, 1, 1.5, 2 and 4 h post-administration were selected as the optimal blood sampling times.

Serum extraction conditions, including extraction solvents (methanol and acetonitrile) and solvent volumes (2, 3, 4, 5, and 6 times), were optimized. All other variables were held constant while one was varied. The results indicated that methanol was more effective at removing impurities than acetonitrile. In addition, extracting four times provided the most optimal conditions, yielding the highest extraction efficiency. No significant increase in yield was observed with further increases in volume. Therefore, methanol and four extractions were selected as the optimal extraction solvent and volume, respectively.

To produce chromatograms with high resolution and sensitivity, composition of the mobile phase was optimized. Initially, acetonitrile was selected as the organic phase because it provided narrower peaks and shorter analysis times. In addition, 0.1% formic acid chosen as the additive for the mobile phase due to its high ionization efficiency, whereas 0.2% formic acid did not yield better results. Thus, the mobile phases selected were acetonitrile and water, both containing 0.1% formic acid and water with 0.1% formic acid.

3.1.2 | Determination of Migration Constituents in Serum

The analyses of migration components of ZZCD was conducted using UHPLC/Q-TOF-MS. The base peak chromatograms (BPCs) of control serum, 1.5-h drug-containing serum, ZZCD solution and standard solutions in both positive and negative ion modes are depicted in Figure 1. The peaks were well-separated and symmetrical, with most achieving baseline separation. A comparison of the chromatograms of the drug-containing serum and ZZCD solution with the control serum sample allowed for the identification of 146 migration constituents in the serum. These were hypothesized to originate from 18 prototype components (Figure S1) and 128 metabolites, where 121 are Phase I and 7 are Phase II metabolites.

The 18 prototype components including daidzin, glycitin, genistin, daidzein, glycitein, genistein, malic acid, p-coumaric acid, shanzhiside, genipin 1-β-gentiobioside, deacetyl asperulosidic acid, deacetyl asperulosidic acid methyl, geniposidic acid, scandoside methyl ester, gardenoside, geniposide, genipin and crocetin in drug-containing serum were hypothesized by comparing their retention times and mass spectra (Figure S2) those of standard solutions. The MS data in both ion modes, along with the maximum absorption times, are detailed in Table S2. The results indicate that maximum absorption for most prototype components occurred 1–2 h after administration. These 18 prototype components were categorized into three groups: isoflavones (6), organic acids (2), and terpenes (10). Isoflavones, primarily derived from SSP, can bind to estrogen receptors and exhibit estrogen-like effects while also regulating endogenous estrogen levels, hence their classification as phytoestrogens. Recent scientific research suggests that consuming bean products such as tofu and natto may reduce the risk of depression during pregnancy due to their estrogen-like effects [41]. Organic acids common in both GJE and SSP, have been widely reported for their antidepressant effects [42]. Terpenoids, primarily derived from GJE, are a diverse class of secondary metabolites known for their extensive biological activities and are clinically used to treat conditions including depression, Alzheimer's disease and cardiovascular diseases [43]. Studies have suggested that the rapid and lasting antidepressant effects of terpenoids may be linked to the activation of the PKA–CREB signalling pathway [44]. On the basis of traditional uses and recent pharmacological activity reports of GJE and SSP, these 18 prototype components absorbed into the bloodstream were considered the material basis of ZZCD's antidepressant effects. This analysis will form the basis for selecting candidate components in subsequent network pharmacological research.

On the basis of the processed and matched peaks provided by MetabolitePilot 1.5 software, a total of 128 metabolites related to the 18 prototype components were inferred in Table S3, including 6 from daidzin, 1 from daidzein, 7 from glycitin, 1 from glycitein, 6 from genistin, 1 from genistein, 1 from malic acid, 5 from p-Coumaric acid, 16 from deacetyl asperulosidic acid, 5 from shanzhiside, 2 from crocetin, 5 from geniposidic acid, 10 from geniposide, 2 from genipin, 19 from scandoside methyl ester or deacetyl asperulosidic acid methyl, 16 from gardenoside and 23 from genipin 1-β-gentiobioside. To characterize these metabolites, the fragmentation rules and reference standards from the literature were utilized. In addition, 62 types of metabolic modes are detailed in Table S3. The peak absorption time for most metabolites was also 1–2 h post-administration. On the basis of the traditional efficacy and the pharmacological activities of GJE and SSP, these 128 metabolites were deemed the material basis of ZZCD for its antidepressant effects. Figures S3-1 through S3-8 illustrate the relationships between the 18 prototype components, their metabolites, and metabolic pathways, elucidating their metabolic processes post-administration.

LC-Q-TOF/MS, as a more advanced and essential tool in analytical science, facilitates the examination of migration constituents in the serum and explores the reactions and metabolism of these compounds more efficiently than traditional extraction and separation methods. This technology simplifies the identification

