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RESEARCH ARTICLE

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Exploring the key ingredients and mechanisms of Banxia Xiexin decoction for the treatment of polycystic ovary syndrome based on network pharmacology and experimental validation

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ABSTRACT

Purpose: This study aimed to investigate the key bioactive constituents and polypharmacological mechanisms of Banxia Xiexin Decoction (BXD) against polycystic ovary syndrome (PCOS) through integrated network pharmacology and experimental validation.

Methods: Network pharmacology was used to determine the key ingredients, potential targets and signaling pathways. 3-week-old female mice were injected subcutaneously with DHEA (6mg/100g body weight) daily to construct a PCOS model and administered different doses BXD and its key ingredients for intervention. Ovarian pathology, vaginal smears, oxidative stress-related indicators, and hub genes were tested to evaluate its therapeutic effects.

Results: We identified 3 key ingredients and 99 potential targets for BXD treatment of PCOS. Biological functions of these targets were mainly enriched in oxidative stress, hormone response and apoptosis. KEGG analysis showed they were mainly involved in signaling pathways such as PI3K-AKT, MAPK, HIF-1 and IL17. By PPI and algorithmic analysis, we identified 8 hub genes, 5 of which (JUN, MAPK1, MAPK3, FOS, TP53) were related to oxidative stress. Further analysis indicated that quercetin, glycyrrhetinic acid A and naringenin are the three key ingredients of BXD, and they have superior binding effects on the hub genes. Animal experiments demonstrated that BXD and its three key ingredients significantly ameliorated the PCOS symptoms, oxidative stress-related indicators and the expression of hub genes.

Conclusions: Five oxidative stress-related hub targets of BXD for PCOS were identified, including FOS, JUN, MAPK3, TP53 and HSP90AA1, while three key ingredients of BXD, quercetin, glycyrrhetinic acid A and naringenin, were uncovered.

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KEYWORDS
Banxia Xiexin decoction; PCOS; traditional Chinese medicine; network pharmacology; oxidative stress

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrine disorder in women of childbearing age, characterized by persistent anovulation, hyperandrogenism, and polycystic ovarian changes [1]. Currently, Western medical treatment options for PCOS mainly include ovulation induction drugs, insulin sensitizers, and anti-androgenic drugs such as clomiphene, metformin, and diane-35 [2–4]. However, these treatments only target the symptoms and have poor long-term efficacy and potential side effects, such as ovarian hyper-stimulation syndrome [5], premature ovarian failure [6], venous thromboembolism [7], and severe gastrointestinal adverse reactions [8]. Therefore, while efforts are being made to develop safe and effective drugs for PCOS, it is necessary to actively seek safe and effective treatment strategies from traditional Chinese medicine (TCM).

Banxia Xiexin decoction (BXD) originated from the book 'Treatise on Febrile Diseases'. The new, improved BXD consists of 9 herbs, including pinellia,

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scutellaria, dried ginger, codonopsis, licorice, coptis, jujube, epimedium, and wolfberry. It is commonly prescribed for cold-heat complicated syndrome [9]. Modern studies have also confirmed that BXD may ameliorate PCOS insulin resistance by regulating intestinal microbiota imbalance and improving metabolic disorders [10]. However, the active ingredients and the exact mechanisms of BXD against PCOS remain unclear.

Network pharmacology is an emerging discipline that uses systems biology theory to analyze biological networks and select specific signal nodes for multi-target drug molecule design [11]. The regulation of multiple signaling pathways is an important approach to new drug development, and it also portends great opportunities for the reform and innovation of TCM formulas. In the present study, we used network pharmacology, bioinformatics, molecular docking and animal model to identify the active ingredients, targets, and molecular mechanisms of BXD in the treatment of PCOS (Figure 1).

2. Materials and methods

2.1. Materials and reagents

The materials and reagents used in this study are shown in Table 1.

The new, improved formula for BXD consists of 9 herbs: Rhizoma Pinelliae (Qing Banxia) 9 g, Scutellaria (Huangqin) 20 g, Rhizoma Coptidis (Huanglian) 10 g, Rhizoma Zingiberis (Ganjiang) 9 g, Codonopsis pilosula (Dangshen) 12 g, Licorice (Gancao) 12 g, Fructus Ziziphi Jujubae (Dazao) 9 g, Epimedium (Xianlingpi) 15 g, and Fruit of Chinese wolfberry (Gouqizi) 30 g. Before use, take one dose of BXD, dissolve in 200 mL warm water, and concentrate using a rotary evaporator at 60 °C to achieve a concentration of 1.062 g/mL of original drug. Based on the dose conversion formula and the dose in rat (Conversion of Animal Doses to Human Equivalent Doses Based on Body Surface Area Data from FDA guidelines (http://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf)), the doses for mice are high, medium, and low doses of 21.24 g/kg, 10.62 g/kg, and 5.31 g/kg respectively.

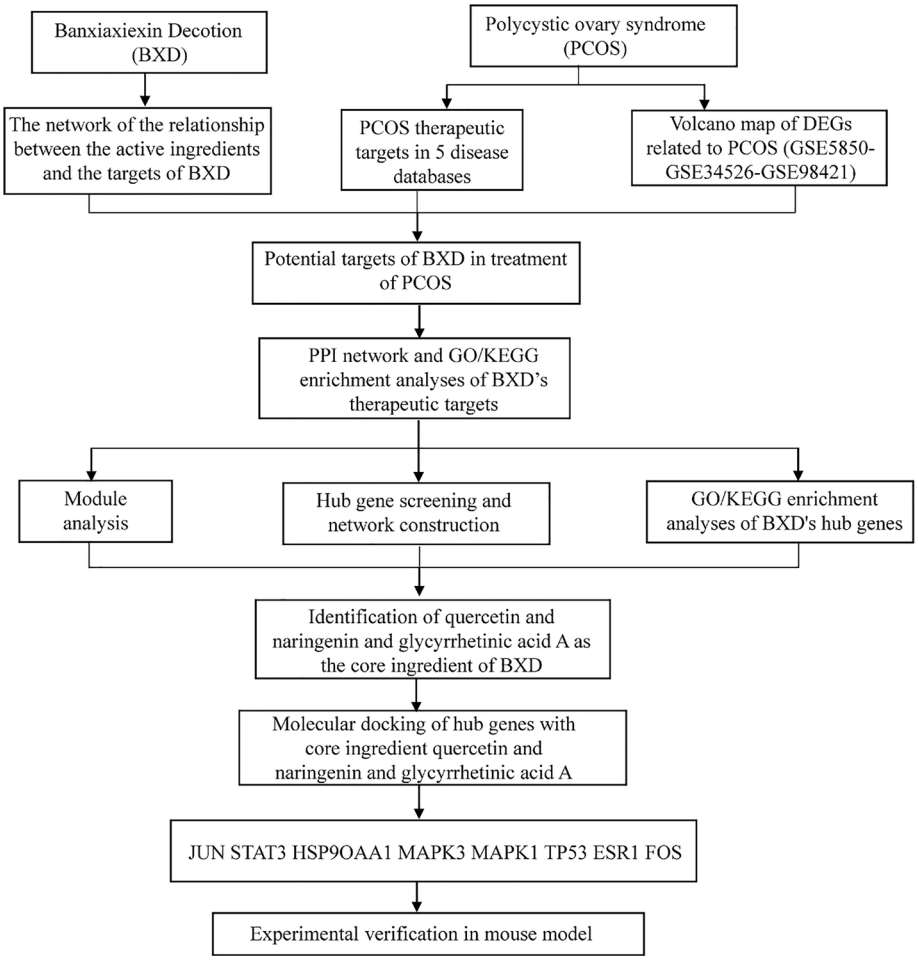


Figure 1. Flow chart.

Table 1. Drugs and test kits.

Reagents	Batch Number	Product source	Country
Quercetin	Q111274	Aladdin	China
Glycyrrhetinic acid	G109797	Aladdin	China
Naringenin	N164488	Aladdin	China
Dehydroepiandrosterone (DHEA)	D106380	Aladdin	China
Testosterone kit	PT872	Beyotime biotechnology	China
MDA kit	S0131S	Beyotime biotechnology	China
SOD kit	S0101S	Beyotime biotechnology	China
3-NT kit	D751015	Sangon Biotech	China
AGEs kit	ab273293	Abcam	England
Reverse transcription kit	12574026	Thermo Fisher Scientific	USA

2.2. Network pharmacology and bioinformatics analysis

2.2.1. Identification of the active ingredients and targets of BXD

Using the TCMSP database (https://old.tcmsp-e.com/tcmspsearch.php), active ingredients and target genes for BXD were obtained based on the drug characteristics of OB ≥ 30%, DL ≥ 0.18, and HL≥ 14. The target names were converted to gene names using the STRING database (https://cn.string-db.org/). The interaction between active ingredients and target genes of BXD was constructed using the Cytoscape software.

2.2.2. Data mining for genes and therapeutic targets for PCOS

'PCOS' and 'Polycystic Ovary Syndrome' were used as search terms to identify therapeutic targets for PCOS from the Disgenet, GeneCards, OMIM, CTD, and GEO databases. Three PCOS-related datasets (GSE5850, GSE34526, and GSE98421) in the Gene Expression Omnibus (GEO) database were merged. Batch correction was performed to remove technical variation from the datasets. Finally, differential expression was performed using the DeSeq2 package in R. Differentially expressed genes (DEGs) were screened using a log2 foldchange > 1 and $p < 0.05$.

The target genes obtained from Disgenet, GeneCards, OMIM, CTD, and GEO were intersected, and target genes that appeared in at least two databases were selected as the treatment targets for BXD in PCOS [11].

2.2.3. Analysis of target gene interactions and functional enrichment analysis

The protein-protein interaction network (PPI) among the treatment targets was constructed using the STRING database and visualized with Cytoscape software. Hub genes were obtained by intersecting the core genes obtained from six algorithms: Degree, EPC, Closeness, MCC, MNC, and Radiality. The core targets were subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using DAVID 6.8 [12]. Enrichment results were considered significant for each enriched item containing at least 2 genes and $p < 0.05$.

2.2.4. Determination of key ingredients of BXD in treating PCOS

The active ingredients of BXD were intersected with the treatment targets of BXD in PCOS, and the active ingredient with the most target overlap was selected as the core component of BXD. The potential treatment targets for the key ingredient were subjected to further bioinformatics analysis.

2.2.5. Molecular docking

The two-dimensional structure of the active ingredients was searched on the PubChem website and converted into the three-dimensional structure with the lowest free energy using the ChemBio3D software. The 3D structure of the core targets was obtained from the PDB database, and water molecules and small molecule ligands were deleted using PyMOL software. The protein and drug components were converted into PDBQL format files using the Auto DockTools software, and the active site was identified. Molecular docking analysis was performed using Vina software.

