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The Polysaccharides from Yiqi Yangyin Complex Attenuated Mammary Gland Hyperplasia: Integrating Underlying Biological Mechanisms and Network Pharmacology

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Abstract

Yiqi Yangyin complex (YYC), the homology of medicine and food, is comprised of *Polygonati Rhizoma, Lycii Fructus, Codonopsis pilosula, and Rehmanniae Radix.* Herein, the YYC polysaccharide treatment effectively attenuated the progression of MGH in a mice MGH model-induced with estrogen and progestogen. YYC significantly relived hormonal disorders by reducing the levels of estrogen receptor α (ERα) and progesterone receptor (PR), and substantially elevated the protein level of BCL2-associated X (Bax) and significantly down-regulated expression of B-cell lymphoma-2 (BCL-2). Finally, the key targets of ERα, PR, Bax and BCL-2 were predicted and significantly enriched on estrogen signaling pathway and apoptosis pathway by network pharmacology. This finding suggests that YYC may influence the sex hormones level through estrogen signaling pathway and then induce apoptosis to balance normal functions of mammary gland. This study thus provided evidences for the potential therapeutic efficacy of YYC on MGH and revealed the correlated regulatory signaling pathways.

Keywords: Yiqi Yangyin complex (YYC), Mammary Gland Hyperplasia (MGH), Sex hormones, Apoptosis, Network pharmacology

1. Introduction

Mammary gland hyperplasia (MGH) is a common disease characterized by pathological hyperplasia for lobules of mammary gland [1]. With increase of work stress and competitive pressure in the fast pace of modern life, the incidence of MGH in middle-aged women is increasing rapidly, and its severe cancerous tendencies to threaten human health [2]. The pathogenesis of MGH is closely related to endocrine disorder, mainly owing to high estrogen release or low progesterone production caused hormones imbalance to increase incomplete differentiation of glandular epithelium, and made the proliferative tissue unredintegration to induce MGH [3]. Although hormones, such as progesterone, tamoxifen and vitamins, are usually used

for treatment of MGH [4], long-term oral administration of such drugs will cause hormone imbalance disorder and various medication discomforts to aggravate the severity of MGH [5]. It is important for us to find new drugs with more convenient, effective, and have few side effects to treat MGH. It has been reported that traditional Chinese medicine (TCM) has the protective effects on MGH by possible biological mechanism [6].

Medicine and Food Homology is regarded as a combination of food and medicine functions, nutritional value, diseases prevention and treatment, and healthcare activities [7]. Yiqi Yangyin complex (YYC), as a medicine and food homology from TCM formula, including *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, *Rehmanniae Radix*, which are used as the common ingredients of the stew soup and famous prescriptions of TCM for replenishing Qi and nourishing Yin, and called as Yi Qi Yang Yin in Chinese. Polysaccharides have been regarded as the main components of the stew soup or water decoction of many herbs for replenishing Qi and nourishing Yin function, which play an important role in exhibiting immunomodulatory activities [8]. *Polygonati Rhizoma* polysaccharide known as an important active compound, has the potential as a drug or dietary adjuvant for the treatment of atherosclerosis and hyperlipidemia [9], which has strong antioxidant, lipid-regulating, anti-inflammatory, and endothelial function improvement effects [10, 11]. *Lycii Fructus* is traditionally used in Chinese home cooking, such as tea, soups, porridge, taste sweet, and in the Chinese pharmacopoeia as an aid for vision and longevity to balance the "Yin" and "Yang" of the body [12]. The polysaccharide of *Lycii Fructus* partly decreased the protein expression of HIF-1α and Bax to regulate the production of inflammatory factors through NF-κB signaling pathway [13]. *Codonopsis pilosula* contained sterol, triterpenes, glycoside, alkaloid, polysaccharide and other components [14], and its polysaccharides had several biological activities, such as tumor growth prevention [15], immune system modulation and anti-oxidant activity [16]. *Rehmanniae Radix* has been traditionally known as lowering blood fever, nourish Yin and promoting the body fluids, curing macula, skin rash, nosebleeds and so on. Meanwhile, *Rehmanniae Radix* could nourish Yin and replenish blood, benefit the essence, and was mainly

used to treat anemia, diabetes, tinnitus and heart palpitations [17]. The above four herbs are usually used for Chinese home cooking and TCM to exerting the function of Reinforcing Qi and Nourishing Yin. However, the efficacy and potential biological mechanism of the polysaccharide formed by a mixture of four herbs (YYC) on MGH have not been completely investigated.

Network pharmacology is an emerging discipline based on the effective mapping of unexplored target space of nature products, which become a novel and powerful method by multi-component and multi-target action mode [20]. Network pharmacology may combine several pharmacological networks with human disease-related genes by multichannel regulation of signaling pathways and revealing disease-related drug targets [21]. It has been successfully applied to decipher the bioactive compounds and synergistic mechanisms of the TCM Li-Ru-Kang (LRK) against MGH from the molecular network level [22]. *Chen Y et al* found that the integrated analysis of network pharmacology and bioinformatics analysis may be used to reveal the potential targets and the molecular mechanism of essential oil from *Rhizoma Curcumae* on liver fibrosis [23]. *Tu C et al*, also found that inflammatory state-dependent dietary supplement hepatotoxicity responses in normal and diseased rats were investigated by network pharmacology [24]. Therefore, network pharmacology may provide new ideas for the potential molecular mechanisms of YYC on MGH.