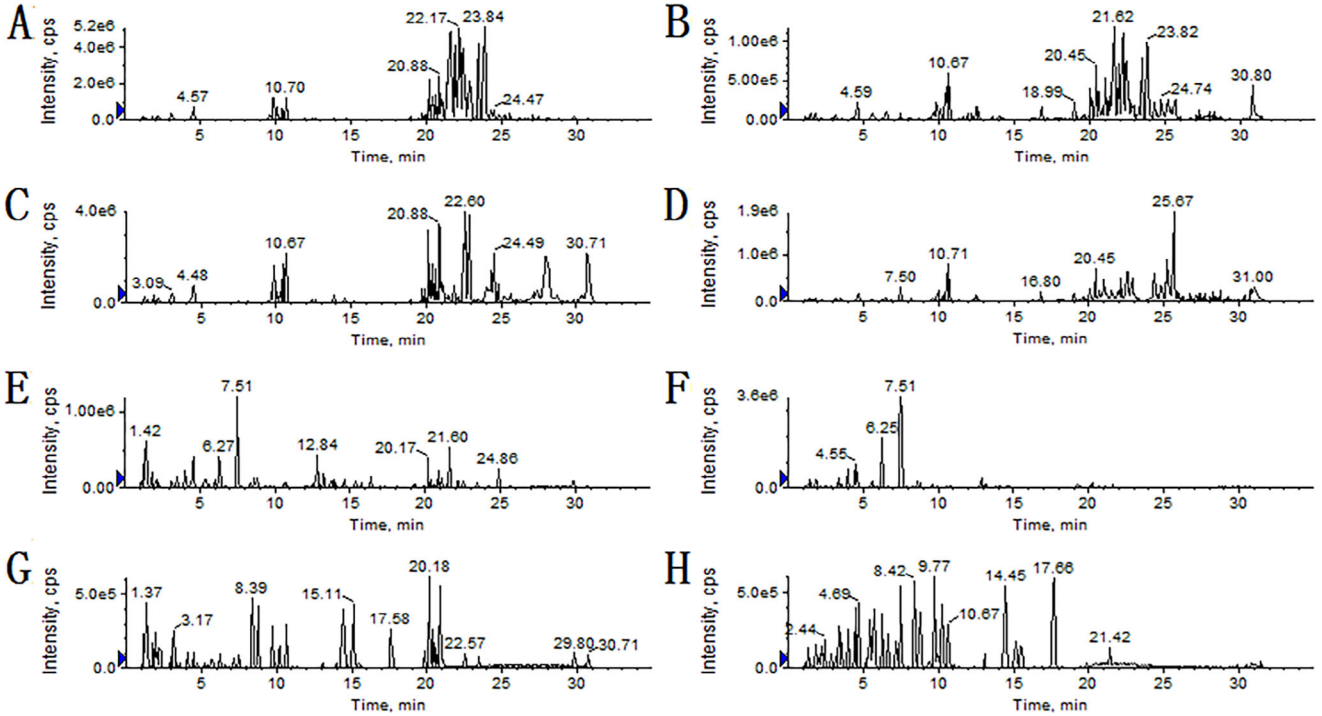

FIGURE 1 | The base peak chromatograms (BPCs) of control serum sample (A and B), 1.5-h drug-containing serum sample (C and D), ZZCD solution (E and F) and standard solutions (G and H) in positive and negative ion modes.

of the material basis of ZZCD by allowing the separation of a single chemical component from a complex system followed by its identification through spectroscopy and mass spectrometry.

3.2 | Network Pharmacology Analysis

3.2.1 | Screening and Docking of Candidate Components and Targets

A total of 16 active constituents of ZZCD were identified using the TCMSP databases, employing mathematical and computational models. Of these, 9 originated from GJE, 9 from SSP, and 5 were common compounds. Integrating the 18 constituents absorbed into the blood from ZZCD with the TCMSP results, the final cluster of ZZCD components was established after eliminating three coincidental components, leaving 31 constituents as candidates listed in Table S4.

Using the PharmMapper server, 538 ZZCD targets were obtained, with their gene names and IDs acquired via the UniProt database. A search across four databases identified 32,514 depression-related targets. Cross-referencing these targets resulted in 321 overlapping predictions.

After docking, 20 out of the 31 candidate components, respectively: p-coumaric acid, kaempferol, 3-methylkaempferol, quercetin, ethyl oleate, mandenol, stigmasterol, isoimperatorin, shanzhiside, scandoside methyl ester, deacetyl asperulosidic acid, geniposide, genipin 1-β-gentiobioside, gardenoside, geniposidic acid, daidzein, daidzin, genistein, genistin, glycitin and their related 85 predicted targets were obtained. They were identified as definitive ingredients and potential targets for further study, as indicated in Table S5, with the number of targets matched by these 20 ingredients ranging from 2 to 8 in Table S5.

3.2.2 | GO Enrichment and KEGG Pathway Annotation Analyses of Targets

The 85 potential targets underwent GO enrichment and KEGG pathway annotation analyses. The bar charts in Figure 2 display the primary terms for biological processes, molecular functions, cellular components and KEGG pathways.

The analysis of biological processes is detailed in Figure 2A and Table S6 highlighting significant processes such as the response to organic substance (19), phosphate metabolic process (18), phosphorus metabolic process (18), intracellular signalling cascade (18), phosphorylation (17), positive regulation of macromolecule metabolic process (17), homeostatic process (16) and protein amino acid phosphorylation (16). Targets were also notably involved in the positive regulation of nitrogen compound metabolic process, cellular carbohydrate catabolic process, carbohydrate catabolic process, fatty acid metabolic process, activation of the immune response, regulation of fatty acid metabolic process and regulation of lipid metabolic processes.

Molecular functions are outlined in Figure 2B and Table S7, with top functions including response to nucleotide binding (37), purine nucleotide binding (32), purine nucleoside binding (31), nucleoside binding (31), adenyl nucleotide binding (31), purine ribonucleotide binding (28), ribonucleotide binding (28), adenyl ribonucleotide binding (27) and ATP binding (24)—and other key activities such as lipid binding, steroid hormone receptor activity, and fatty acid transporter activity.