2.3. Animal experiments

2.3.1. Animals

C57BL/6J female mice were purchased from Xuzhou Medical University Animal Centre and housed in an SPF-grade IVC system, and in light (12-hour light & 12-hour dark) and 25 °C. Mice were provided with adequate food and fresh water. All animal experiments were approved by the Ethics Committee for Animal Experimentation of Xuzhou Medical University (license number 202209S077). We confirmed that we have followed the ARRIVE guideline for the reporting of studies involving animals. Mice were housed Mice are administered by subcutaneous injection using a 1 ml syringe, and gastric lavage using a No. 8 gastric tube (No. 8 gastric tube specifications: inner diameter 0.8 mm; ball head diameter 1.3 mm; total length of needle head 45 mm). The individual drug is prepared as a storage solution, diluted with saline to the appropriate concentration for gastric lavage.

PCOS mouse model was constructed as previously described [13]. Briefly, 3-week-old female mice were injected subcutaneously with DHEA (6 mg/100 g body weight) or an equivalent dose of sesame oil daily. PCOS mice were randomly divided into three groups: the blank group, the control group, the PCOS model

group and the treatment group. The treatment group was treated with different doses of BXD, quercetin, Glycyrrhetinic acid A, and naringenin ($n = 5$/dose/group). The detailed groupings are as following:

Blank: Normal mice with no treatment, $n = 5$; Control: Subcutaneous injection with sesame oil, $n = 5$; PCOS: Subcutaneous injection with DHEA (6 mg/100g body weight), $n = 5$; PCOS+BXD: DHEA+ BXD (5.31 g/kg, 10.62 g/kg, 21.24 g/kg), $n = 5$/group; PCOS+quercetin: DHEA+ quercetin (100 mg/kg, 200 mg/kg), $n = 5$/group; PCOS+naringenin: DHEA+ naringenin (20 mg/kg, 40 mg/kg), $n = 5$/group; PCOS+glycyrrhetinic acid A: DHEA+ naringenin (100 mg/kg, 200 mg/kg), $n = 5$/group.

2.3.2. Sample collection and biochemical indicators testing

The estrous cycle of mice was assessed by vaginal smears for 10 consecutive days. Mouse estrous cycle was determined by microscopic analysis of the main cell types in vaginal smears using Wright staining. After the end of the treatment, blood was collected from the eyes, and ovarian tissue was collected for testing and analysis. Serum testosterone, MDA, 3-NT, SOD and AGEs testing were performed with kits listed in Table 1.

2.3.3. Hematoxylin and eosin (H&E) staining

Ovarian tissue was fixed in 4% paraformaldehyde for 24 h, then embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin and sealed with neutral resin. Five sections were taken for each mouse to determine the number of dominant and small sinus follicles.

2.3.4. Gene expression estimation with real-time quantitative PCR (qPCR)

The gene testing with qPCR was performed according to our previous methods [14,15]. Briefly, mouse ovarian tissue was extracted using the TRIzol method and reverse transcribed using a cDNA kit. Gene expression was measured using real-time fluorescence quantitative PCR instrumentusing (Roche 480, Roche, Switzerland) with Glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal control. The mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were listed in Table S1.

2.4. Statistical analysis

Data were analyzed with GraphPad Prism8 (GraphPad Software Inc., La Jolla, CA, USA) and expressed as means and standard errors (SEM). Statistical significance of differences between two groups was assessed by Student's t-test, while multiple groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test. Values of $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate statistical significance.

3. Results

3.1. Active ingredients and targets regulatory network of BXD

Based on the screening criteria, 158 active ingredients and 259 therapeutic targets of BXD were obtained from the TCMSP database. The therapeutic targets were converted into gene names, and a regulatory network was constructed using the 'Cytoscape' software. As shown in Figure 2, the surrounding circles represent the various active ingredients of BXD, with different colors representing different ingredients and the blue rectangles representing the therapeutic targets of various Chinese medicines. The degree value indicates the number of edges connected to the node. The top three drug ingredients based on degree value are quercetin, glycyrrhetinic acid A, and naringenin.

3.2. The therapeutic targets and related genes of PCOS

After batch correction using the 'limma' package in R (Figure 3a,b), differential gene expression was analyzed to generate a volcano plot and heatmap (Figure 3c,d). In the volcano plot, blue dots on the left indicate genes with low expression in PCOS samples, and red dots indicate genes with high expression in PCOS samples. The heatmap shows the differentially expressed genes between control and PCOS samples, with the top 20 upregulated genes and the top 20 downregulated genes.

Using 'PCOS' and 'polycystic ovary syndrome' as keywords, 733, 3222, 644, 10378, and 316 therapeutic targets were obtained from the Disgenet, GeneCards, OMIM, and CTD databases. The PCOS-related target genes were obtained by taking the intersection of the five databases with target genes that appeared at least twice (Figure 3e). The intersection with the target genes of BXD yielded 99 potential therapeutic targets for PCOS (Figure 3f).

3.3. Functional enrichment analysis

GO and KEGG enrichment analyses were performed on the potential therapeutic targets using R to further explore the mechanism of action of BXD in treating PCOS. As shown in Figure 4a, the target genes were

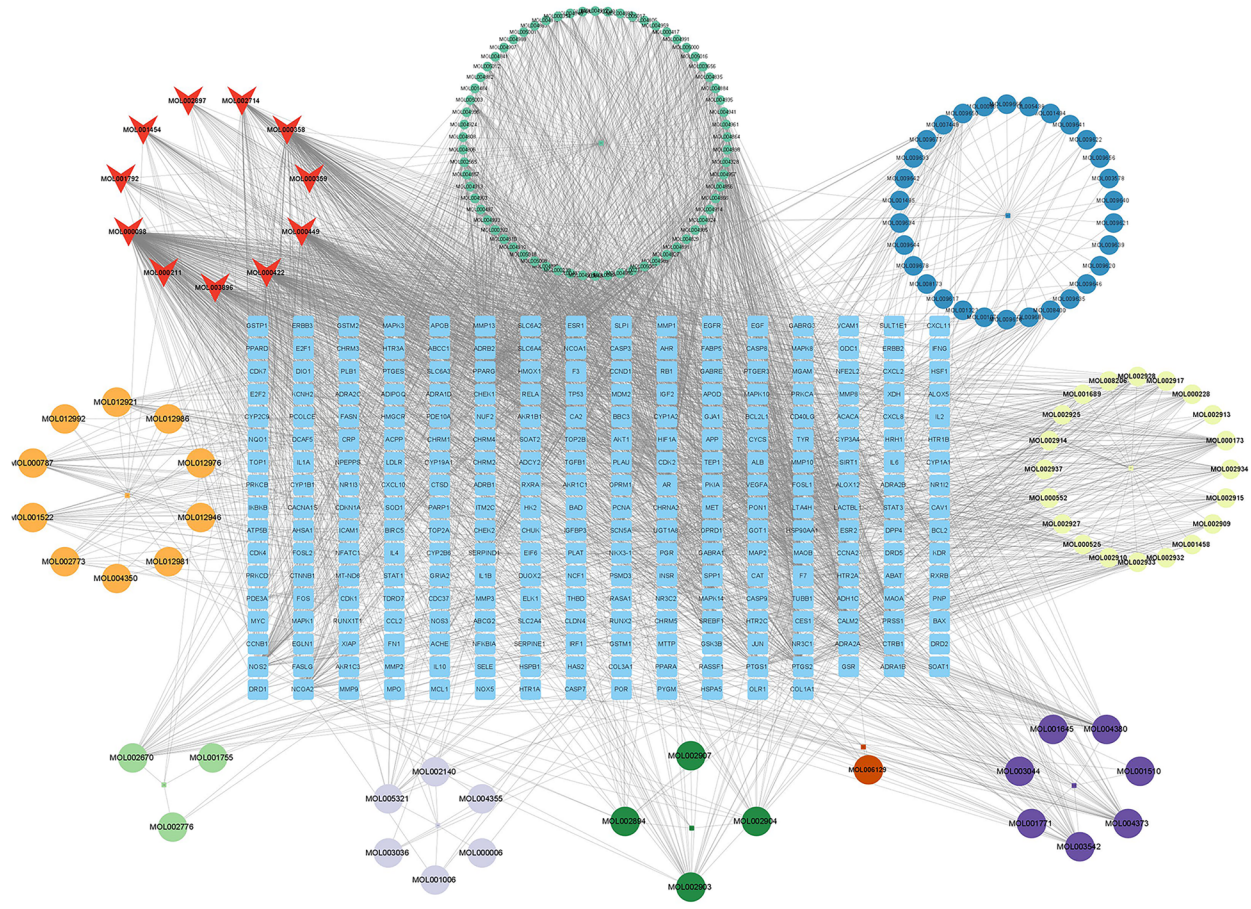


Figure 2. The network of the relationship between the active ingredients and the targets of BXD.

mostly enriched in processes such as response to reactive oxygen species and cellular response to chemical stimulus in biological processes. KEGG enrichment analysis showed enrichment in pathways such as the PI3K-AKT, AGE-RAGE, and MAPK signaling pathway (Figure 4b).

3.4. Protein–protein interaction network of target genes

Figure 5a shows the PPI network of potential therapeutic targets, where darker colors indicate larger node size and higher node degree. The target genes were divided into 7 modules using Cytoscape's algorithm for module analysis. The core therapeutic targets were obtained by taking the intersection of the top-ranked genes using six algorithms (Degree, EPC, Closeness, MCC, MNC, and Radiality) in Cytoscape, resulting in 8 genes (Figure 5b). GeneMANIA was used to analyze the biological functions of the core targets (Figure 6). Results showed that 5 of the 8 hub genes were associated with oxidative stress. The 5 genes were subsequently validated in animal experiments.

3.5. Key ingredients of BXD

Taking the intersection of each active ingredient's targets with the potential therapeutic targets of BXD, quercetin, naringenin, and glycyrrhetinic acid A were identified as the top three ingredients of BXD. Quercetin has 92 targets, with 72 overlapping with the potential therapeutic targets of BXD in treating PCOS (Figure 7a). PPI network analysis of the 92 targets revealed 5 core genes that overlap with BXD (Figure 7b). GO and KEGG enrichment analysis showed an 82% repetition rate, with enrichment in processes such as reactive oxygen species metabolic process, response to steroid hormone, reproductive structure development, regulation of apoptotic signaling pathway, Ovarian steroidogenesis, GnRH secretion, IL-17 signaling pathway, TNF signaling pathway, MAPK signaling pathway and PI3K-AKT signaling pathway (Figure 7c,d).

