Network Pharmacology-based Research of the Therapeutic Effects of Epimedium Brevicornu Maxim on Diminished Ovarian Reserve

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Research

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Abstract

Background: Diminished ovarian reserve (DOR) is a common gynecological disease. As a traditional Chinese medicine, Epimedium brevicornu maxim (EBM) is often used to treat sexual dysfunction and irregular menstruation. But whether EBM could alleviate the symptoms of DOR is unclear.

Methods: We constructed the DOR rat models and determined the effect of EBM on the development of DOR. Next, we used the network pharmacology to analyze the active ingredients of EBM and the targeted genes of these ingredients. And the critical genes were selected for the next research. ELISA were performed to detect the expression of these genes in the serum of rats.

Results: We found that the EBM treatment suppressed the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the ovary tissue of rats. Meanwhile, the levels of estradiol (E2), anti-mullerian hormone (AMH) and Inhibin B (INHB) was promoted after the EBM treatment. The HE staining showed that the treatment of EBM enhanced the formation of follicles. Based on the network pharmacology analysis, we found that EBM has the potential to relieve symptoms of DOR by modulating the levels of steroid hormone receptor.

Conclusion: In conclusion, EBM relieved the symptoms of DOR by modulating the steroid hormone receptors, such as promoting the secretion of androgen receptor (AR) and estrogen receptor (ESR1).

Background

Diminished ovarian reserve (DOR) is one kind of the gynecological disease. The symptoms of DOR are decreasing quantity and quality of stored follicles in the ovary, decreasing levels of sex hormone, loss of reproductive function [1]. Furthermore, the continuous development of DOR could finally lead to the menstrual disorder, ovulation failure, amenorrhea and infertility [2]. If the effective treatment is not taken in time, the premature ovarian failure of female patients could lead to loss of reproductive capacity. Therefore, it is urgent to develop the new drug treatment schemes to alleviate the damage of DOR to the reproductive system of female patients.

Epimedium brevicornu maxim (EBM) is a traditional Chinese medicine, which has the effect of nourishing kidney and liver. And the EBM also used for the treatment of osteoporosis in postmenopausal women [3]. Furthermore, the EBM was also used alone or in combination with other Chinese medicine to treat a variety of diseases, including inflammation response, sexual dysfunction, menopausal symptoms and irregular menstruation. And EBM could also enhance the secretion of the glands of hypothalamus-pituitary-gonadal axis, promote sex hormone secretion, improve function of sexual, improve cerebrovascular function and upregulate the expression of neurotransmitter receptor [4, 5]. The study also revealed that the extractive of EBM could impact the hypothalamus-pituitary-gonadal axis and increase the weight of the ovaries of rats [6]. But whether the EBM could relieve the DOR is still unknown.

In recent years, network pharmacology has been widely used to analyze the relationship between complex components of drugs and diseases. Since the active ingredients of traditional Chinese medicine monomers and compound traditional Chinese medicine are very complicated, it is necessary to analyze the active ingredients in these medicines, and predict their target genes and mechanism of action through the theory of network pharmacology [7]. Network pharmacology could systematically analyze the network relationship between active ingredients in Chinese medicine and genes which could induce the occurrence of diseases. And this will further clarify the specific mechanism of active ingredients in drugs which could relieve the symptoms of corresponding diseases.

In this study, we established the DOR rat models. And then we determined whether the EBM treatment could alleviate the symptoms of DOR. Finally, we used the methods of network pharmacology to explore the molecular mechanism of the efficacy of EBM on DOR. And these results could reveal that the effect of EBM on DOR and the specific molecular mechanism.

Materials And Methods

Animal assays

The 8 weeks old female rats was obtained from laboratory animal center of Guangdong province (Guangdong, China). These experimental rats were bred normally for one week. After that, the vaginal smear was performed and the estrous cycle was observed. Then, 24 rats with two normal estrous cycles were selected for the next experiments. These rats were divided into three groups (Control, Model and EBM treatment groups). Next, the gavage was performed to give the tripterysium glycosides (50 mg/kg.d) to the rats of model and EBM treatment groups for two weeks. And the estrous cycle was observed from the eighth day after the gavage.