In this study, the anti-hyperplasia biological mechanism of the polysaccharides from the four herbs and YYC on mice with MGH were identified, and the potential key targets and possible signaling pathways were investigated by network pharmacology-based prediction and verification (Figure 1). The present study may provide a useful reference for exploring the potential mechanism and action pathways of the function food from Reinforcing Qi and Nourishing Yin herbs are helpful for the healthcare of MGH.

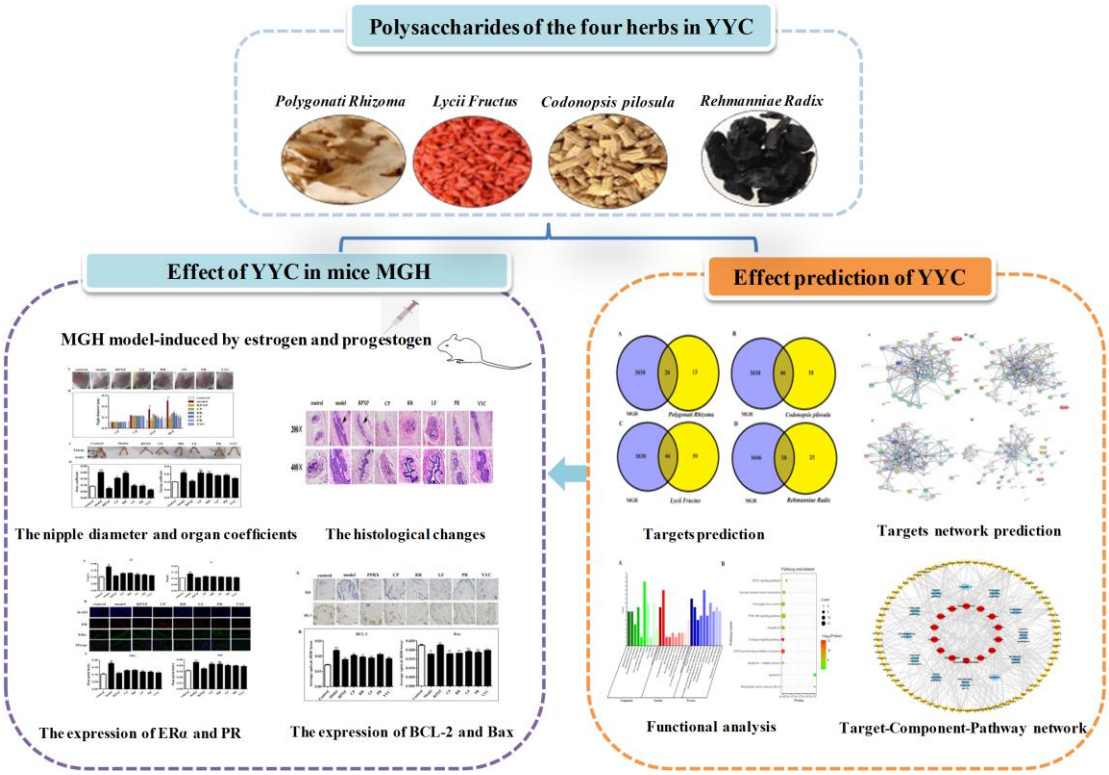


FIGURE 1 The biological mechanisms of MGH. The establishment of animal experimental model and network pharmacology-based computational predictions.

2. Materials and methods

2.1 Preparation of YYC and analysis of polysaccharides

Polygonati Rhizoma (Sichuan, China), *Lycii Fructus* (Qinghai, China), *Codonopsis pilosula* (Gansu, China), and *Rehmanniae Radix* (Henan, China) were purchased from Tianfangjian (China) Pharmaceutical Co. LTD. The four raw materials, including *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, and *Rehmanniae Radix* were extracted in boiling water for 2 h and then further extracted in boiling water for 1 h. All collected filtrates were processed with vacuum concentration to obtain extracts. The concentrated extracts were purified with 75% ethanol and contained 30-45% solid contents. The extracts were followed by drying in a 70～100 ℃ oven to obtain the polysaccharides of four raw materials. Finally, the four polysaccharides were mixed to obtain YYC. The proportion of *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, and *Rehmanniae Radix* in YYC were 25% (w/w), 25% (w/w),

25% (w/w), and 25% (w/w), respectively.

2.2 Determination of sugar and protein content

The basic physicochemical properties of four polysaccharides and YYC were performed. The total sugar contents of YYC were determined with the phenol sulfuric acid assay [25]. The protein contents were determined using BCA assay [26]. The molecular weight distributions of YYC were determined by high performance gel-permeation chromatography (HPGPC) [27].