Naringenin had 35 targets, with 13 overlapping with the potential therapeutic targets of BXD in treating PCOS (Figure 8a). PPI network analysis of the 35 targets revealed 4 core genes that overlap with BXD (Figure 8b). GO and KEGG enrichment analysis showed a 62% repetition rate, with enrichment in metabolic

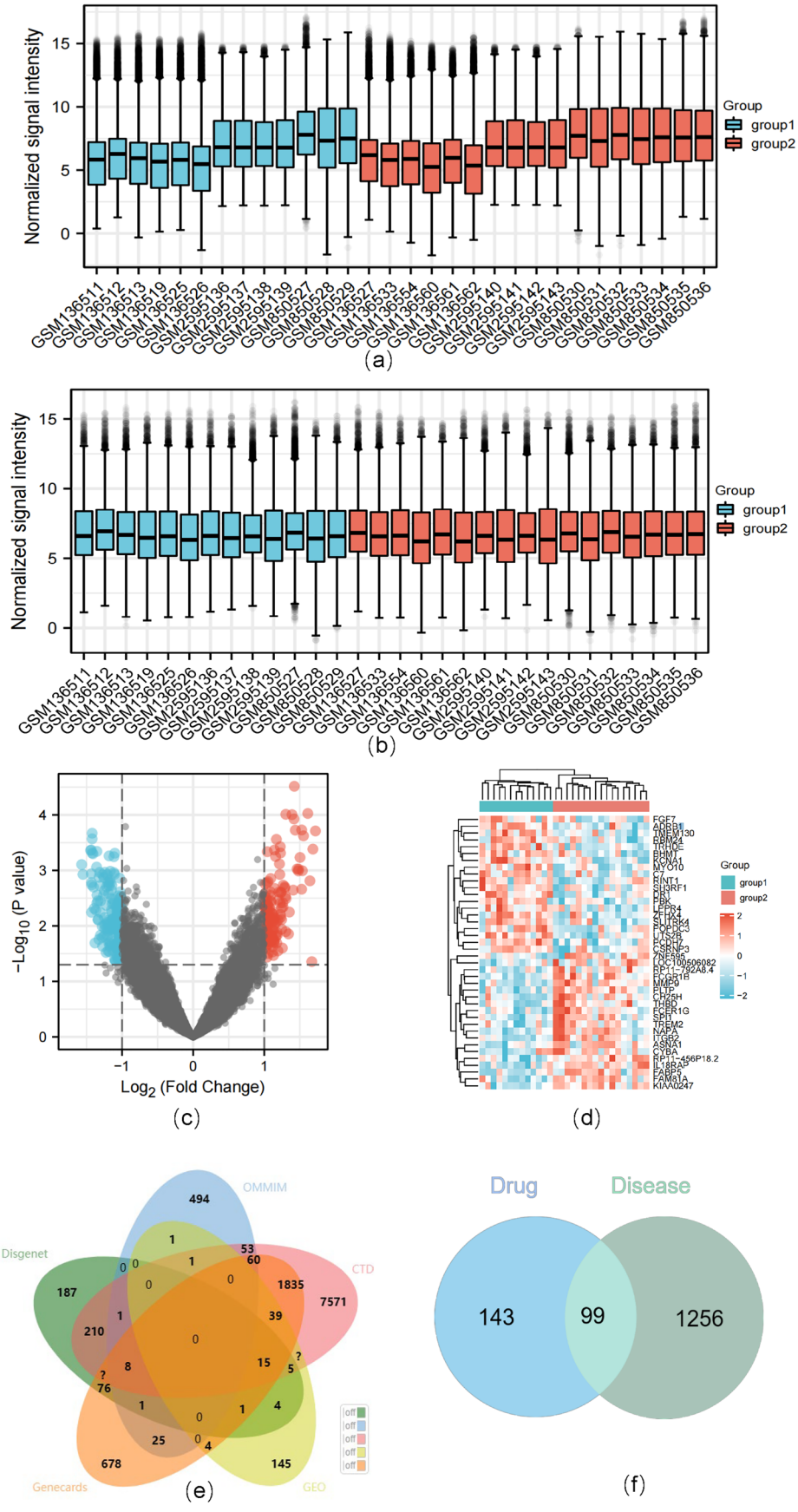
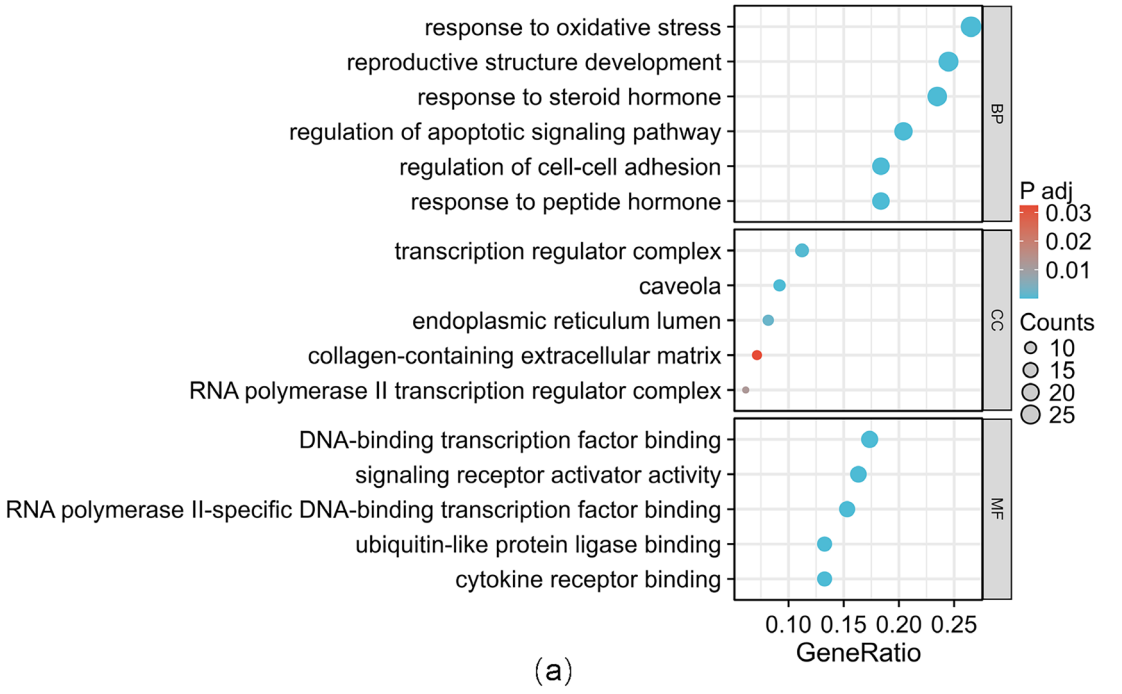


Figure 3. Identification of targets of BXD for the treatment of PCOS. (a) Distribution of the dataset before removal of batch effects. (b) Distribution of the dataset after removing the batch effect. (c) Volcano map of DEGs related to PCOS. (d) Heat map of DEGs related to PCOS. (e) The Venn diagram of PCOS therapeutic targets in 5 disease databases and GEO data sets. (f) The Venn diagram of the targets in at least two databases in A and the therapeutic targets of BXD.



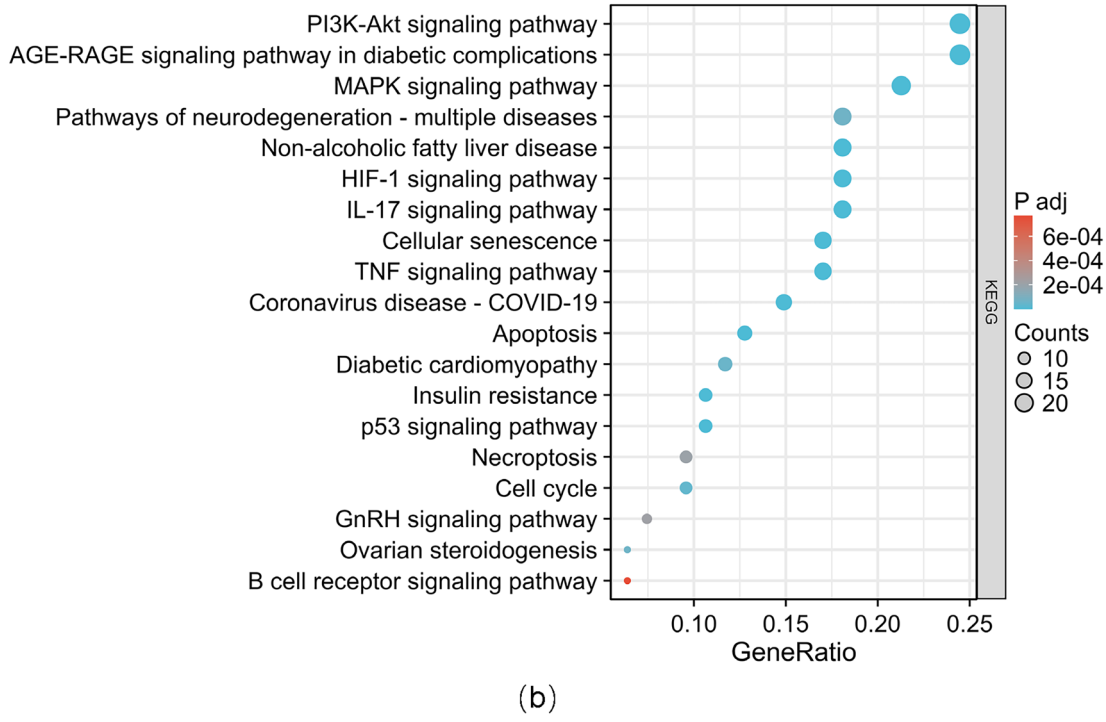
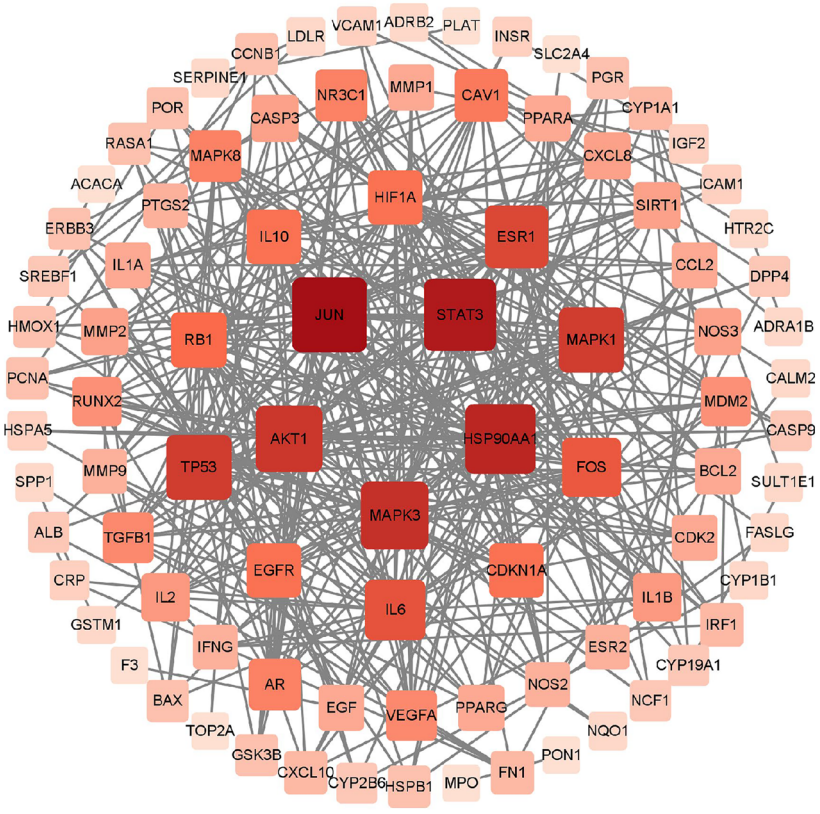