When the estrous cycle disorder occurs in these rats, the establishment of DOR model is successful. When the DOR models was constructed, the extractive of EBM (110 mg/kg.d) was dissolved in the saline solution and given to the rats of EBM treatment group by gavage. The rats of control and model groups were given with the equal volume of normal saline solution. These rats were fasted for 24 hours after the last treatment, then these rats were anesthetized and the blood was collected for the next research. After that, the ovaries were quickly gathered and weighed. At last, half of the ovarian tissues was fixed in 4% paraformaldehyde solution for HE staining. Granulosa cells were extracted from the other half of the ovarian tissues for subsequent analysis.

Elisa Assays

The blood was centrifuged using a high-speed centrifuge (Beckman Coulter, USA) and then isolated the serum. The serum was collected into the sterilized tube for the ELISA assays. The ovary granulosa cells were separated from the ovarian tissue and lysed with the RIPA buffer (Beyotime, China). After that, the FSH Rat ELISA kit (Senbeijia Bio, SBJ-R0712), LH Rat ELISA kit (Runyu Bio, China), E2 ELISA kit

(Baiaolaibo Bio,ARB12674), AMG antibody (Abcam, ab54507) and INHB Rat ELISA kit (Jianglai Bio, China) were used for the detection of the levels of corresponding indicators in serum of rats from diverse groups. Next, the androgen receptor (AR) Rat ELISA kit (Tongwei Bio, China), estrogen receptor (ESR1) Rat ELISA kit (Jianglai Bio, China), CYP19A1 Rat ELISA kit (Boshikang Bio, China) were used for the detection of the levels of the corresponding indicators in the granulosa cells from these rats in different groups. All the operations in these assays was followed the guidelines.

He Staining

The procedures of HE staining followed the routine protocols [8]. Firstly, the deparaffinization and rehydration were performed for the staining of the ovarian tissue. After that, 5 μm tissue sections was stained with hematoxylin solution for 6 minutes. Then these sections were steeped in the 1% acid ethanol and washed with the distilled water. Next, these sections were stained with the eosin solution for 5 minutes. At last, these sections were dehydrated with the graded alcohol and washed with the xylene. The photographs were taken with the fluorescence microscope (Olympus, Tokyo, Japan).

Chemical Composition Database And Active Ingredient Screening

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://lsp.nwu.edu.cn/) and the Traditional Chinese Medicines Integrated Database (TCMID, http://119.3.41.228:8000/) were used to search the information of active ingredients in EBM. Because the composition of traditional Chinese medicine is complicated, not all ingredients will play the role in human bodies. Therefore, the active ingredients of EBM were selected according to the pharmacokinetic parameters including the oral bioavailability (OB) ≥ 30%, drug-likeness (DL) ≥ 0.18 and permeability of Caco2 > 0 [9, 10]. Oral bioavailability (OB) is the percentage of orally drugs that entered the systemic circulation, which is one of the most commonly used pharmacokinetic properties in drug screening. DL value is mainly used to indicate the similarity of related active ingredients with known drugs in the compound [11]. The absorption of oral drugs is mainly in the small intestine. The presence of villi and microvilli greatly increases the absorption rate. The absorption rate of drugs in the intestine is the crucial factor which could affect the bioavailability of drugs. Furthermore, gastrointestinal epithelial cells are the only way for the absorption of oral drugs. The Caco-2 cell model is an intestinal transport model and an epithelial transport model which were widely used in laboratories to evaluate the absorption of drug in vitro. Compounds are impermeable when the values of Caco-2 less than 0, so the threshold of Caco-2 permeability is set to 0.

Disease-related Targets

The 19 compounds screened in the previous step were imported into PharmaMapper (http://lilab-ecust.cn/pharmmapper/) to predict the potential therapeutic targets of EBM. According to the keywords “diminished ovarian reserve”, we searched the DOR related genes in the GeneCards database (https://www.genecards.org/). At last, the VENNY2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used for the screening of the potential targets which was affected by EBM active ingredients during the development of DOR.