2.3 Animal experiments

Eight-week-old female KunMing mice weighing 18-20 g (license number: SCXK2018-0002) were commercially obtained from the Experimental Animal Centre of Guangdong Province. The mice were housed at a controlled room (23±1℃, humidity 60±5%, 12 h day/light). They were acclimated under climate-controlled conditions for 7 days before the experiments began. Mice were randomly divided into seven groups with six mice in each group, including a control group (without treatment), model group (0.5 mg/kg/d estrogen for first 25 days and 5 mg/kg/d progestogen for last days), positive group (250 mg/kg/d, Rupixiao Pian, RPXP), *Codonopsis pilosula* group (67 mg/kg/d, CP), *Rehmanniae Radix* group (67 mg/kg/d, RR), *Polygonati Rhizoma* group (67 mg/kg/d, PR), *Lycii Fructus* group (67 mg/kg/d, LF) and YYC group (*Polygonati Rhizoma, Lycii Fructus, Codonopsis pilosula,* and *Rehmanniae Radix*, at a ratio of 0.25:0.25:0.25:0.25, 67 mg/kg/d). The dose of polysaccharides were used according to the guidelines of the Chinese Pharmacopoeia (2015). Mice except for control group were injected with estrogen (0.5 mg/kg/d) into the muscle of hind leg for consecutive 25 days, and followed with progestogen (5 mg/kg/d) for another 5 days [28]. For positive group, CP group, RP group, PR group and YYC group, mice were treated with once daily intragastric administration before intramuscular injection for 30 days. For control group and model group, mice were intragastrically administered with equal volume of saline. Mice were sacrificed 24 h after the last polysaccharides administration. The nipple height was firstly detected,

and mice were weighed. The blood were collected from eyeball extraction, and then the mice were sacrificed. The mammary glands were immediately removed and fixed 4% paraformaldehyde. The blood was centrifuged at 3000 rpm for 15 min to separate the serum without hemolysis, and then stored at -80℃. This study was approved by the Animal Care and Use Committee of Guangdong University of Technology.

2.4 Determination of nipple diameter and organ coefficients

The diameter of the mice's nipple was measured on 1, 7, 15, 30 day (d). The ovary and uterus were collected and weighted at the end of this experiment. The uterus and ovary index were calculated by uterus or ovary weight divided by body weight [29, 30].

2.5 Biochemical analysis and enzyme-linked immunosorbent assay (ELISA)

Blood was collected by eyeball extraction, and then centrifuged at 3000 rpm for 15 min to obtain the serum. The serum was collected and stored at -80 ℃ for hormone assays. The concentrations of E2, P in serum were measured by commercial detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6 Histological analysis

Fourth inguinal mammary glands (n=6) was obtained for histopathological examination and fixed in 4% paraformaldehyde for 48 h. After processed in a series of graded ethanol and dimethyl benzene, the tissues were embedded in paraffin and cut into 4 μm thick sections, and then stained with hematoxylin and eosin (H&E). Finally, pathological changes were observed by using SZX10 microscope (Olympus Corp., Tokyo, Japan).

2.7 Immunofluorescence assays

Each mammary gland tissue block was sectioned at 4 μm on the graded slide. Slices were dried overnight and washed with PBS for 5 min. Sections were blocked with BSA for 1 h at room temperature on a shaker. The samples were incubated at 4℃ overnight with primary antibodies ERα (1:200), or PR (1:200) (Danvers, MA, United

States) and incubated at 4℃. After being washed with PBS, the sections were treated with the secondary antibody conjugated with horseradish peroxidase for 1 h and then DAPI was added into slices for nuclear counter-staining for 5 min [28]. The sections were captured by microscope (Olympus, Tokyo, Japan). The mean integrated optical density (IOD) of these areas was measured by image analysis software Image J.

2.8 Immunohistochemistry assay

In situ expression of Bcl-2 and Bax in mammary gland was performed as follows. Paraffin-embedded sections (4 μm) were dewaxed in xylene, sequentially rehydrated in alcohol and incubated in 3% H_2O_2 for 20 min. The sections were heated twice in a microwave oven for 5 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval and followed by overnight incubation at 4℃ with the primary antibodies Bcl-2 (1:50) and Bax (1:50) (Danvers, MA, United States) [30]. The sections were washed and incubated with the HRP conjugated secondary antibody for 30 min at 37℃. After staining with DAB, the tissue slides were counterstained with hematoxylin, dehydrated with a graded ethanol series, and sealed with neutral gum in the end. The sections were captured by microscope (Olympus, Tokyo, Japan). The mean integrated optical density (IOD) of these areas was measured by software Image J.

2.9 Database construction

The chemical constituents from four polysaccharides were obtained from TCMSP. Known targets of single polysaccharide were collected from Herbal Ingredients' Targets Database (HIT), and the putative targets from these were screened out from Therapeutic Targets Database (TTD) through structural similarity comparison. Gene and protein targets associated with MGH were collected from the Online Mendelian Inheritance in Man (OMIM) database and GeneCards server. The targets of interactive proteins were obtained from Database of Interacting Proteins (DIP) and ID types of the proteins were converted to UniProt IDs. Based on the previous steps, the targets were prepared, namely, drug-related genes and disease targets. The crossed genes were screened by the R software by using the Venn Diagram.

2.10 Target protein-protein interaction (PPI) network construction

To provide the scientific and reasonable interpretation of the complex relationships between chemical constituents and targets associated with MGH, network analysis was performed. The single polysaccharide-target network was constructed by using candidate substance and significant targets for MGH. The network was performed by using Cytoscape 3.5.1 software. The topological features of each node in the network were calculated by "Degree", "Betweenness centrality", and "Closeness centrality" ("Degree" values were two fold greater than the median value of all the network nodes, "Betweenness centrality" and "Closeness centrality" value were greater than the median value of all the network nodes). Targets with higher value were screened as the candidates for MGH.