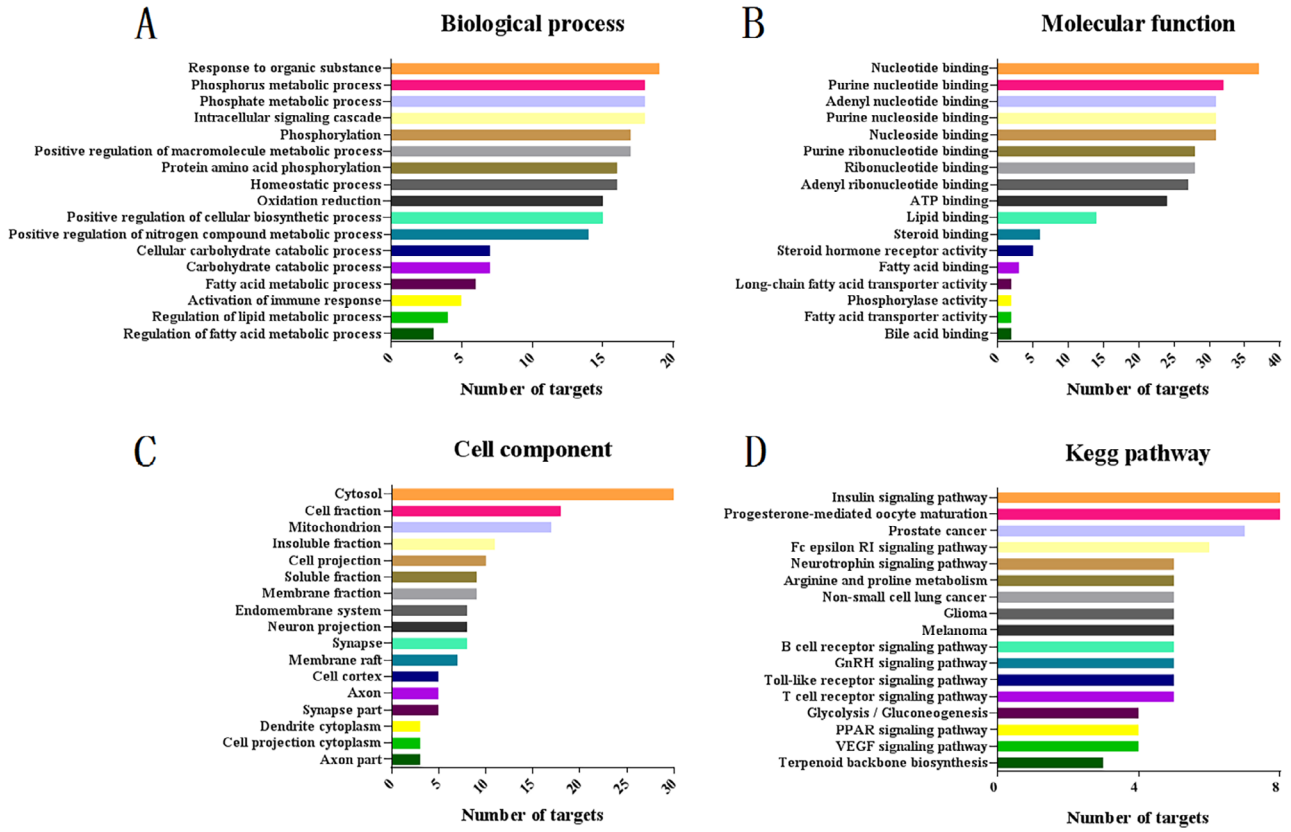

FIGURE 2 | Bar charts of Biological process (A), Cellular components (B), Molecular function (C) and KEGG pathway annotation (D) of potential targets docked with definitive ingredients in ZZCD against depression.

Cellular components are detailed in Figure 2C and Table S8, where the cytosol (30) accounted for the largest percentage, followed immediately by cell fraction (18), mitochondrion (17) and insoluble fraction (11). Notable associations were found with neuron projection, synapse, axon and dendrite cytoplasm.

This analysis indicates that depression involves numerous biological processes associated with various proteinases in vivo. Meanwhile, ZZCD may alleviate depression by enhancing these biological processes. The involvement of amino acid metabolism, neurotransmitter metabolism, carbohydrate metabolism, lipid metabolism and genetic information processing provides validation and supplements our previous metabonomic findings [9]. Further investigations into the neuro–endocrine–immune network could elucidate ZZCD's role against depression.

To confirm the involvement of depression further, 40 of the 85 potential targets and their corresponding 21 pathways were identified as pathway-associated targets and signalling pathways in Table S9 through KEGG pathway analysis. Among which insulin signalling pathway, prostate cancer, fc epsilon RI signalling pathway, prion diseases, peroxisome proliferator activated receptor (PPAR) signalling pathway, arginine and proline metabolism, melanoma, vascular endothelial growth factor (VEGF) signalling pathway, non-small cell lung cancer, glioma, signalling pathway, T-cell receptor signalling pathway, B-cell receptor signalling pathway, terpenoid backbone biosynthesis, one carbon pool by folate, neurotrophin colorectal cancer, endometrial cancer, Gonadotropin-releasing hormone (GnRH) signalling pathway, toll-like receptor (TLR) signalling pathway and glycolysis/gluconeogenesis were all directly or indirectly related to the depression (Figure 2D).