Construction Of Target Proteins Network

The String database (http://string-db.org) was used for the analysis of target proteins network. The species of these proteins are limited to “Homo sapiens”. In the network, the green elements represent active ingredients in EBM, while the yellow elements represent disease-related proteins. The red line represents the targeted effect of the active ingredients in the EBM for these proteins, and the blue line represents the interaction between these proteins. We used Network Analyzer to analyze the connectivity of each protein to other proteins. The higher values of connectivity implied that numerous proteins interacted with this protein. It also showed that this protein played the critical role in the occurrence and development of disease.

Enrichment Analysis For Target Proteins

The Gene Ontology (GO) enrichment analysis was used for the analysis of the function of target genes. Therefore, we further predicted that EBM affected the occurrence and development of DOR by affecting the biological function of the corresponding proteins and signaling pathways. And the lower of P values implied that the close relationship between the targeted protein and DOR. And values of P less than 0.05 is considered statistically significant.

Statistical analysis

All the experiments in this study was repeated for three times. And the data was presented as mean ± SD. All the data was analyzed with the Graphpad Prism7.0 (GraphPad Software, La Jolla, CA, USA). And the comparison between diverse groups in histograms was performed with the student’s t test. When the P value is less than 0.05, the difference is considered statistically significant.

Results

EBM relieved the symptoms of DOR

Major symptoms of DOR is the injury of ovarian reserve. Therefore, the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), anti-mullerian hormone (AMH) and Inhibin B (INHB) in the serum of rats was determined with the ELISA. Results (Table 1) showed that compared to

the control group, the levels of FSH and LH was increased while the secretion of E2, AMH and INHB was inhibited in the serum of rats from model groups. However, after the treatment of EBM, the expression of FSH and LH was suppressed and the levels of E2, AMH and INHB was increased compared to the model group. Next, the HE staining was performed to observe the corpus luteum and follicle in the ovarian tissue. As shown in Fig. 1, compared to the control group, the number of follicles and corpus luteum was significantly decreased in the ovarian tissue of rats from the model group. Nevertheless, the formation of follicles and corpus luteum was enhanced after the treatment of EBM. After that, the numbers of follicles and corpus luteum was calculated according to the images of HE staining. The amount of corpus luteum, mature follicles, grown follicles and atresia follicles was counted in five photographs which were captured with the highly magnifying lens of microscopes. We found that compared to the control groups, the number of corpus luteum, mature follicles and grown follicles was decreased, while the atresia follicles were increased in ovarian tissues of rats in model group. After the treatment of EBM, the amount of corpus luteum, mature follicles and grown follicles was recovered and the number of atresia follicles was decreased (Table 2).

The Screening Of Active Ingredients

The information of active ingredients of EBM was acquired from the TCMSP and TCMID database. Based on the search results (Table 3 and Fig. 2), we received 19 ingredients of EBM. And these ingredients all conformed to the screening conditions (OB ≥ 30%, DL ≥ 0.18, Caco-2 > 0).

Establishment Of Drug-active Ingredient-targeted Gene Network

In the next research, we used the PharmaMapper to predict the targeted genes of these ingredients. Meanwhile, the DOR associated genes was selected with the GeneCards. Then, these genes were imported into the VENNY2.1 to obtain these genes which was targeted by the active ingredients of EBM (Fig. 3A). After that, these genes were analyzed with the String and the network documents were imported into the Cytoscape 3.7.1 software to draw the network diagram (Fig. 3B). There are 48 targeted genes and 19 active ingredients in this diagram. The green diamond nodes represent the active ingredients of the EBM. And the yellow oval nodes represent targeted genes. Furthermore, each active ingredient targets multiple target genes (red lines) and each gene interacts with other targeted genes (blue lines). According to this result, we found that the EBM could relieved the symptoms of DOR by targeting multiple genes. Next, the Network Analyzer was used for the calculate of the degree of these targeted genes. And the degree means that the number of other targeted genes which was associated with this gene. The higher levels of degree of targeted gene indicated that this gene plays the critical role in the occurrence and development of DOR. According to the result (Fig. 4A), we found that these genes (ERBB2, ESR1, CCND1, EP300, NOTCH1, AR, HSP90AA1, VEGFA, TP53 and ALB) were the crucial modulators during the development of DOR. In the next study, the GO enrichment analysis was performed to analyze the

biological function of targeted genes. And we arranged the result in descending order of the P values. As shown in Fig. 4B, the biological function of targeted genes was mainly associated with the steroid hormone receptor activity, proximal promoter sequence specific DNA binding, RNA polymerase II transcription factor binding and P53 binding. We selected steroid-hormone active receptor related target genes (AR, ESRRB and ESR1) for subsequent research.