2.11 Go gene enrichment analysis and KEGG pathway

To elucidate the function of the four polysaccharides target compounds and its role in signal transduction, the Database for Annotation, Visualization and Integrated Discovery (DAVID) database were used to analyze the GO and KEGG pathway enrichment. The biological processes, cellular components, molecular functions for GO enrichment and the pathways were also described.

2.12 Statistical analysis

The data were expressed as the mean values ± standard error of mean (SEM). Statistical analysis was performed by GraphPad Prism 5.0 software, using student t-tests or one-way analysis of variance (ANOVA). Difference with *P*-value ($P < 0.05$) was considered as significance and drew the diagrams.

3. Results

3.1 The physicochemical properties of YYC

These polysaccharides had significant differences in total sugars and proteins. Among which the content of total sugar of *Polygonati Rhizoma, Lycii Fructus, Codonopsis pilosula, Rehmanniae Radix* and YYC respectively 22.9%, 16.21%, 19.2%, 18.4%,

22.31% (Table 1). The protein content of *Polygonati Rhizoma, Lycii Fructus, Codonopsis pilosula, Rehmanniae Radix* and YYC are respectively 4.28%, 17.67%, 22.19%, 10.68%, 9.72% (Table 1). The components of *Polygonati Rhizoma* with molecular weight less than 5×10^3 Da had 92% of the peak area. The components of *Lycii Fructus* with molecular weight between 5×10^3 Da and 4.8×10^4 Da had 92% of the peak area. The components of *Rehmanniae Radix* with molecular weight less than 5×10^3 Da had 84% of the peak area. The components of *Codonopsis pilosula* with molecular weight between 5×10^3 Da and 3×10^5 Da had 80% of the peak area. The components of YYC with molecular weight between 5×10^3 Da and 2×10^4 Da had 70% of peak area by HPGPC (Table 1, supplementary material Fig. S1).

Table 1 Physicochemical composition of *Polygonati Rhizoma, Lycii Fructus, Codonopsis pilosula, Rehmanniae Radix* and YYC

Samples	Total sugar %	Protein %	Molecular Weights (Da)
Polygonati Rhizoma	22.9%	4.28	$< 5\times 10^3$ Da
Lycii Fructus	16.21%	17.67	5×10^3 Da - 4.8×10^4 Da
Codonopsis pilosula	19.2%	22.19	5×10^3 Da - 3×10^5 Da
Rehmanniae Radix	18.4%	10.68	$< 5\times 10^3$ Da
YYC	22.31%	9.72	5×10^3 Da - 2×10^4 Da

3.2 YYC improved the nipple diameter and organ coefficients of mice with MGH

In order to assess the therapeutic efficacy of YYC on MGH, a MGH model- induced by estrogen and progesterone in mice was firstly established. The efficacy of YYC on MGH within 30 days was observed. As shown in Figure 2, there was no significant difference with nipple diameter between the model group and YYC group in 7 d. After 14 or 30 days' administration of polysaccharides, the nipple diameters were obviously suppressed in RPXP ($p < 0.05$), LF ($p < 0.05$), PR ($p < 0.05$) and YYC group ($p < 0.05$) (Figure 2A and Figure 2B). This result suggested that LF, PR and YYC significantly relieved the nipple diameter in mice with MGH.

The mammary gland as the target organ for sex hormones is closely related to the

endocrine status of the ovary, while the uterus index and ovary index reflect the changes of the uterus and ovary [4]. Compared with the control group, ovary and uterine coefficient were significantly increased in model group. Compared with the model group, the ovary index was markedly inhibited in RPXP (p <0.01), CP (p <0.05), LF (p <0.01), PR (p <0.01) and YYC group (p <0.001), and uterine coefficient was significantly reduced in RPXP (p <0.01), LF (p <0.05) and YYC group (p <0.05) (Figure 2C and Figure 2D). Taken together, this result suggested that YYC significantly down-regulated the ovary index and uterine coefficient to relieve the symptom of mice with MGH.