The biological significance of these 12 pathways was detailed based on information from the KEGG website and related literature:

Neurotrophin signalling pathway (*brain-associated*): Neurotrophins, part of the neurotrophic factors (NTFs) family, significantly influence the survival and differentiation of nerve cells, including neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT-4), and nerve growth factor (NGF). By binding to either the p75 neurotrophin receptor (p75NTR) or receptor tyrosine kinases, neurotrophins activate their biological functions. The NTF/Trk signal is modulated through various intracellular signalling cascades, including the PLC, PI-3 kinase and MAPK pathways, which transmit positive signals that enhance growth and survival. However, p75NTR can simultaneously transmit positive and negative signals, playing a critical role in neurodevelopment and other higher-order functions such as memory and learning [45].

VEGF signalling pathway (*brain-associated*): VEGF, a vital NTF produced by nerve cells, glial cells or inflammatory cells, participates in neuronal regeneration alongside BDNF and promotes nerve cell growth and synaptic plasticity. Therefore, it is emerging as a new focus for depression treatment [46].

GnRH signalling pathway (*brain-associated*): GnRH, secreted by the hypothalamus, primarily modulates the anterior pituitary's receptors to regulate the secretion of gonadotropins, including luteinizing hormone and FSH. GnRH levels play a critical role in sperm and egg development. Recent research indicates that nuclear transcription factor (NF-kB) in the hypothalamus becomes more active with age in mice, leading to a reduction in GnRH levels, a neuropeptide that regulates sex hormones. Consequently, aging mice exhibit senescent characteristics. Injecting GnRH into the hypothalamus has been shown to promote neuron formation and delay the aging process [47].

Melanoma (*brain-associated)*: Glioma is the most common primary brain tumour, comprising astrocytomas, oligodendrogliomas, various gliomas and ependymomas [48].

T-cell receptor signalling pathway (*immunity-associated*): T lymphocytes, crucial components of the human immune system, can eliminate target cells, assist B-cells in antibody production, produce cytokines, and respond to specific antigens. T lymphocyte activation is central to an effective immune response, involving gene transcription and expression, intracellular enzyme activation, signal transmission, stimulation, and cell growth. This process not only combats malignant cells and infections but also prevents autoimmune diseases [49].

B-cell receptor signalling pathway (*immunity-associated*): B lymphocytes, derived from pluripotent stem cells in the bone marrow, play a vital role in adaptive immunity. Upon antigen stimulation, B-cells can differentiate into plasma cells, synthesize antibodies, and contribute to humoral immunity. They can also function as antigen-presenting cells, capturing antigens and presenting them to T lymphocytes. Cellular processes mediated by B-cell antigen receptors primarily involve cell proliferation and apoptosis, abnormalities in which can lead to impaired humoral immune responses [50].

TLR signalling pathway (*immunity-associated*): TLRs were essential proteins in non-specific immunity, acting as a bridge between non-specific and specific immunity. They recognize and activate immune cell responses when microorganisms breach body barriers such as skin and mucosa. TLR4 is crucial in the pathophysiology of depression; it promotes the expression of inflammatory factors and induces an inflammatory response by activating relevant signal transduction pathways. In addition, it can inhibit serotonin production, disrupt the hypothalamic-pituitary-adrenal axis, interfere with nerve regeneration in the hippocampus and cortex and lead to autophagy in the hippocampus [51].

Fc epsilon RI signalling pathway (*immunity-associated*): The Fc epsilon RI signalling pathway enhances NF-κB activity by stimulating various intracellular pathways such as NADPH oxidase and protein kinase. This activation promotes the release of inflammatory factors such as TNF-α and interleukins 1, 4, 5, 6; it might also activate phospholipase A2, releasing membrane lipids and triggering the production of lipid mediators such as prostaglandins and leukotrienes (LTC4, LTD4 and LTE4), thus, contributing to the inflammatory response [52].

PPAR signalling pathway (*lipometabolism-associated*): Fatty acids and their derivatives activate the nuclear hormone receptor known as PPAR, a nuclear hormone receptor that regulates genes involved in lipid metabolism and lipogenesis, making it a significant target for metabolic disorders. PPAR also exerts an anticancer effect in various human tumours and maintains inflammation and metabolic balance. It is linked to many pathophysiological processes, including tumours, ankylosing spondylitis (AS), obesity, insulin resistance, hypertension and Type-2 diabetes. Three PPAR subtypes capable of binding arachidonic and fatty acids have been identified: PPARα, which is highly expressed in the liver, skeletal muscle, kidney, heart and vascular wall, aids in the scavenging of circulating or cellular lipids; PPARβ/δ, expressed in the brain, stomach and colon, may assist in lipid oxidation and cell proliferation; PPARγ, often found in adipose tissue, myocardial tissue and vascular smooth muscle, promotes adipocyte differentiation and enhances glucose uptake [53].

Arginine and proline metabolism (*amino acid metabolism-associated*): Arginine is essential for body function and healing, enhancing immune system health and disease resistance. Low levels of arginine can lead to a decline in body function and healing capacity. The regulation of different cytokines and neurotransmitters is directly linked to the process of amino acid metabolism itself [54].

Glycolysis/gluconeogenesis (*glycometabolism-associated*): Glycolysis, a central metabolic pathway, converts glucose into pyruvate, producing a small amount of energy and is present in almost all organisms. It also generates essential precursor metabolites, such as pyruvate, phosphoenolpyruvate, glycerol, glyceraldehyde and glyceric acid. Gluconeogenesis, the pathway for synthesizing glucose from noncarbohydrate precursors, is essentially the reversal of glycolysis [55].