Treatment of EBM alleviated the lower levels of AR, ESR1, ESRRB and CYP19A1 in DOR rats

In the last part, we determined the levels of AR, ESR1, Estrogen related receptor β (ESRRB) and CYP19A1 in ovarian granular cells. These targeted genes are all associated with the normal growth of ovarian tissue and formation of follicular. As shown in Fig. 5, compared to the control groups, the levels of AR, ESR1, ESRRB and CYP19A1 were decreased in the ovarian granular cells of rats of model group. However, the expression of AR, ESR1, ESRRB and CYP19A1 was rescued after the treatment of EBM.

Discussion

Diminished ovarian reserve (DOR) is the critical reason of female infertility. On the other side, the main symptoms of DOR are the decreasing of the number and quality of oocytes, reduced probability of pregnancy for women and the increasing probability of miscarriage [12, 13]. Relevant statistics showed that the incidence of DOR in young women is about 10% [14, 15]. Although the incidence of DOR is higher, the exact cause of DOR remains unclear. Aging is considered to be a crucial cause of diminished ovarian reserve, while chemotherapy, radiation therapy, autoimmune diseases and genetic factors are also considered to be critical reasons for the recession of ovarian reserve function [16].

EBM is a traditional Chinese medicine, mainly used to treat various diseases such as sexual dysfunction and osteoporosis [17–19]. Furthermore, due to the protective effects of EBM on the reproductive system, immunity, cardiovascular, bone and nervous system, EBM has been added to various beverages in China. On the other side, EBM also has multiple biological effects such as induction the biological effects of estrogen and nerve regeneration [20, 21]. From these results, we found that EBM could alleviate the ovarian-related dysfunction of women. In this study, we also found that the treatment of EBM relieved the DOR induced higher levels of FSH and LH and promoted the secretion of E2 which was suppressed during the development of DOR. According to the photographs of HE staining, we also found that the EBM therapy alleviated the reduction of the follicles and corpus luteum during the development of DOR. These results also indicated that the treatment of EBM relieved the symptoms of DOR.

The Network pharmacology is a research method which could analyze the target genes of active ingredients in drugs and genes associated with the occurrence of disease to build a network, and then clarify the specific mechanism of treatment efficacy of this drug on diseases. Recently, this method is widely used in pharmacological analysis of traditional Chinese medicine. In this study, we selected that 19 active ingredients in the EBM such as kaempferol, quercetin and luteolin. The kaempferol was proved that could stimulate the development of follicular in sheep [22]. Quercetin was revealed that could

strengthen the antioxidant capacity of ovary in rats [23]. Therefore, these results suggested that EBM has the potential to treat DOR.

Next, the targeted genes of the active ingredients of EBM and genes related to the occurrence of DOR were analyzed to clarify the specific molecular mechanism of effect of EBM on DOR. And the Network Analyzer was used to select some critical factors in this process such as ESR1, AR, NOTCH1 and VEGFA. Among them, ESR1 is an estrogen receptor which could promote the proliferation and differentiation of breast cells by binding to estrogen [24]. AR is one kind of the androgen receptors which existed in the tissues of prostate, testes, ovary and fat [25]. There is study revealed that the levels of ESR1 and AR alleviated the symptoms primary ovarian insufficiency [26]. Furthermore, the higher levels of NOTCH1 was proved that could relieve the premature ovarian failure [27]. And the VEGFA could also impact the growth of ovary by promoting the formation of blood vessels [28]. These results indicated that the EBM alleviated the symptoms of DOR by modulating the expression of these targeted genes such as ESR1, AR, NOTCH1 and VEGFA.

Therefore, the results of GO enrichment analysis showed that the treatment effect of EBM on DOR was associated with the steroid hormone receptors. The AR and ESR1 could ensure normal development of ovary [29]. In our study, we also found that the treatment of EBM relieved the decreasing levels of AR and ESR1 during the development of DOR. These results also indicated that the EBM relieved the symptoms of DOR by regulating the secretion of steroid hormone receptors.