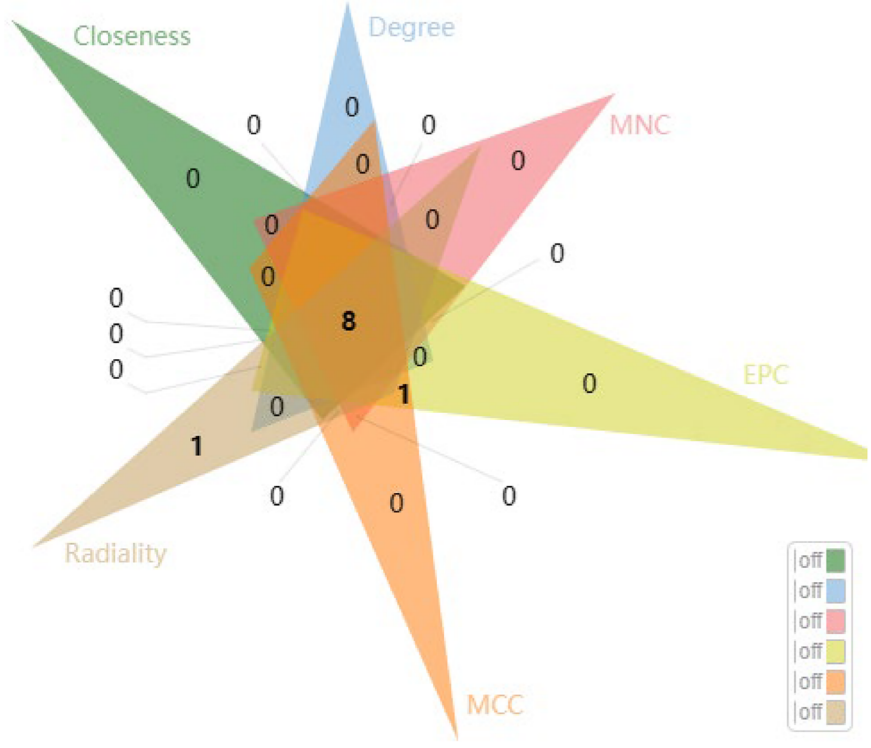
Figure 4. Enrichment analyses of BXD's therapeutic target. (a) GO enrichment analysis (including BP, CC, and MF) of BXD's therapeutic target. (b) KEGG enrichment analysis of BXD's therapeutic target.

processes, cell differentiation, regulation of oxidative stress, extrinsic apoptotic signaling pathway, and intrinsic apoptotic signaling pathway (Figure 8c,d).

Glycyrrhetinic acid A has 31 targets, with 16 overlapping with the potential therapeutic targets of BXD in treating PCOS (Figure 9a). PPI network analysis of the 31 targets revealed 4 core genes that overlap with BXD (Figure 9b). The repetition rate of the GO and KEGG enrichment analysis results reached 60.4%, with enrichment in processes such as cell proliferation, negative regulation of apoptotic signaling pathway, cell differentiation, and intracellular receptor signaling pathway (Figure 9c,d).



(a)



(b)

Figure 5. PPI network and gene module analysis of BXD's targets for PCOS. (a) The PPI network was constructed through the String website and visualized with Cytoscape. (b) Six algorithms for the intersection screening of hub genes.

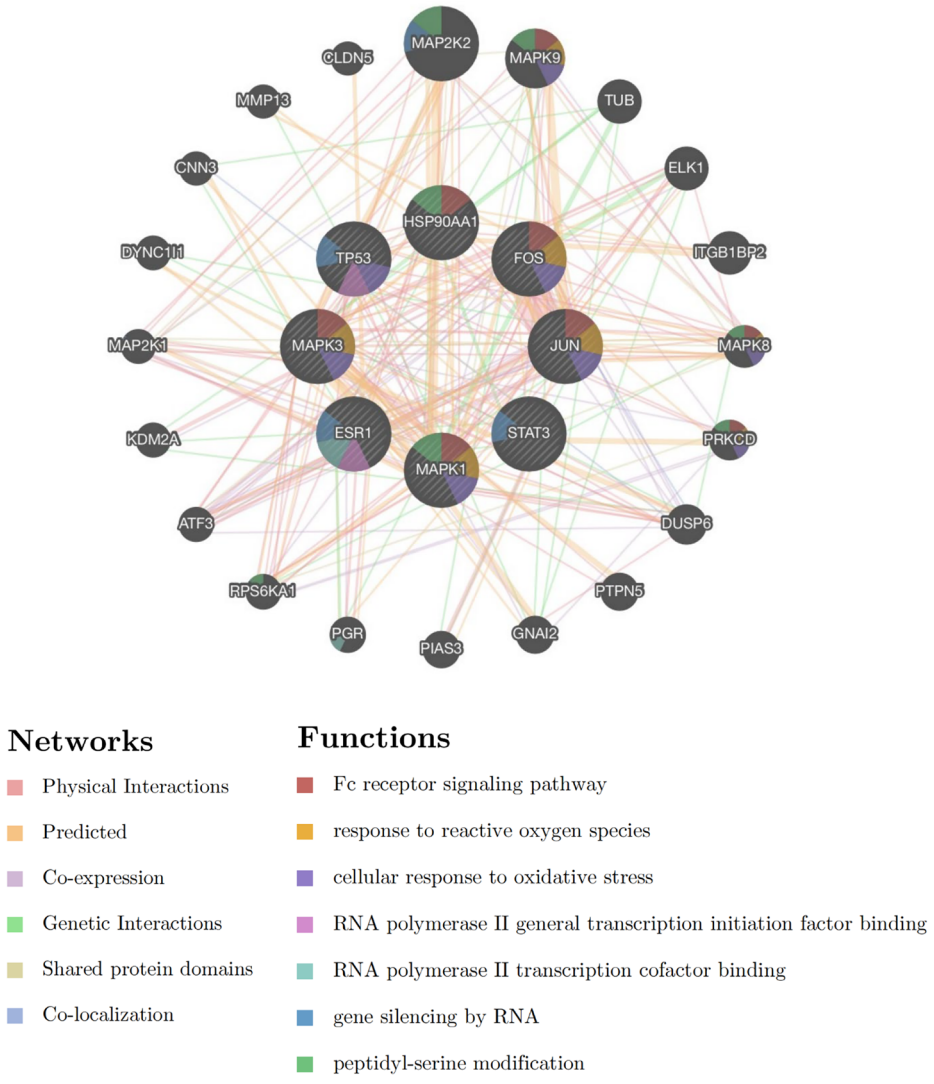


Figure 6. Hub gene co-expression network and functional analysis with GeneMANIA.

3.6. Results of molecular docking

To elucidate the interaction of quercetin, glycyrrhetinic acid A, and naringenin with the core targets, we obtained the docking results according to the steps of molecular docking, from finding ligands to docking. We found that the three key active ingredients had the highest binding potential to ESR1, FOS, HSP90AA, MAPK3, TP53, STAT3, and MAPK1, with binding energies less than −6.0 kcal/mol (Figure 10).

3.7. Therapeutic effects of quercetin in PCOS mice were confirmed in animal experiments

To investigate the therapeutic effects of the three key ingredients, a PCOS mouse model was constructed and treated with different doses of the BXD and its active ingredients. HE staining showed that 10.62 mg/kg of BXD, 100 mg/kg of quercetin, 20 mg/kg of glycyrrhetinic acid A and 100 mg/kg of naringenin had significant efficacy (Figure 11). PCOS mice showed significant improvement in the number of dominant and small antral follicles after treatment (Figure 12a,b). Testosterone levels in PCOS mice were significantly reduced with the treatment (Figure 12c). Compared to the control group, the estrous cycle was disturbed in PCOS mice and was significantly restored by BXD, quercetin, glycyrrhetinic acid A and naringenin (Figure 12d–j). These results show that quercetin, glycyrrhetinic acid A, and naringenin are significantly effective in the treatment of PCOS.

Moreover, we tested oxidative stress-related indicators. It was found that BXD, quercetin, glycyrrhetinic acid A, and naringenin reduced oxidative stress-related indicators, such as MDA, 3′-NT, AGEs, and SOD levels, in