Insulin signalling pathway (*glycometabolism-associated*): The binding of insulin to its receptor triggers the phosphorylation of insulin receptor substrate (IRS), activating the PI3K/Akt pathway, which is a crucial insulin signalling route. Akt1 promotes cell survival and prevents apoptosis; Akt2 is a key component in the insulin signalling pathway; Akt3 is predominantly expressed in the brain. Some studies suggest that imbalances in the brain's insulin signalling pathway could serve as an early indicator and new therapeutic target for brain diseases, and repairing insulin signalling might ameliorate cognitive impairments [56].

According to the findings of KEGG pathway annotation and GO enrichment analysis, the key components of ZZCD may be linked to these 12 signalling pathways and are responsible for imparting therapeutic effects on depression.

3.2.3 | Construction of Ingredients–Targets–Pathways Network and PPI Network

To intuitively determine the pathways in which the targets were involved, the signalling pathways from the KEGG analysis were integrated into a network diagram using Cytoscape software.

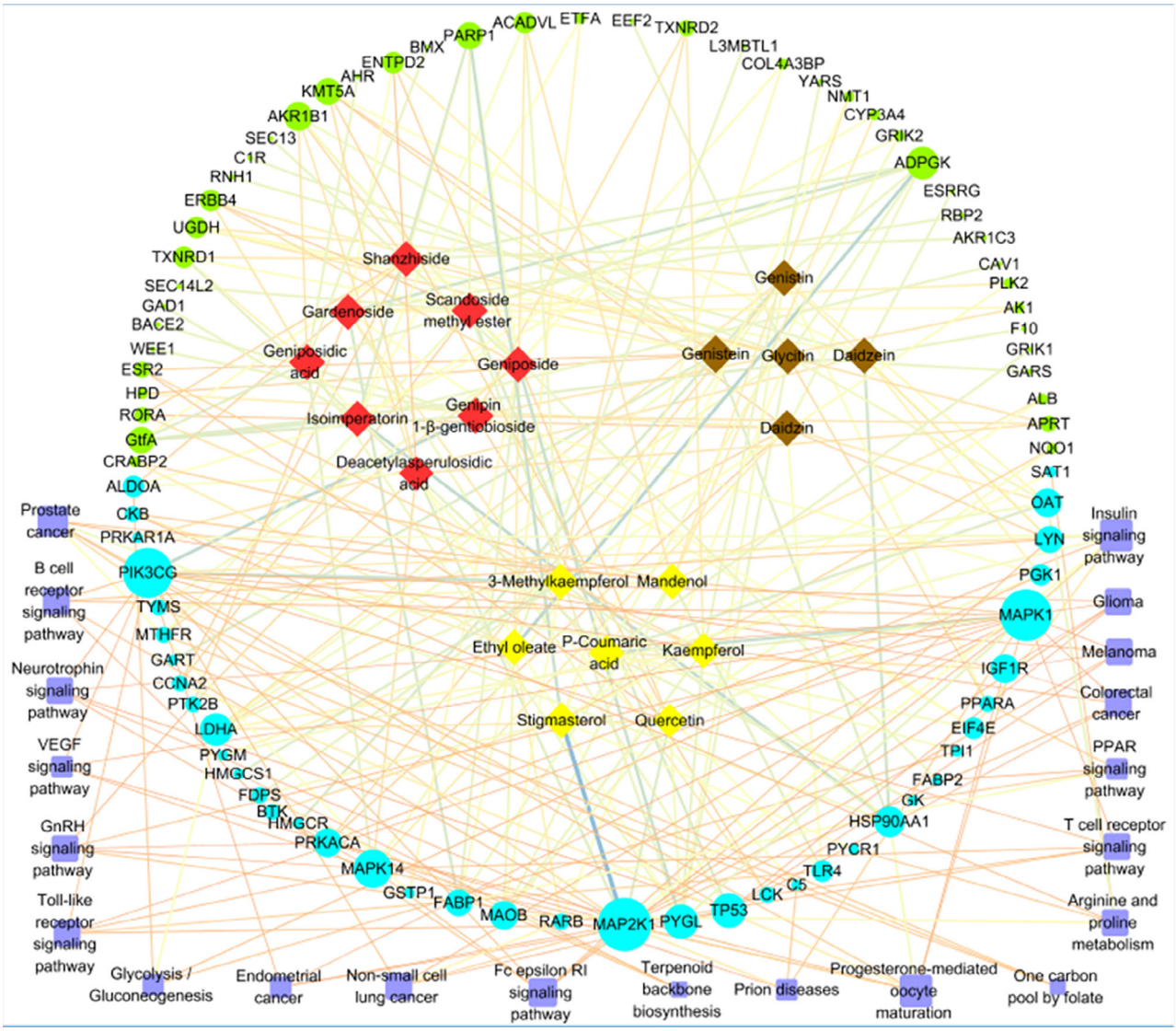

FIGURE 3 | ZZCD pharmacology network of depression. The red, brown and yellow diamonds represent definitive ingredients of GJE, SSP and the common ones, respectively. The blue circles denote the pathway-associated targets and the green circles delineate nonassociated ones. The purple rectangles represent the signaling pathways.

This integration resulted in a pharmacology network of ZZCD depicted in Figure 3, illustrating the relationships among the 20 definitive ingredients, 85 potential targets and 21 signalling pathways related to depression. This network provides a preliminary understanding of ZZCD's mechanism in alleviating depression. In the diagram, the red, brown and yellow diamonds represent the definitive ingredients from GJE, SSP and common sources, respectively. Blue circles indicate pathway-associated targets, whereas green circles denote nonassociated targets. Purple rectangles represent the signalling pathways. The edges illustrate interactions between ingredients and corresponding targets or between targets and their pathways, with the size proportional to their correlation. This arrangement highlights that the potential targets related to ZZCD's definitive ingredients are distributed across various signalling pathways, suggesting a synergistic effect as the mechanism for relieving depression.