Conclusions

Above all, we detected the effect of EBM on DOR and explored the molecular mechanism with the network pharmacology. And all the results suggested that the EBM alleviated the symptoms of DOR by modulating the levels of steroid hormone receptors such as upregulating the levels of AR and ESR1.

List Of Abbreviations

Diminished ovarian reserve (DOR); Epimedium brevicornu maxim (EBM); Follicle-stimulating hormone (FSH); Luteinizing hormone (LH); Inhibin B (INHB); Androgen receptor (AR); Estrogen receptor (ESR1); Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP); Traditional Chinese Medicines Integrated Database (TCMID); Drug-likeness (DL); Gene Ontology (GO); Estrogen related receptor β (ESRRB).

Declarations

Ethics approval and consent to participate

Our research involved the utilization of laboratory animals under the supervision of the Shenzhen Peking University – The Hong Kong University of Science and Technology Medical Center Animal Care and Use

Committee.

Consent for publication

All authors have approved this manuscript for publication.

Availability of data and material

The analytical data in this study can be obtained from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Conceived and supervised the experiments: DL. Performed the experiments: SH, YN, SJ and DZ. Analyzed the data: SH, YN and YX. Technical support: SJ and YX. Wrote the paper: DL and SH. All authors read and approved the final manuscript.

Acknowledgement

Not applicable

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Figures

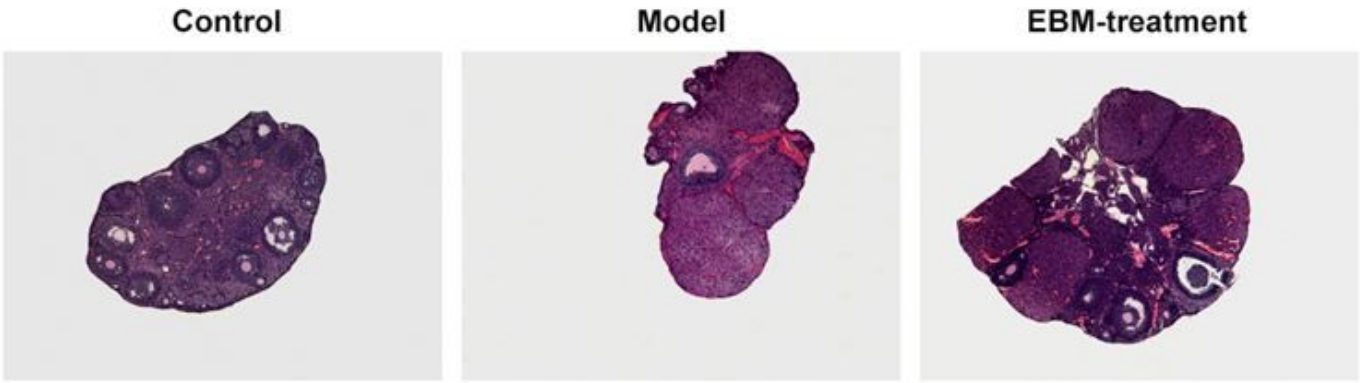

Figure 1

Application of EBM alleviated the symptoms of DOR. Representative images of the HE staining of ovarian tissues of rats from different groups.EBM, Epimedium brevicornu maxim; DOR, Diminished ovarian reserve.