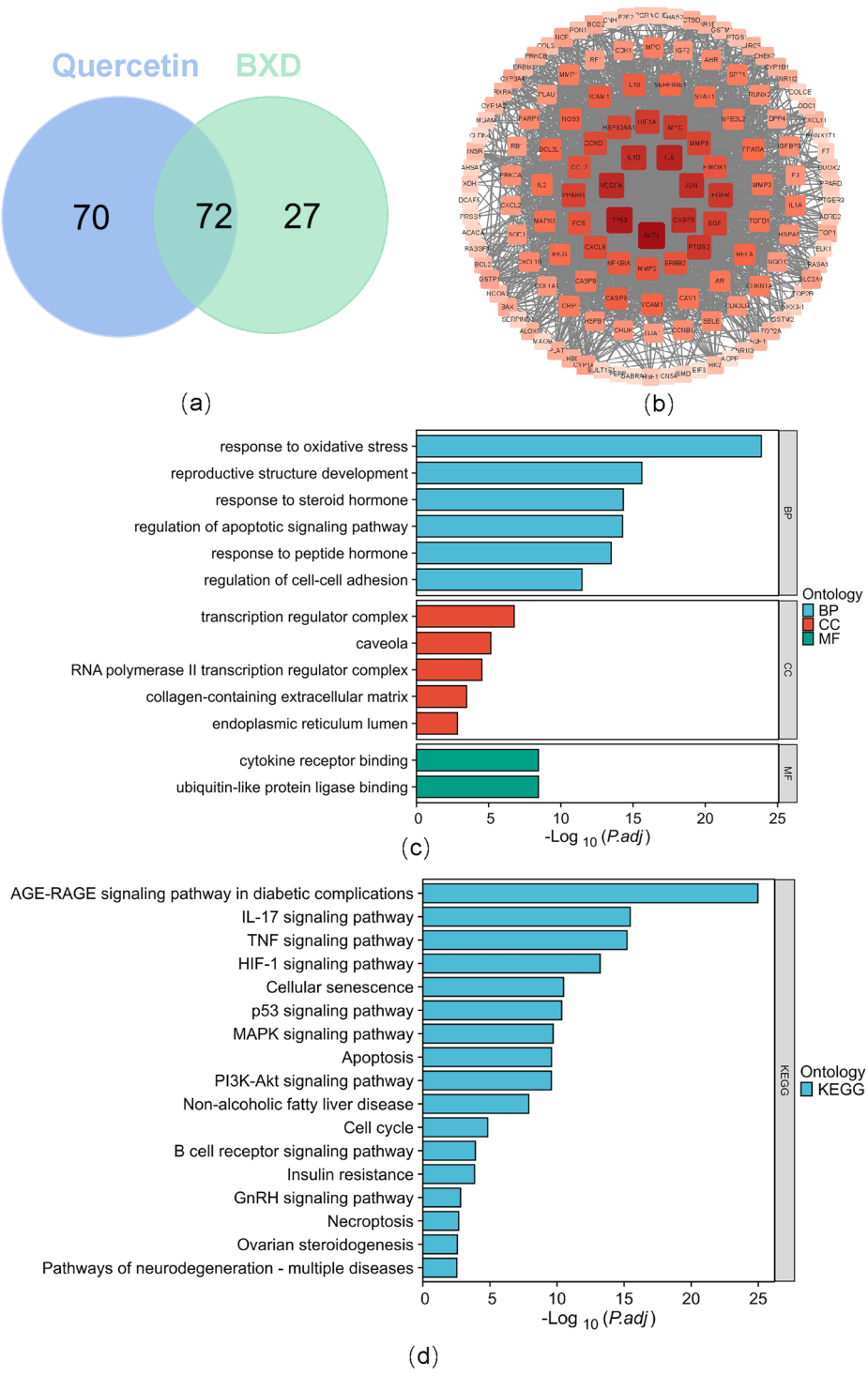


Figure 7. Analysis of quercetin's potential therapeutic target. (a) Venn diagram of the potential therapeutic targets of quercetin and the therapeutic targets of BXD. (b) PPI network of quercetin's therapeutic targets. (c) GO analysis of quercetin's therapeutic targets. (d) KEGG analysis of quercetin's therapeutic targets.

the ovaries of PCOS mice (Figure 13a–d). Furthermore, we found that the three ingredients could restore the expression of 5 oxidative stress-related hub genes in the PCOS mouse model (Figure 13e–i). These results suggest that BXD, quercetin, glycyrrhetinic acid A, and naringenin may ameliorate PCOS by inhibiting oxidative stress.

4. Discussion

Traditional Chinese Medicine (TCM) has been used to treat PCOS for a long time, and BXD is one such formulation that targets the underlying causes of PCOS. Its mechanism of action involves improving kidney function, promoting blood circulation, regulating

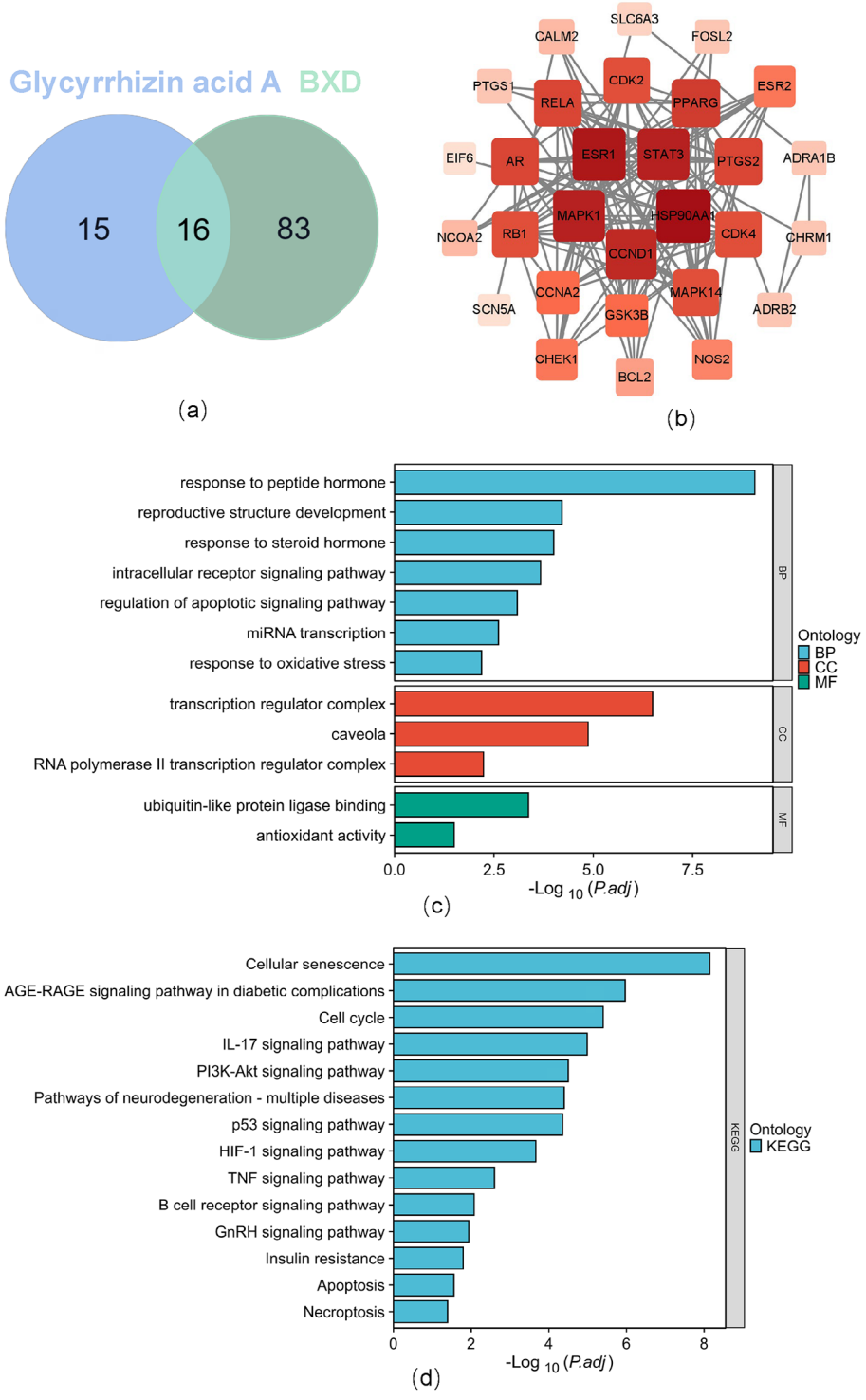


Figure 8. Analysis of glycyrrhetinic acid A's potential therapeutic target. (a) Venn diagram of the potential therapeutic targets of glycyrrhetinic acid A and the therapeutic targets of BXD. (b) PPI network of glycyrrhetinic acid A's therapeutic targets. (c) GO enrichment analysis of glycyrrhetinic acid A's therapeutic targets. (d) KEGG enrichment analysis of glycyrrhetinic acid A's therapeutic targets.

estrogen levels, and enhancing ovarian function. However, due to the complex nature of TCM formulations with multiple ingredients and targets, it is challenging to examine their molecular mechanisms. Fortunately, network pharmacology and bioinformatics have provided an opportunity to systematically study the effects of TCM formulations like BXD on PCOS at the molecular level. This study combined these approaches with experiment validation to explore the mechanism of BXD in PCOS treatment.

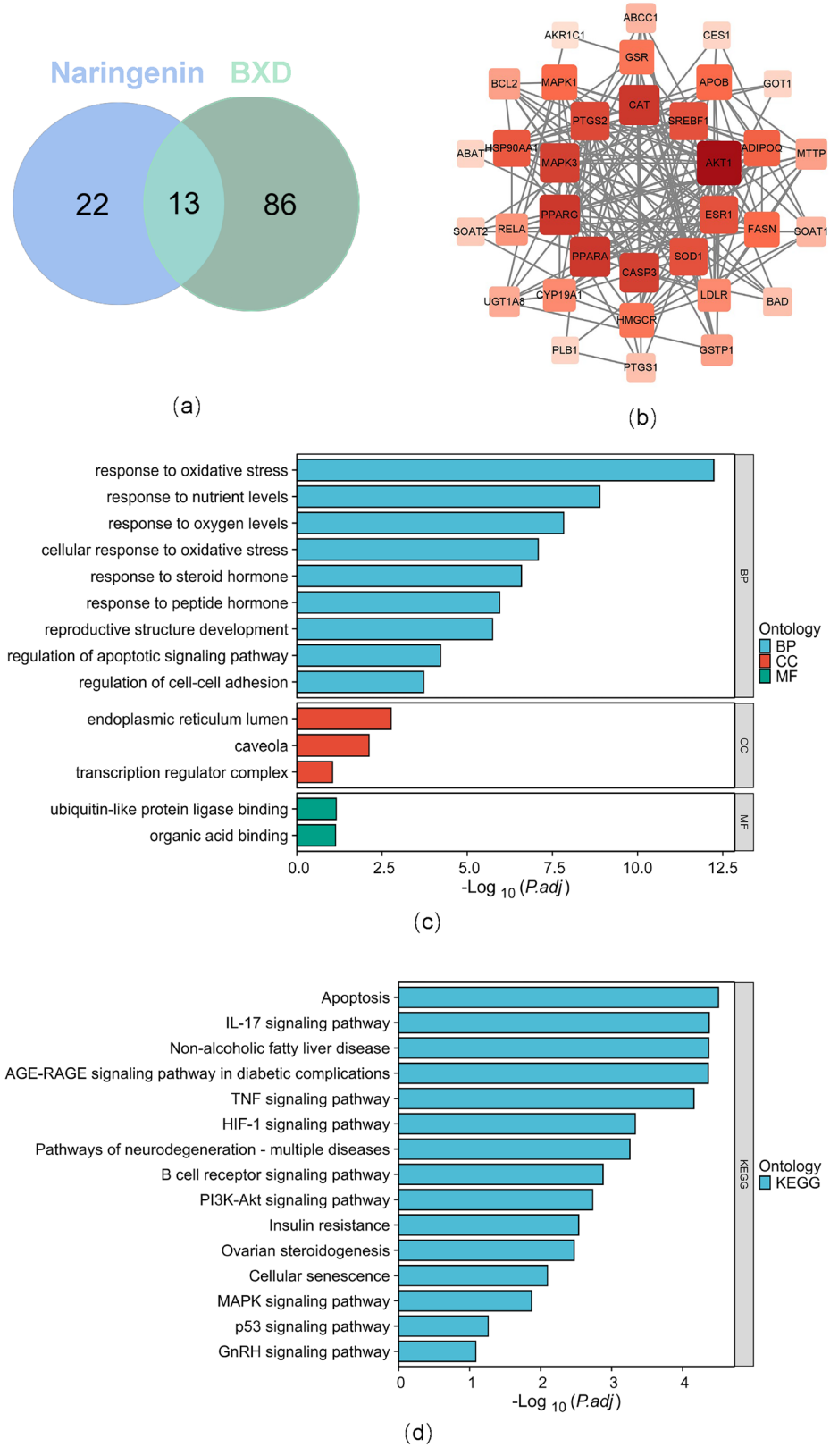


Figure 9. Analysis of naringenin's potential therapeutic target. (a) Venn diagram of the potential therapeutic targets of naringenin and the therapeutic targets of BXD. (b) PPI network of naringenin's therapeutic targets. (c) GO enrichment of naringenin's therapeutic targets. (d) KEGG enrichment of naringenin's therapeutic targets.