To extract potential biological information from ZZCD's complex pharmacology network, key topological parameters such as network degree and betweenness were analysed among specific nodes (ingredients, targets and related pathways).

Figure 4 displays the Venn diagram of definitive ingredients distribution and their degree bar charts. Of these, p-Coumaric acid, 3-methykaempferol, kaempferol, quercetin, mandenol, ethyl oleate and stigmasterol were common to both GJE and SSP, exhibiting a high degree of match; Genipin 1-β-gentiobioside, scandoside metyl ester, gardenoside, deacetyl asperulosidic acid, geniposide, geniposidic acid, shanzhiside and isoimperatorin originated exclusively from GJE, among which scandoside metyl ester, geniposide, gardenoside and deacetyl asperulosidic acid had a high matching degree. Genistin, daidzin, glycitin, daidzein and genistein were solely from SSP, among which, genistin, daidzin and glycitin had a high matching degree. The quantitative analysis of the entire formula revealed that daidzin, genistin, daidzein, genipin 1-β-gentiobioside, deacetyl asperulosidic acid methyl ester, deacetyl asperulosidic acid, shanzhiside, gardenoside and geniposide were the principal chemical constituents of

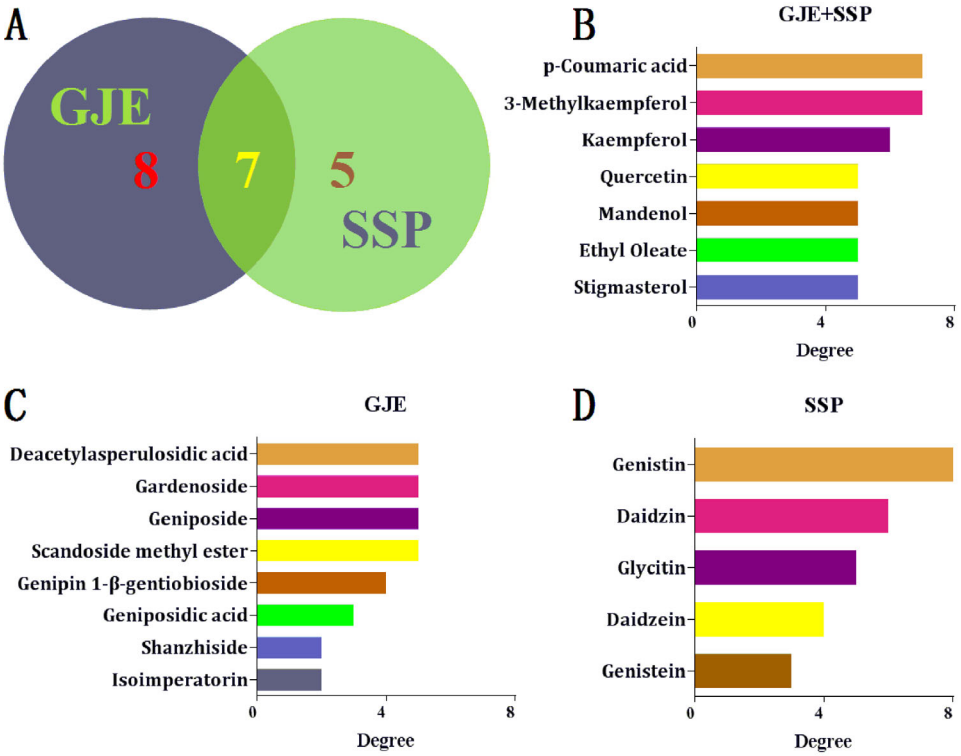

FIGURE 4 | The Venn diagram of definitive ingredients distribution (A) and their degree bar charts (B-common sources, C-GJE, D-SSP) in ZZCD.

ZZCD. In addition, geniposide and genipin 1-β-gentiobioside have been identified as the primary effective components and lead compounds in GJE [57]. This lends support to the reliability of our results to some degree.

Table S10 presents the degrees and betweenness of pathway-associated targets, highlighting the top 10 associated targets as MAP2K1, MAPK1, PIK3CG, MAPK14, PYGL, TP53, LDHA, OAT, MAOB and HSP90AA1.

The definitive ingredients and signalling pathways related to the top 10 associated targets in detail are illustrated in Tables S11 and S12, respectively.

Table S9 indicates that among the ingredients common to GJE and SSP, p-coumaric acid, 3-methykaempferol and kaempferol were linked to the top 10 associated targets with higher degrees and betweenness. Ingredients exclusive to either GJE or SSP were also associated with these top targets. Consequently, these seven definitive ingredients are hypothesized to be the main contributors to the pharmacodynamic effects.

On the basis of Table S12, the 12 signalling pathways associated with the top 10 targets and depression, characterized by high degrees and betweenness, were identified as Fc epsilon RI signalling pathway, neurotrophin signalling pathway, TLR signalling pathway, T-cell receptor signalling pathway, glycolysis/gluconeogenesis, VEGF signalling pathway, insulin signalling pathway, glioma, B-cell receptor signalling pathway, GnRH signalling pathway, arginine and proline metabolism and PPAR signalling pathway. According to KEGG target and pathway annotations, it can be inferred that the 20 definitive ingredients may exert a synergistic effect on alleviating depression by targeting these 10 points. These are subsequently involved in signalling pathways including PPAR signalling pathway related to lipid metabolism, arginine and proline metabolism related to amino acid metabolism, insulin signalling pathway and glycolysis/gluconeogenesis related to glucose metabolism, GnRH signalling pathway, neurotrophin signalling pathway and glioma associated with the nervous system as well as T-cell receptor, Fc epsilon RI, B-cell receptor, TLR and VEGF signalling pathways linked to the immune system. This greatly demonstrates effects of ZZCD's multi-ingredient, -target, and -pathways against depression.