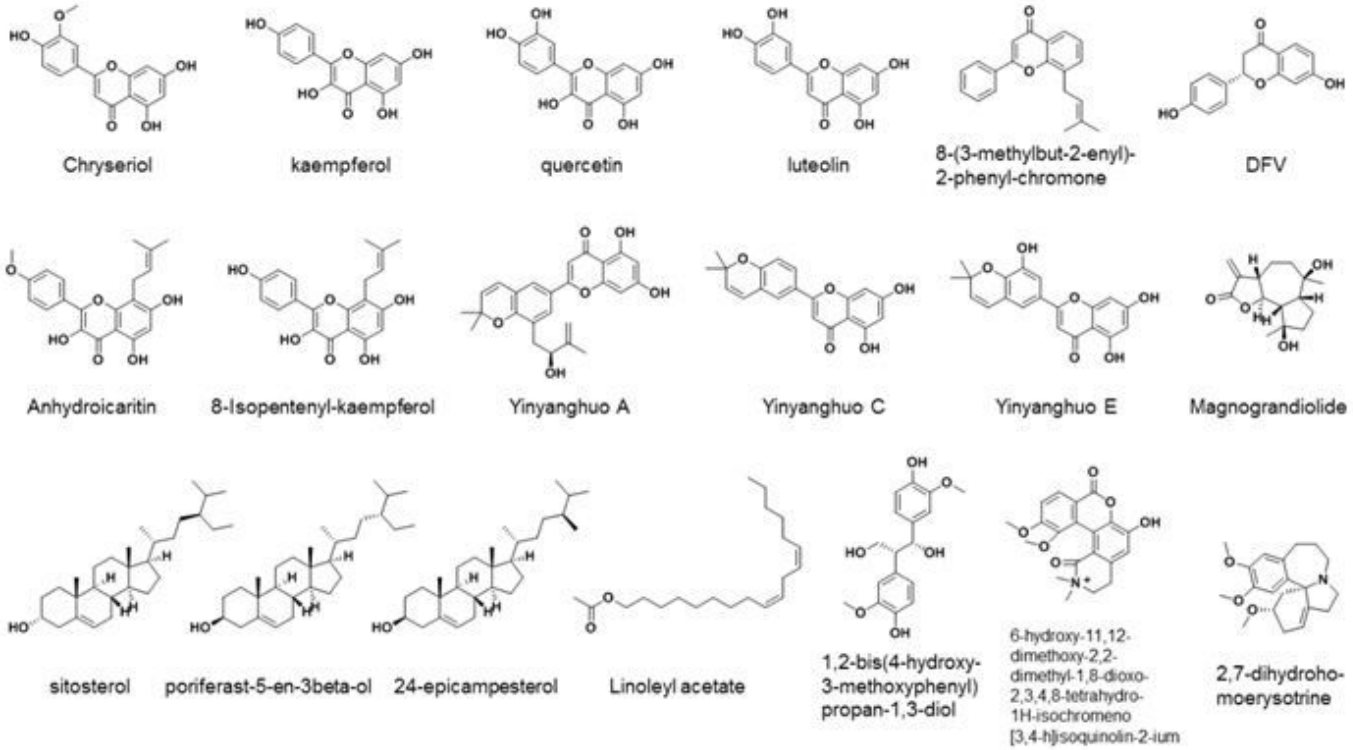

Figure 2

Theactive ingredients of EBM which was selected from the TCMSP and TCMID database. These active ingredients of EBM were selected from the database according to the screening criteria (OB≥30%, DL≥0.18, Caco-2>0). EBM, Epimedium brevicornu maxim.