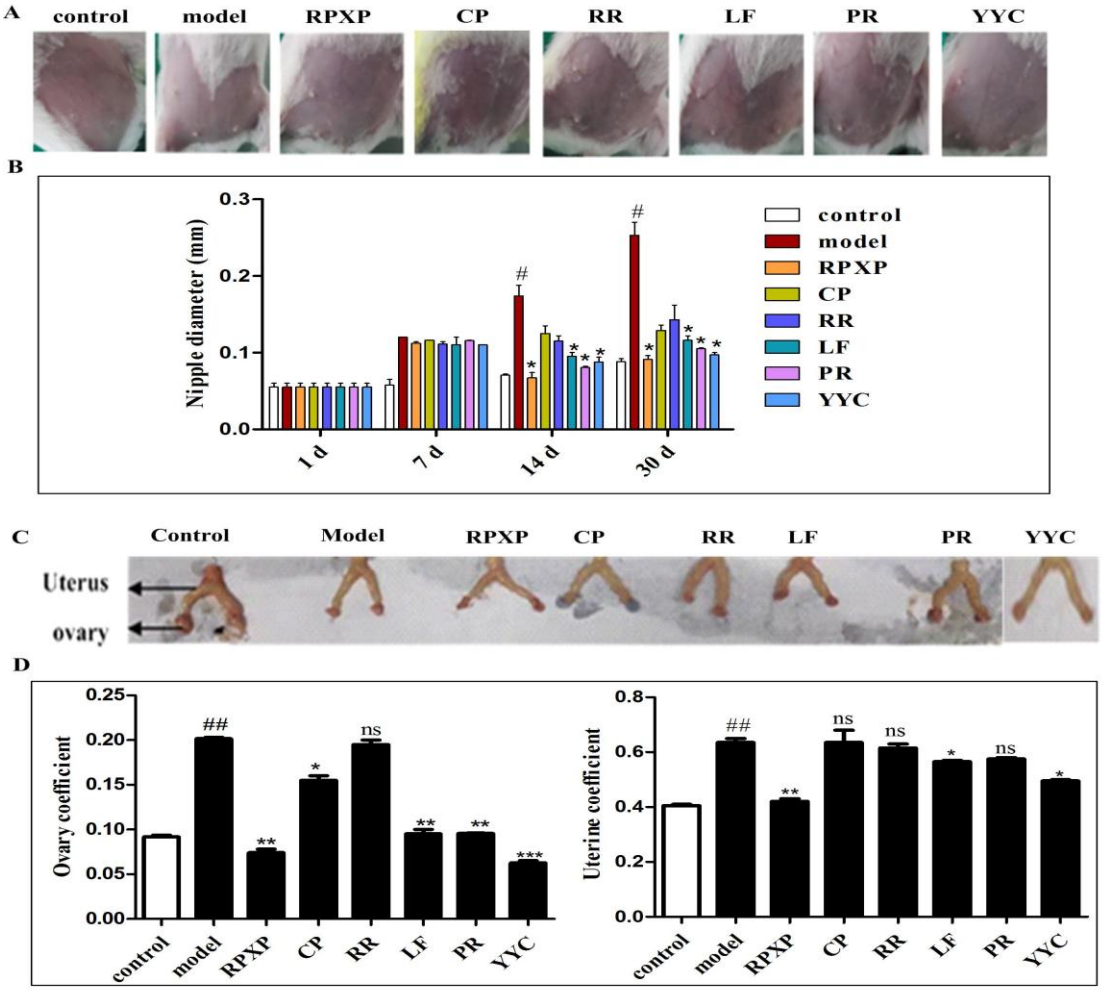


FIGURE 2 Effects of polysaccharides on the nipple diameters in MGH. (A) The pathological features of mammary gland tissues in different group. (B) Diameter of nipples in different group. (C) The pathological features of uterus and ovary. (D) The ovary and uterus coefficient in

different group. Control group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP), *Codonopsis pilosula* (CP), *Rehmanniae Radix* (RR), *Polygonati Rhizoma* (PR), *Lycii Fructus* (LF), Yiqi Yangyin Complex (YYC). Data are expressed as the mean± SEM. $^{\#}$ represents MGH model group vs control group ($^{\#}$ $p < 0.05$); * indicated significant difference in polysaccharide treatment group vs MGH model group (* $p < 0.05$).

3.3 Effect of YYC on the histological changes in mice with MGH

In order to verify whether the YYC could alleviate MGH, HE staining was used to detect the pathological changes of mammary gland tissue. As shown in Figure 3, compared with the control group, there was obvious proliferative lesions in mammary epithelial cell tissue, including lobules hyperplasia, increase of count of acini and ducts, and irregular arrangement and obvious expansion of duct lumen in model group. Compared with the model group, administration of PRPX and YYC for consecutive 30 days significantly inhibited the typical histological patterns, whereas treatment of CP, RR and PR were also capable to decrease the area of proliferative lesions and counts of mammary acini and ducts in different degrees (Figure 3). This result showed that YYC had therapeutic efficacy on mice with MGH.

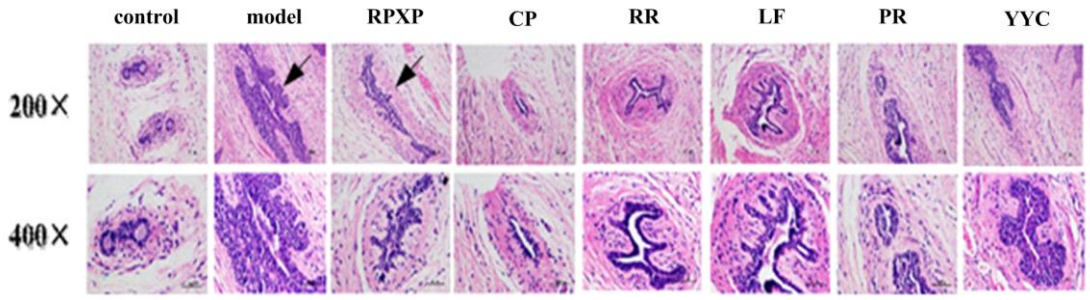


FIGURE 3 Analysis of histopathological for mammary gland tissue with polysaccharides. Control group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP), *Codonopsis pilosula* (CP), *Rehmanniae Radix* (RR), *Polygonati Rhizoma* (PR), *Lycii Fructus* (LF), Yiqi Yangyin Complex (YYC). (Arrow indicated hyperplasia of ductal epithelial cells.)