The gene IDs of the 85 potential targets were imported into the STRING database to create the PPI network, which was then saved in TSV format and visualized using Cytoscape software. Figure S4 depicts the network comprising 77 nodes and 325 edges. The node colour and size represent its DC value, with larger, darker nodes indicating higher DC values, highlighting their strong association with the disease targeted by ZZCD. Similarly, edge colour and size denote BC, where thicker edges with deeper colours suggest higher BC values, indicating robust interactions among the targets. A total of 13 potential core nodes, including TP53, ALB, HSP90AA1, PPARA, MAPK1, TPI1, TXNRD1, MAPK14, MAP2K1, LDHA, GSTP1, CCNA2 and AKR1B1 were identified based on DC $\geq$ 8 (the medium of ranking), BC $\geq$ 0.01 and CC $\geq$ 0.497 in Table S13. A comparison of these core nodes in the PPI network with the top 10 pathway-associated targets in the ingredients–targets–pathways network found matches for TP53, HSP90AA1, MAPK1, MAPK14, MAP2K1 and LDHA in Figure 5, indicating their significant roles in antidepressant activity.

According to the PPI results, the clustered protein groups primarily participated in metabolic processes. Analysing protein

interaction networks, where diseases often result from disruptions in PPIs, is one of the main challenges in biology focussed on how genes influence phenotypes. The pathogenic pathways differ depending on the induction methods and cell types involved. TLR4, as predicted by the pharmacology network, and MAP2K1, MAPK1, and MAPK14, as predicted by the PPI network, were associated with the TLR4-MyD88-ERK/MAPK-NF-κB pathway involved in the LPS-stimulated BV2 cells inflammatory model [58], and the predicted TP53, PPARA and PPAR-γ pathway were also linked to neuroinflammation [59]. This suggests that the preliminary results can be further validated through additional anti-inflammatory experiments.

Serum pharmacology examines the actual components of ZZCD in the body, whereas network pharmacology screens for active substances in ZZCD from a holistic perspective. Integrating both approaches provides a more accurate reflection of ZZCD's effects within the body, overcoming the limitations of TCM research and offering a new methodology for studying ZZCD components.

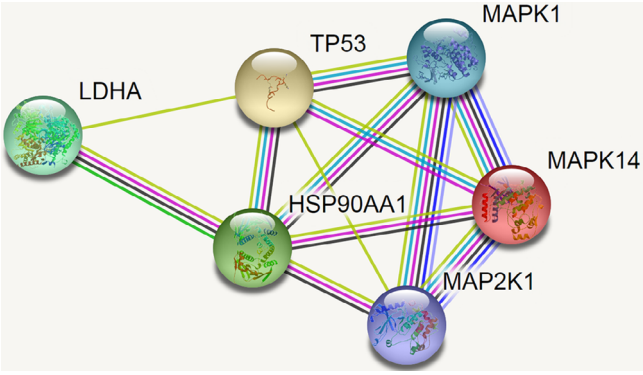

FIGURE 5 | Targets interaction of the six core nodes matched by pharmacology and PPI network involved in ZZCD against depression.

3.3 | Anti-Inflammatory Effect Evaluation

3.3.1 | Effects of ZZCD Samples on Cell Viability of BV2 Microglia

An initial MTT assay assessed the cytotoxicity of ZZCD samples prior to their anti-inflammatory evaluation. Figure 6A revealed that all test groups, including the solvent group, did not affect cell viability, except for the high-dosage group of the complete prescription. This result allowed the selection of 10 groups for further anti-inflammatory experiments due to their negligible impact on cell vegetative status.

3.3.2 | Effects of ZZCD Samples on LPS-Induced Production of TNF-α, IL-6 and IL-1β

To assess the anti-inflammatory effects of ZZCD samples, their impact on the production of TNF-α, IL-6 and IL-1β was measured. Results depicted in Figure 6B–D showed that these cytokines were elevated in the model group compared to the control group ($p < 0.01$). Nevertheless, treatment with ZZCD samples reduced their levels in a concentration-dependent manner relative to the model group. These findings confirm the therapeutic anti-inflammatory potential of ZZCD samples.

As illustrated in the figures, the anti-inflammatory effect of AIG 1 at medium dosage was comparable to that of the complete