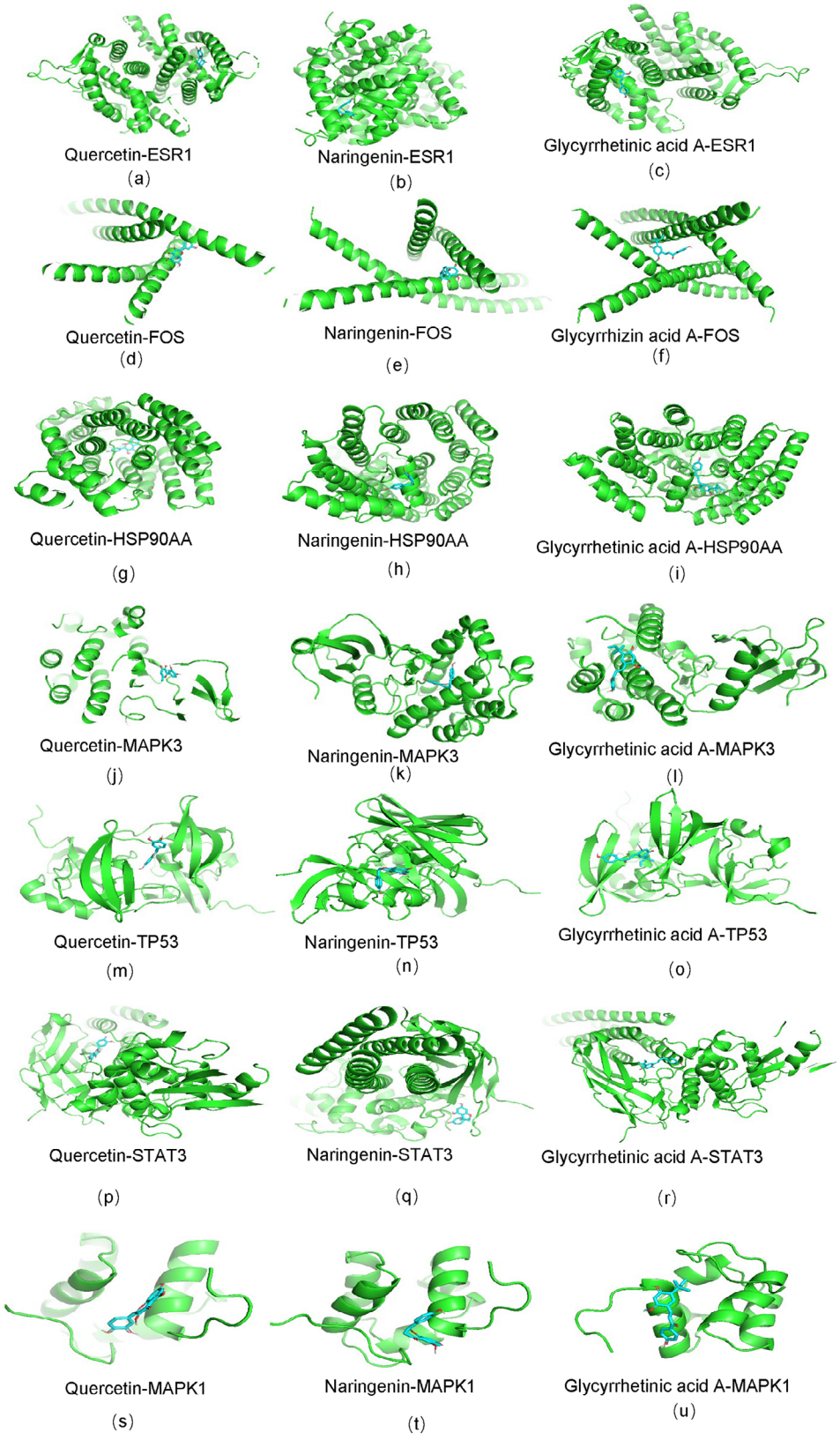


Figure 10. Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with hub genes. (a–c) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with ESR1. (d–f) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with FOS. (g–i) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with HSP90AA. (j–l). Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with MAPK3. (m–o) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with TP53. (p–r) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with STAT3. (s–u) Molecular docking of quercetin, glycyrrhetinic acid A and naringenin with MAPK1.

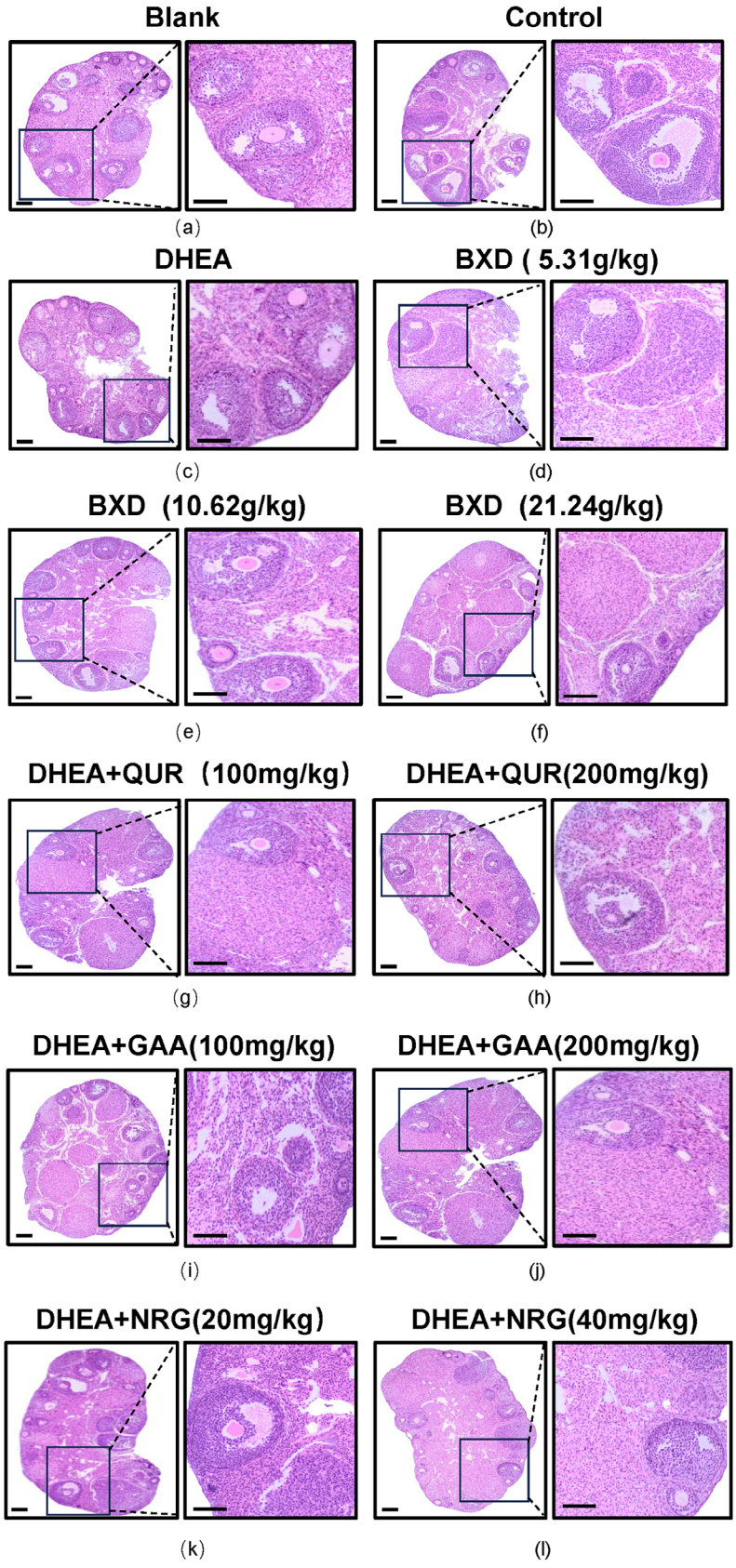


Figure 11. Ovarian morphology of BXD, quercetin, glycyrrhetinic acid A and naringenin on PCOS-like mice. HE staining showed the morphology of ovarian tissue in blank (a), control (b), PCOS-like mice (DHEA-induced mice) (c), BXD treated PCOS-like mice (d-f), quercetin-treated PCOS-like mice (g and h), glycyrrhetinic acid A-treated PCOS-like mice (i and j) and naringenin-treated PCOS-like mice (k and l). BXD, Banxia Xiexin Decoction, QUR, quercetin; GAA, glycyrrhetinic acid A; NRG, naringenin.