3.4 YYC modulated the serum biochemical parameters and protein expression of ERα and PR

Estrogen receptors play a critical role in regulating cell proliferation and

differentiation in mammary glands, which may be as important nuclear transcription factors activated by E2 or P [31]. As shown in Figure 4, compared with control group, the level of Estrogen 2 (E2) was significantly elevated ($p < 0.05$) in model group. Compared with model group, the secretion of E2 was significantly decreased in RPXP, CP, RR, LF, PR and YYC group ($p < 0.05$). However, there were no significant differences in the level of progesterone (P) between all groups (Figure 4A).

To investigate the effect of YYC on Estrogen receptors, the levels of ERα and PR were detected by immunofluorescence assay. The fluorescence intensity of ERα ($p < 0.05$) and PR ($p < 0.01$) in model group were significantly elevated in comparison with control group (Figure 4B and Figure 4C). Compared with model group, the fluorescence intensity of ERα was markedly inhibited in all group ($p < 0.05$), while the fluorescence intensity of P was significantly decreased in RPXP ($p < 0.01$), LF ($p < 0.05$), PR ($p < 0.05$) and YYC ($p < 0.05$) group (Figure 4B and Figure 4C). This result illustrated that LF, PR and YYC could regulate the estrogen receptors on the mice with MGH-induced by estrogen and progestogen.