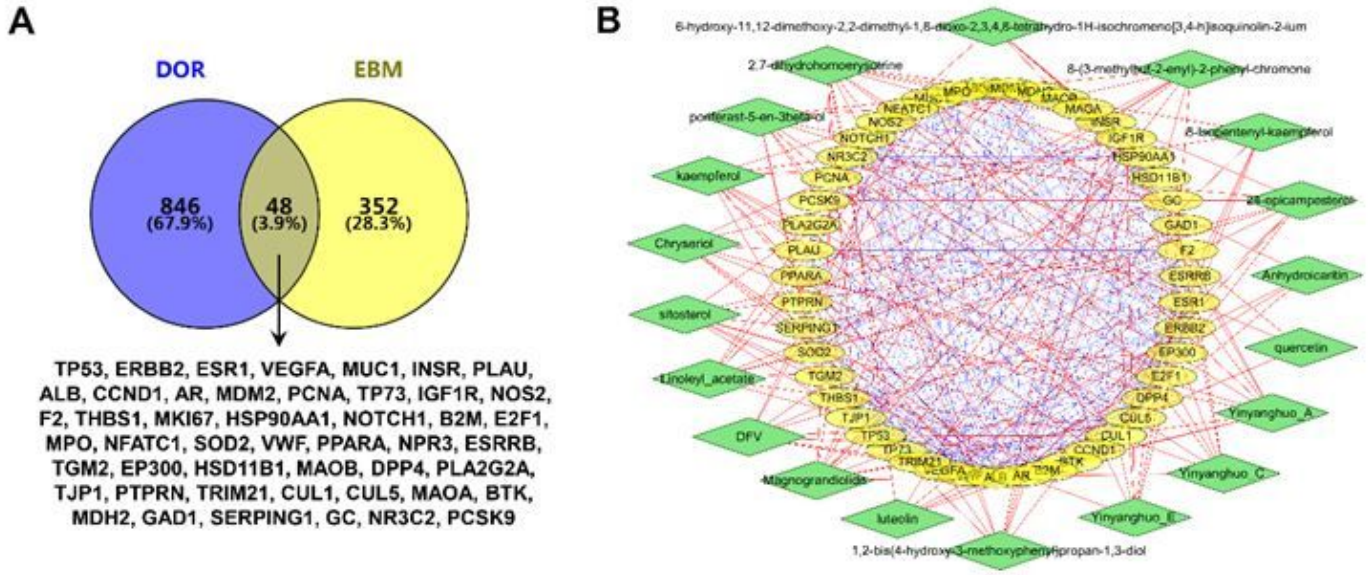

Figure 3

Construction of drug-active ingredient-targeted gene network. (A) The VENNY2.1 was used to select these genes which was associated with the occurrence of DOR and targeted with the active ingredients of EBM. (B) Cytoscape 3.7.1 software was used to draw the network diagram to exhibit the relationship between the active ingredients of EBM and targeted proteins. DOR, Diminished ovarian reserve;EBM, Epimedium brevicornu maxim.

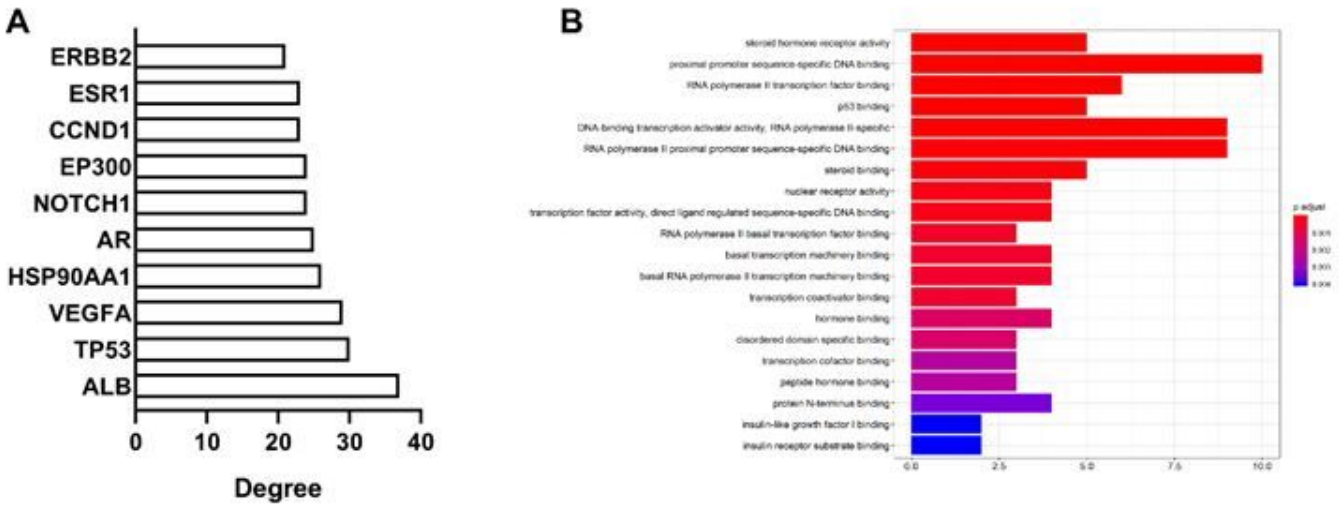

Figure 4

Related pathways was analyzed with the GO enrichment analysis. (A) The Network Analyzer was used to detect the relationship between the protein and other proteins. (B) The GO enrichment analysis was performed to explore the function of these targeted genes.