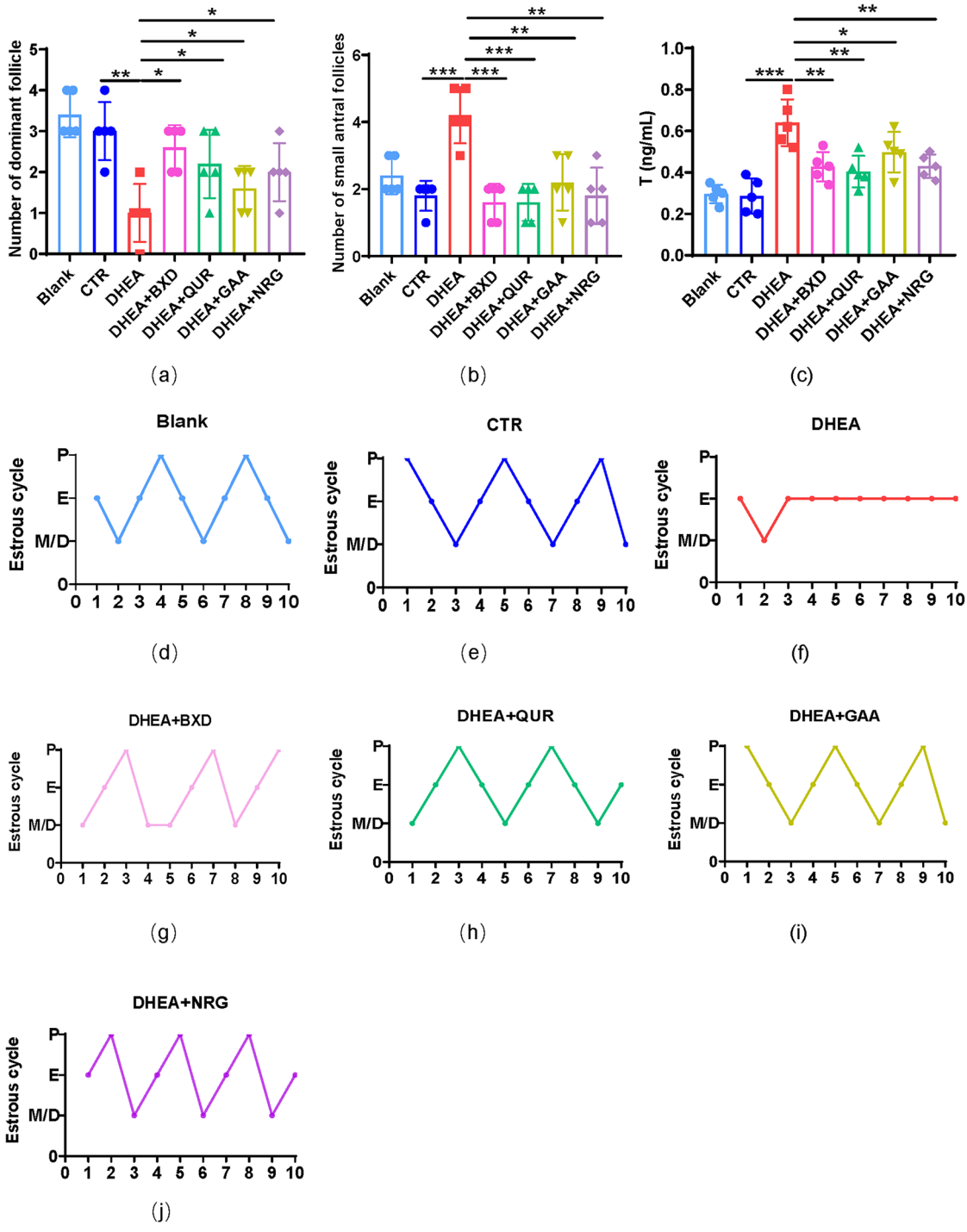


Figure 12. Therapeutic effects of BXD, quercetin, glycyrrhetinic acid A and naringenin on PCOS-like mice. (a) Number of small antral follicles in the ovaries of mice in each group were counted. (b) Number of dominant follicles in the ovaries of mice in each group were counted. (c) Serum T was measured by radioimmunoassay (n = 5/group). (d–j) The estrous cycle was assessed by vaginal cytology for 10 consecutive days. P, proestrus; E, estrus; M, metestrus; D, diestrus. BXD, Banxia Xiexin Decoction, QUR, quercetin; GAA, glycyrrhetinic acid A; NRG, naringenin; ns: $P > 0.05$; $*P < 0.05$, $**P < 0.01$, and $***P < 0.0001$.

By analyzing the therapeutic targets of BXD and PCOS using a bioinformatics database, we identified 99 potential therapeutic targets for treating PCOS with BXD. Using these potential targets, we constructed a PPI network with GeneMANIA from which we identified 8 core targets – STAT3, AKT1, MAPK3, JUN, HSP90AA1, FOS, TP53, and ESR1 – that may be particularly important in treating PCOS with BXD. Furthermore, we validated the expression of oxidative stress-related genes STAT3, MAPK3, JUN, HSP90AA1, FOS and TP53 using a mice model of PCOS.

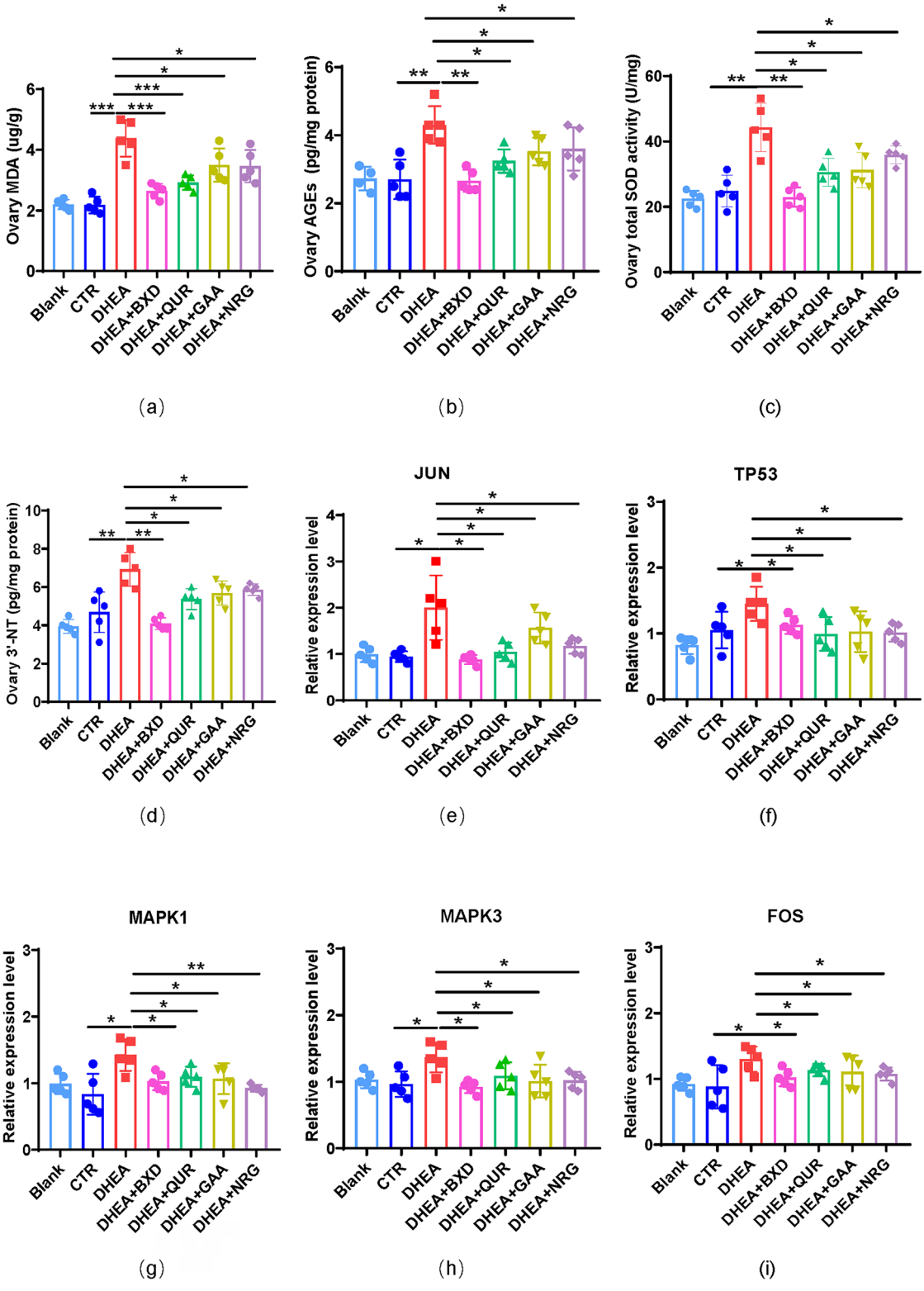


Figure 13. Evaluation of oxidative stress-related indicators and hub gene. (a) Ovary MDA levels in mice ovarian. MDA, Malondialdehyde. (b) Ovary AGEs levels in mice ovarian. AGEs, Advanced Glycation End Product. (c) Ovary total SOD levels in mice ovarian. SOD, Superoxide Dismutase. (d) Ovary 3'-NT levels in mice ovarian. 3'-NT, 3-Nitrotyrosine. (e) Relative expression levels of JUN in mice ovarian. (f) Relative expression levels of TP53 in mice ovarian. (g) Relative expression levels of HSP90AA1 in mice ovarian. (h) Relative expression levels of MAPK3 in mice ovarian. (i) Relative expression levels of FOS in mice ovarian. ns: $P > 0.05$; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

STAT3 belongs to the family of transcription factors known as STATs, which are crucial in regulating gene expression by transmitting signals from cell surface receptors to the nucleus [16]. Some studies showed that the intraovarian JAK/STAT pathway might be involved in follicular development [17]. In PCOS rats, the activation of the p-JAK2/p-STAT3 signaling pathway played a role in follicular development by increasing the expression of LHCGR and CYP17a while decreasing the expression of FSHR and CYP19 [18].

AKT (protein kinase B or PKB) is a serine/threonine-specific protein kinase that plays a key role in the PI3K/AKT signaling pathway. This pathway is activated by growth factors and other extracellular signals, and is involved in regulating many important cellular processes, including survival, proliferation, growth, metabolism, and migration [19]. Also, AKT plays a crucial role in regulating various stages of oocyte maturation. It is involved in the control of meiosis resumption and regulates polar body emission and spindle organization during the metaphase II stage. Disruption of AKT activity can have negative effects on preimplantation embryo development, leading to conditions such as infertility. Furthermore, AKT dysregulation has been linked to the development of ovarian cancer and other human diseases [20]. It has been reported that increased expression of AKT1 and AKT2 in combination with androgens could contribute to granulosa-lutein cell (GC) dysfunction in hyperandrogenic (+HA) PCOS patients [21].

About 50% to 70% of women with PCOS patients have variable degrees of insulin resistance [22]. The MAPK/ERK pathway, as the most common and typical subset of the MAPK family, plays a crucial regulatory role in cell proliferation, differentiation, apoptosis, and inflammatory response. However, when the MAPK/ERK pathway is abnormally activated, it can cause a series of metabolic disorders, including impaired insulin signaling and insulin resistance [23]. Berberine has been shown to improve insulin resistance in rats with polycystic ovary syndrome (PCOS) by regulating the PI3K/AKT and MAPK pathways involved in glucose metabolism and insulin signaling [24].

JUN, an important member of the MAPK signaling pathway, plays a crucial role in various physiological and pathological processes, such as embryonic development and apoptosis [25]. A study showed that combining metformin and pioglitazone is highly effective in improving PCOS by modulating the AMPK/PI3K/JNK pathway [26].

Heat shock protein 90α (Hsp90α), encoded by the HSP90AA1 gene, is the stress-inducible isoform of the molecular chaperone Hsp90 [27]. It has been suggested that dysregulation of HSP90 may play a role in the pathogenesis of PCOS by altering steroid hormone signaling and promoting aberrant follicular development [28]. A research result showed that modifying a specific protein called HSP90a through S-palmitoylation could be a promising target for developing new treatments for ovarian hyperandrogenism [29].

GO analysis showed that the targets of BXD for PCOS were significantly enriched in oxidative stress, hormone response, and apoptosis. Several studies have confirmed that the increased apoptosis in ovarian GCs might be closely related to the occurrence and development of abnormal follicles in PCOS cases [30]. The lower apoptotic rates observed in PCOS patients were associated with decreased levels of caspase-3. Additionally, higher levels of cellular inhibitor of apoptosis proteins-2, which promote cell survival, were detected in the PCOS group [31]. In the current study, the core targets STAT3 and TP53 are both common key genes involved in apoptosis, while the enriched PI3K-AKT is a significant signaling pathway that regulates cell growth and apoptosis [32,33].