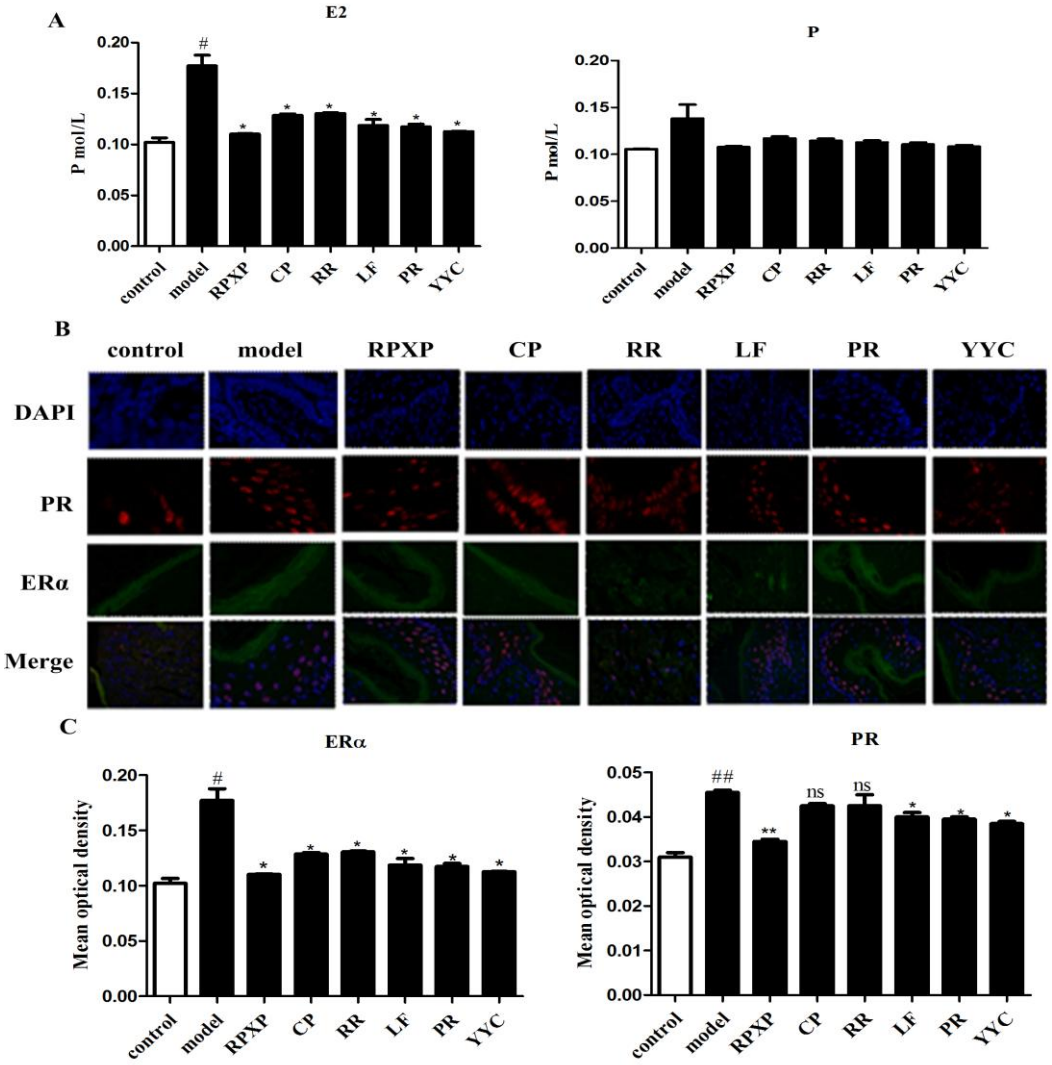


FIGURE 4 Effect of polysaccharides on sex hormones levels and the expression of ERα and PR in mice's mammary gland tissue. (A) Sex hormones level for E2 and P in serum. (B) Immunofluorescence analysis for ERα and PR in mammary gland tissue. Red signals represents PR expression, green signals represents ERα expression, blue signals represents nuclei. (C) The quantification of immunofluorescence signals in different groups. Data are expressed as the mean ± SEM. Control group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP), *Codonopsis pilosula* group (CP), *Rehmanniae Radix* group (RR), *Polygonati Rhizoma* group (PR), *Lycii Fructus* group (LF), Yiqi Yangyin Complex group (YYC). Data are expressed as the mean± SEM. $^{\#}$ represents MGH model group vs control group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$); * indicated significant difference in polysaccharide treatment group vs MGH model group. (* $p < 0.05$, ** $p < 0.01$), ns indicated no significant difference.

3.5 Effect of YYC on expression of BCL-2 and Bax in mice with MGH

Apoptosis plays an important role in maintaining tissue homeostasis and cancer prevention [32]. In order to verify the effect of YYC on apoptosis in MGH, the apoptotic protein Bax and anti-apoptotic protein BCL-2 were detected by immunohistochemical assay. Compared with the control group, the levels of BCL-2 ($p < 0.01$) were significantly up-regulated, whereas Bax expression ($p < 0.05$) were markedly down-regulated in model group. Meanwhile, we found that the level of BCL-2 was significantly down-regulated in RPXP ($p < 0.01$), CP ($p < 0.05$), LF ($p < 0.01$), PR ($p < 0.05$), RR ($p < 0.01$) and YYC group ($p < 0.01$) in comparison with model group, while Bax expression was up-regulated in RPXP ($p < 0.01$), and YYC group ($p < 0.01$) (Figure 5A and Figure 5B). This result suggested that YYC might be involved in regulating the symptom of mice with MGH via apoptosis.

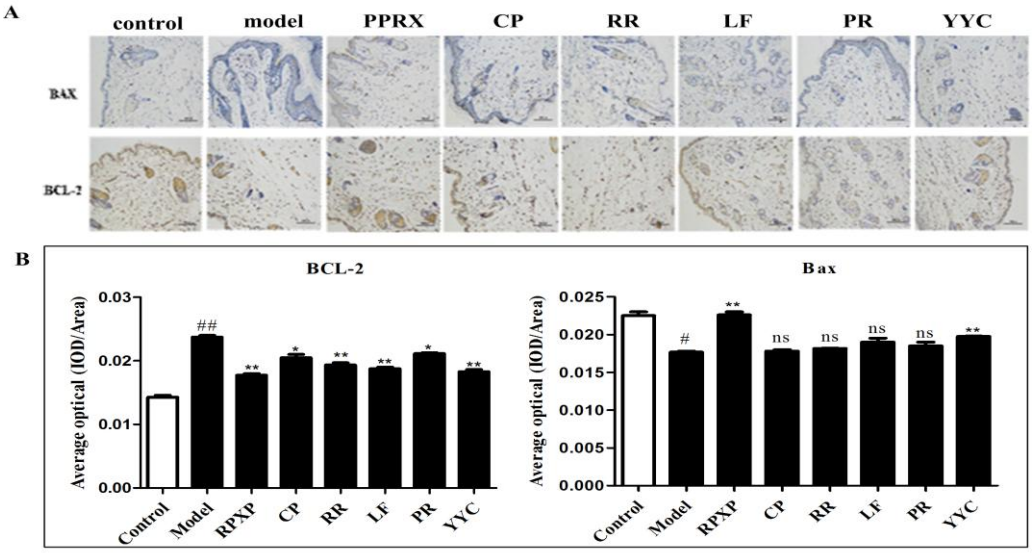


FIGURE 5 Effects of polysaccharides on level of BCL-2 and Bax in mammary gland with MGH. (A) Immunohistochemical analysis of BCL-2 and Bax in different group. (B) The quantification of immunohistochemistry signals in different groups. Control group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP), *Codonopsis pilosula* group (CP), *Rehmanniae Radix* group (RR), *Polygonati Rhizoma* group (PR), *Lycii Fructus* group (LF), Yiqi Yangyin Complex group (YYC). Data are expressed as the mean± SEM. $^{\#}$ represents MGH model group vs control group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$); * indicated significant difference in polysaccharide group vs MGH model group (* $p < 0.05$, ** $p < 0.01$). ns indicated no significant difference.

3.6 The targets of four polysaccharides affecting MGH were predicted by network pharmacology

A total of 3684 targets for MGH were firstly predicted by the GeneCards server. Meanwhile, the targets of four polysaccharides were predicted by Swiss-target-prediction server. After the duplicate genes have been removed, the targets for *Polygonati Rhizoma* polysaccharide, *Codonopsis pilosula* polysaccharide, *Lycii Fructus* polysaccharide, *Rehmanniae Radix* polysaccharide are respectively 41, 76, 63, 84. Finally, the Venn diagram was constructed by predicted targets for MGH and targets of four polysaccharides. The targets of affecting MGH for *Polygonati Rhizoma* polysaccharide, *Codonopsis pilosula* polysaccharide, *Lycii Fructus* polysaccharide, *Rehmanniae Radix* polysaccharide were 26, 46, 38, 46 (Figure 6).