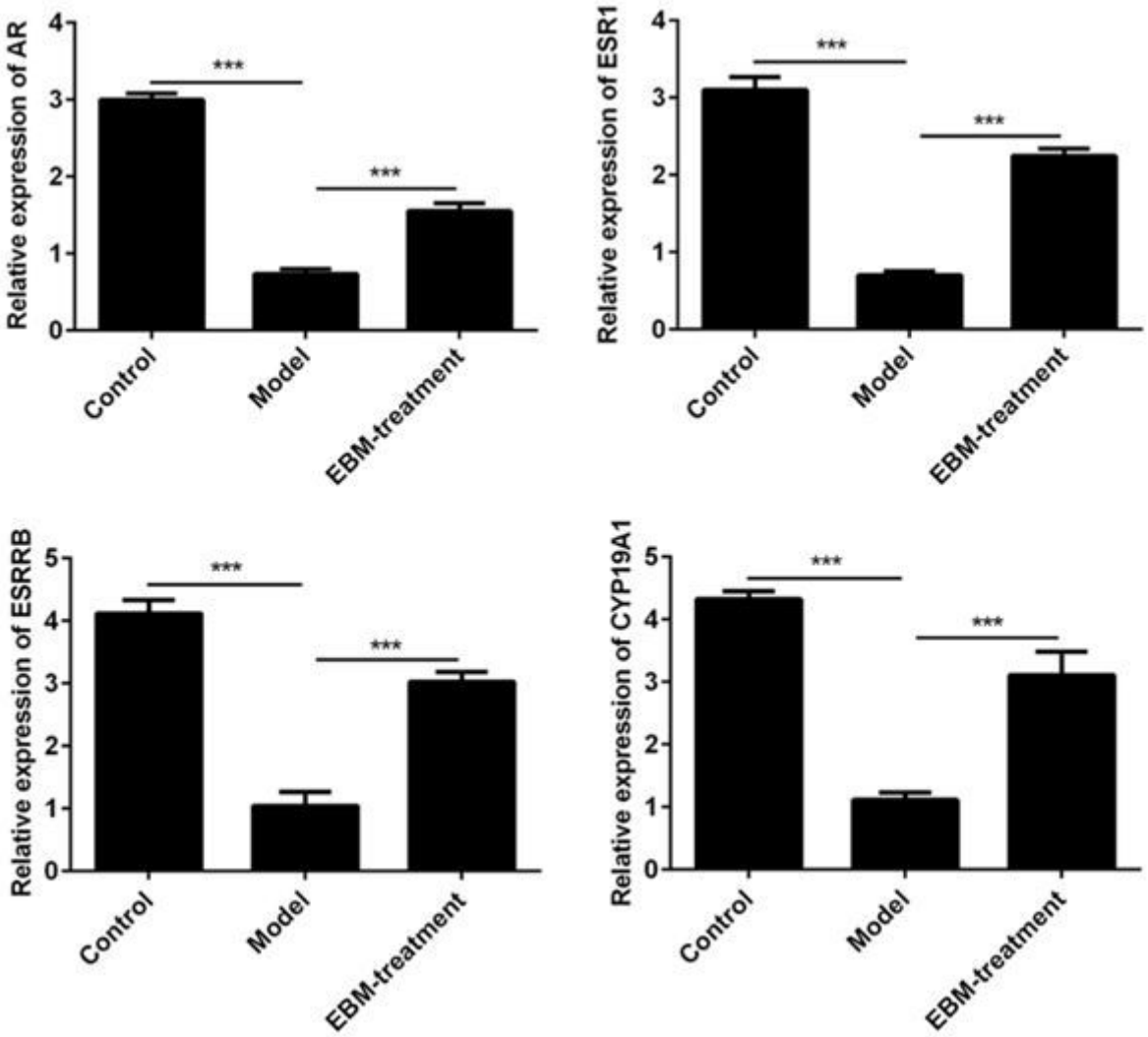

Figure 5

EBM alleviated the downregulation of AR, ESR1, ESRRB and CYP19A1. The levels of AR, ESR1, ESRRB and CYP19A1 in ovarian granular cells was determined with the ELISA assays. ***p<0.001.AR, androgen receptor;ESR1, estrogen receptor; ESRRB,Estrogen related receptor β.