In addition, the oxidative stress has been demonstrated to play an important role in the development of PCOS by regulating the balance of oxidative and antioxidant activity in the body [34]. Research has shown that the pro-inflammatory conditions triggered by oxidative stress can potentially lead to insulin resistance and, consequently, the development of cardiovascular disease in PCOS [34–36]. Similarly, this study identified the enrichment of the MAPK signaling pathway and HIF1 pathway through KEGG analysis. The HIF-1 (Hypoxia-Inducible Factor 1) signaling pathway plays a vital role in maintaining the balance of oxygen levels within the body. HIF-1 can be triggered by factors beyond oxygen deprivation, such as nitric oxide and various growth factors [37]. Previous research clarified that HIF-1a-mediated ET-2 inhibition *via* increased prolyl hydroxylase activity was an important mechanism mediating the formation of PCOS in rats [38].

The role of BXD in managing conditions like PCOS and insulin resistance appears to be extensive and multifactorial. BXD has been observed to exert beneficial actions under high glucose conditions, commonly seen in patients with insulin resistance and conditions like PCOS. It achieves these effects by modulating various cellular pathways and processes. In HT22 cells, a model for neuronal function, BXD treatment reduced the levels of proteins such as JNK, Foxo3a, SIRT1, ATG7, Lamp2 and LC3 that are associated with oxidative stress and autophagy—a process crucial for cell survival and homeostasis. The presence of excessive

oxidative stress is a well-known pathogenic feature of PCOS and insulin resistance, and the upregulation of these proteins by BXD suggests a capacity to counteract this stress, thereby promoting cell survival and function. Interestingly, the protective effects of BXD were partially reversed by the JNK inhibitor SP600125, while quercetin, a natural flavonoid, was found to enhance BXD's beneficial effects in high glucose settings, demonstrating the complexity and potential synergism in BXD's mechanism of action [39]. Animal studies have provided additional insights into BXD's systemic effects. Moreover, BXD administration was shown to lower blood glucose levels and enhance insulin sensitivity, two key factors related to both PCOS and the development of insulin resistance. These effects may be mediated by BXD's ability to suppress inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and interleukin-17 (IL-17), while concurrently promoting Akt phosphorylation, a sign of improved insulin signaling [40]. Besides, this multifaceted approach of BXD is further supported by research indicating its ability to inhibit apoptosis in pancreatic β-cells, which are essential for insulin production. By promoting insulin secretion and lowering blood glucose levels, BXD addresses the core issues in insulin resistance. Its regulatory influence on lipid profiles augments its therapeutic potential, making it a valuable candidate for addressing the dysregulated lipid metabolism often seen in PCOS patients [41]. In summary, its therapeutic effects encompass the modulation of cellular stress responses, inflammatory cytokines, and insulin signaling pathways, all of which are critical for normalizing metabolic functions and potentially mitigating the progression of associated conditions. These findings suggest that BXD holds considerable promise as a complementary approach to conventional treatments for metabolic dysfunctions such as PCOS and insulin resistance.

Chronic inflammation is considered a key factor in the pathogenesis of PCOS, mainly manifested by abnormal elevation of inflammatory factors such as NF-κB and TGF-β1 [42]. NF-κB signaling pathway significantly influences inflammation and regulates diverse biological functions, such as elevated androgen levels, insulin resistance, cardiovascular complications and endometrial irregularitie [43]. Olaniyi found that PCOS induces cardiac inflammation which are associated with elevated PCSK9/NF-kB. Acetate could attenuates cardiac inflammation by suppressing PCSK9/NF-kB [44]. Women with PCOS was treated with cyproterone acetate/ethinyloestradiol with addition of metform could reduce the level of NF-κB, TGF-β1 and HOMA-IR and increased the level of TSP-1 [42]. Quercetin showed promising effects in reducing blood insulin levels and inflammation markers in an insulin-resistant PCOS rat model. It also inhibited NF-κB translocation in granulosa cells and expression of inflammation-related genes in ovarian tissue, improving insulin resistance and showing therapeutic potential for PCOS. The mechanism may involve targeting the Toll-like receptor/NF-κB signaling pathway and enhancing the ovarian tissue's inflammatory microenvironment [45]. Wang found that glycyrrhizic acid could suppressed the activation of NF-κB and the functions of PI3K p110δ and p110γ variants, leading to a decrease in the secretion of LPS-induced TNF-α, IL-6, and IL-1β [46]. A study investigated licorice extract's anti-inflammatory effect on PCOS mice. Treatment with licorice extract for 21 days improved ovarian morphology and returned the estrus cycle to normal. While some markers like TGF-ß expression increased, others like TNF-α and COX-2 levels remained unchanged. Licorice extract (key component: glycyrrhizic acid) may help improve histological and immunological changes in PCOS due to its anti-inflammatory properties [47]. Naringenin has anti-inflammatory and anti-diabetic effects. Xiang found that naringenin could inhibit the expression of PKGIα to alleviate IR that occurs in PCOS [48]. These compounds could be valuable for treating inflammatory conditions in PCOS model.

The active ingredients and monomers of traditional Chinese medicine play an important role in the treatment of PCOS. In this study, we identified 3 herb monomers-quercetin, glycyrrhetinic acid A, and naringenin in BXD formulation for PCOS treatment. Importantly, we found that they significantly improved symptoms in PCOS mice. Quercetin can significantly reduce the levels of testosterone and LH in patients with obese PCOS [49]. It can also reduce aromatase expression in infertile patients, which helps with ovulation and conception [50]. Besides, quercetin is a flavonoid compound extensively studied for its antioxidant properties. It exerts its antioxidant effects by scavenging free radicals and reactive oxygen species (ROS), which are generated as part of normal metabolism or in response to environmental stressors such as pollution and toxins. Additionally, quercetin can enhance the activity of several antioxidant enzymes, including glutathione peroxidase and catalase, which help to neutralize harmful ROS. By maintaining oxidative balance, quercetin may protect against oxidative stress-related diseases, such as cancer, cardiovascular disease, and neurodegenerative disorders [51]. In our study, GO analysis showed that the target of BXD was significantly enriched for oxidative stress. It is suggested by these analyses above that BXD may improve PCOS by inhibiting oxidative stress.

Glycyrrhetinic acid A (GAA) is a bioactive compound found in licorice root. GAA has anti-diabetic properties, including the ability to reduce blood glucose levels, decrease serum insulin levels, enhance insulin sensitivity, and regulate lipid metabolism [52,53]. Admittedly, increased blood glucose and serum insulin levels, decreased insulin sensitivity and dysregulated lipid metabolism are complications of PCOS. Studies have shown that GA-A given to female rats with PCOS for 30 days significantly improved their lipid profile, blood glucose levels, and estrous cycle. Specifically, triglyceride levels decreased by 50.61%, total cholesterol levels decreased by 29.04%, blood glucose levels decreased by 20.32%, and over half of the treated rats had a normalized estrous cycle [54]. Therefore, GA may be a potential candidate for the treatment of PCOS.

Naringenin was also reported to be effective in the treatment of PCOS. Naringenin treatment in a rat model of PCOS significantly increased the levels of the reactive oxygen species (ROS) scavenging enzymes CAT, SOD, and GPX ($p < 0.05$) and prevented weight increase. Naringenin treatment resulted in a significant reduction in serum glucose levels ($p < 0.05$), normalized estradiol and testosterone levels, steroidogenic enzyme activity, and maintained the normal anatomy of the ovaries [55]. Treatment with naringenin in the PCOS animal groups increased ovulation potential and decreased cystic follicles and levels of androgens. Naringenin improves ovulation and suppresses androgens and cystic follicles, involving AKT activation [56].

To enhance the validity of network pharmacology findings, molecular docking was utilized to validate the robust binding interaction between the crucial bioactive constituents and central target molecules. The docking studies showed that three essential components of BXD, including quercetin, glycyrrhetinic acid A, and naringenin, could regulate/inhibit target receptors such as ESR1, FOS, HSP90AA, MAPK3, TP53, STAT3 and MAPK1 to alleviate the pathological consequences of polycystic ovary syndrome (PCOS). Similar studies had shown that quercetin could bind well with JUN, TP53, and ESR1 [57], glycyrrhetinic acid A could bind well with MAPK1 and FOS with MAPK3 [58], and naringenin had a high affinity with TP53, HSP90AA1, MAPK3, and MAPK1 [59]. This investigation offers a more profound understanding of the underlying mechanisms of PCOS and holds the potential to facilitate the development of novel medications and therapeutic strategies for PCOS treatment.

Notably, our study offered some novel insights into BXD in the therapy of PCOS and suggested plausible molecular pathways and prospective pharmacological targets of PCOS for the first time. However, future confirmation of these findings will require recruiting patients with PCOS. Also, additional *in vivo* and *in vitro* studies are necessary to confirm the hypothesized mechanisms and pharmacological targets to confirm the potential therapeutic application of BXD for PCOS.

5. Conclusion

In this study, we used network pharmacology and bioinformatics to systematically study the effects of TCM formulations BXD on PCOS at the molecular level. We identified 8 core targets, including FOS, JUN, STAT3, MAPK3, ESR1, TP53, and HSP90AA1 of BXD for PCOS. The biological functions of these targets are mainly related to oxidative stress, the development of reproductive structures, hormonal response, and apoptosis. Signaling pathways were mainly enriched in PI3K-AKT, MAPK, HIF1, IL17, cellular senescence, TNF signaling, insulin resistance, necroptosis, GnRH, ovarian steroidogenesis, etc. Based on the target and functional analysis, we mined three key ingredients of BXD quercetin, glycyrrhetinic acid A, and Naringenin. Molecular docking analysis revealed their superior binding properties to the core targets, suggesting that they are potential targets for the treatment of PCOS. We validated the inhibition effect of BXD, quercetin, glycyrrhetinic acid A, and Naringenin on oxidative stress in an animal model. In general, these results suggest that BXD and three key ingredients may alleviate the development of PCOS by affecting oxidative stress, which provides a theoretical basis and new insight for BXD in the clinical treatment of PCOS.

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Ethics approval

All animal experiments were approved by the Ethics Committee for Animal Experimentation of Xuzhou Medical University (approval number 202209S077), and all procedures were conducted following the Guidelines for the Care and Use of Laboratory Animals.

Consent for publication

All authors approved the final manuscript and gave their consent for publication.

Authors contributions

The study was designed by Mingming Wang. Data preparation and analysis were performed by Hai Bai, Yingying Zhang. Jing Huang conducted molecular docking. The manuscript was revised and refined by Richard Mprah. All authors read and approved the final manuscript.

Disclosure statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The data are available within the article.

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