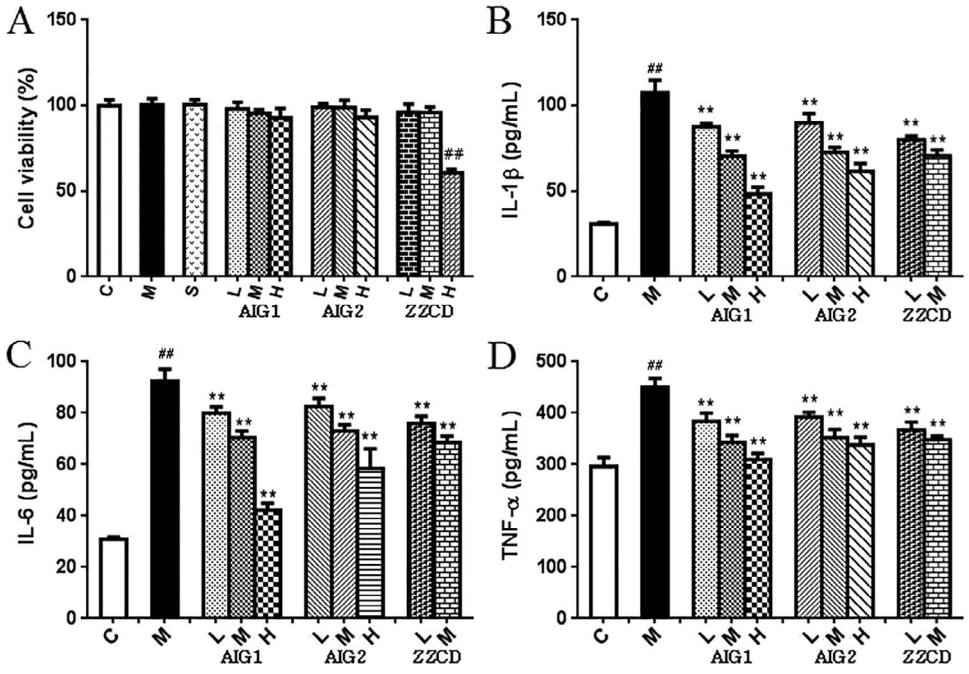

FIGURE 6 | Anti-proliferative effect (A) and anti-inflammatory effect on IL-1β (B), IL-6 (C), TNF-α (D) of ZZCD samples. All values are expressed as mean ± S.E.M for three independent experiments. C: control group, M: model group, S: solvent containing group, L: low-dosage group, M: medium-dosage group, H: high-dosage group. $^{\#}$: $p < 0.05$ vs. control group, $^{\#\#}$: $p < 0.01$ vs. control group, *: $p < 0.05$ vs. model group, **: $p < 0.01$ vs. model group.

prescription, suggesting that AIG 1 could be a key pharmacodynamic component in inhibiting the three inflammatory cytokines, corroborating previous network pharmacology findings. The anti-inflammatory effect of AIG 2 was evident but slightly less than that of AIG 1, thus AIG 2 is considered a principal pharmacodynamic component of AIG 1, aligning with network pharmacology speculation. High-dosage groups of AIG 1 and AIG 2 exhibited significant anti-inflammatory effects with minimal impact on cell proliferation, whereas the high-dosage group of the complete prescription affected cell viability, indicating that AIGs with lesser cytotoxicity hold greater research value than the full recipe. Ultimately, AIG 1 was designated as the 'antidepressant component' of ZZCD, hypothesized to offer higher medicinal value than the complete prescription in certain aspects.

4 | Conclusions

In this study, 146 migration constituents in serum and their attribution were hypothesized and characterized via ZZCD serum pharmacochemistry, identified as 18 prototype components and 128 metabolites—121 being Phase I and 7 being Phase II metabolites. A ZZCD pharmacology network was constructed, illustrating the relationships among the 20 definitive ingredients, 85 potential targets, and 21 signalling pathways associated with the depression. The targets predicted by the pharmacology and PPI network were linked to neuroinflammation, suggesting the need for further anti-inflammatory experiments. Notably, the anti-inflammatory effect of AIG 1, composed of the p-coumaric acid, quercetin, scandoside methyl ester, geniposide, shanzhiside, deacetyl asperulosidic acid, genipin 1-β-gentiobioside, gardenoside, geniposidic acid, daidzein, daidzin, genistein, genistin, glycitin, closely matched that of the entire recipe. Consequently, AIG 1 was hypothesized to be the critical pharmacodynamic component of ZZCD for inhibiting inflammatory factors and was defined as the 'antidepressant components' of ZZCD, thereby verifying the results of the pharmacology network.

The most significant aspect of this study is the integrated strategy that combines network biology and serum pharmacochemistry using advanced LC–MS technology, which breaks the limits of traditional analytical techniques for constructing depression-related networks and the action network of ZZCD.

Serum pharmacology focussed on the actual components of ZZCD in the body, whereas network pharmacology screened for active substances in ZZCD from an overall perspective. The combination of both approaches more accurately reflects the real effects of ZZCD in the body. In addition, when combined with the validation through the evaluation of inflammatory factors as pharmacological indicators, this integrated method overcomes the limitations of TCM research and provides a new perspective and approach for studying the components of ZZCD.

As a result, the 'multi-component, multi-target and multi-pathway' mechanism of ZZCD was preliminarily clarified, screening out the 'antidepressant components' to provide a solid scientific basis and new directions for developing new drugs and intervention strategies for depression treatment, as well as the advancement of analytical techniques.

Authors contributions

Chuan Chai: conceptualization, data curation, formal analysis, methodology, resources, writing–original draft. **Bo Jin:** conceptualization, data curation, software, writing–review and editing. **Jinghan Bi:** formal analysis, methodology. **Yuhan Cui:** validation. **Xiaobing Cui:** resources, supervision. **Chenxiao Shan:** validation. **Sheng Yu:** supervision, validation. **Hongmei Wen:** conceptualization, funding acquisition, resources, writing–review and editing.

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The authors have nothing to report.

Ethics Statement

All animal experiments were approved by the animal experiment ethics committee of Nanjing University of Chinese Medicine, under ethics application number 201905A031, which complied with the Ministry of Health's Detailed Rules for the Implementation of the Administration of Medical Laboratory Animals, State Science and Technology Commission's Regulations on the Administration of Laboratory Animals, and ARRIVE guidelines and the AVMA euthanasia guidelines 2020.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.