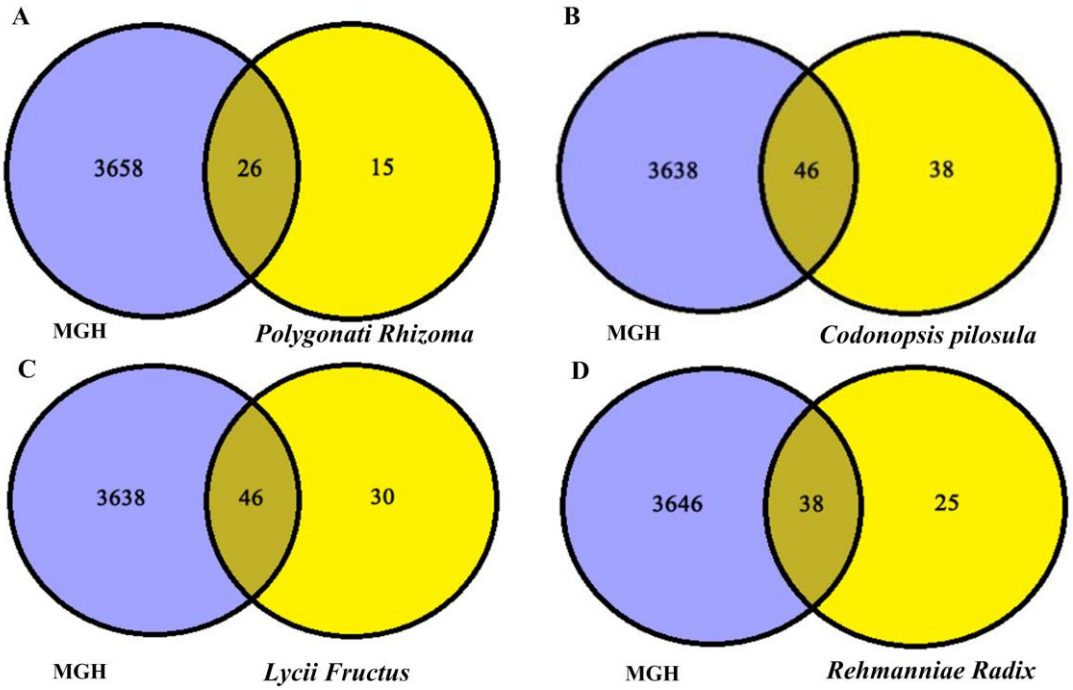


FIGURE 6 The targets of affecting MGH for four polysaccharides. A-D: Venn diagram of the candidate targets in four polysaccharides (*Polygonati Rhizoma* (A), *Codonopsis pilosula* (B), *Lycii Fructus* (C), *Rehmanniae Radix* (D)) and MGH; Note: Purple circle represents targets for MGH; yellow circle represents targets for four single polysaccharide.

3.7 Network construction and analysis of target protein-protein interaction

To study the target of combined polysaccharides affecting MGH, the cytoscape

software was used to predict the target protein interaction. The target protein-protein interaction network was constructed (Figure 7). Through the analysis of topological parameters, the 16 direct targets of *Polygonati Rhizoma* included heat shock protein HSP 90-alpha (HSP90AA1), signal transducer and activator of transcription 3 (STAT3), estrogen receptor (ESR1), BCL2, cyclin-dependent kinases1 (CDK1), cyclinB1 (CCNB1), SH2 domain-containing transforming protein C1 (SHC1), Bcl-2-like protein 1 (BCL2L1), vascular endothlial growth factor (VEGFA), progesterone receptor (PGR), salt overly sensitive 1 (SOS1), growth factor receptor-bound protein 2 (GRB2), cell Division Cycle 25A (CDC25A), protein kinase C delta type (PRKCD), protein kinase C alpha (PRKCA), PPP2CA. The 21 direct targets of *Codonopsis pilosula* included steroid receptor coactivator (SRC), EGFR, HSP90AA1, BCL2, STAT3, ESR1, BCL2L1, VEGFA, SHC1, caspase 3 (CASP3), GRB2, PRKCD, fibroblast growth factor 2 (FGF2), CDK1, breast cancer anti-estrogen resistance 1 (BCAR1) , PRKCA, CCNB1, PGR, SOS1, fibroblast growth factor 1 (FGF1), Cell Division Cycle 37 (CDC37). The 23 direct targets of *Lycii Fructus* included SRC, endothlial growth factor receptor (EGFR), HSP90AA1, BCL2, STAT3, BCL2L1, VEGFA, CASP3, Src homology 2 domain containing (SHC1), BAX, GRB2, PRKCD, CDK1, FGF2, PRKCA, BCAR1, CCNB1, PGR, FGF1, CDC37, SOS1, caspase 7 (CASP7), caspase 1 (CASP1). The 12 direct targets of *Rehmanniae Radix* included SRC, EGFR, BCL2, VEGFA, STAT3, BCL2L1, FGF1, FGF2, KDR, CDK1, BAX, CCNB1 (Degree >10).

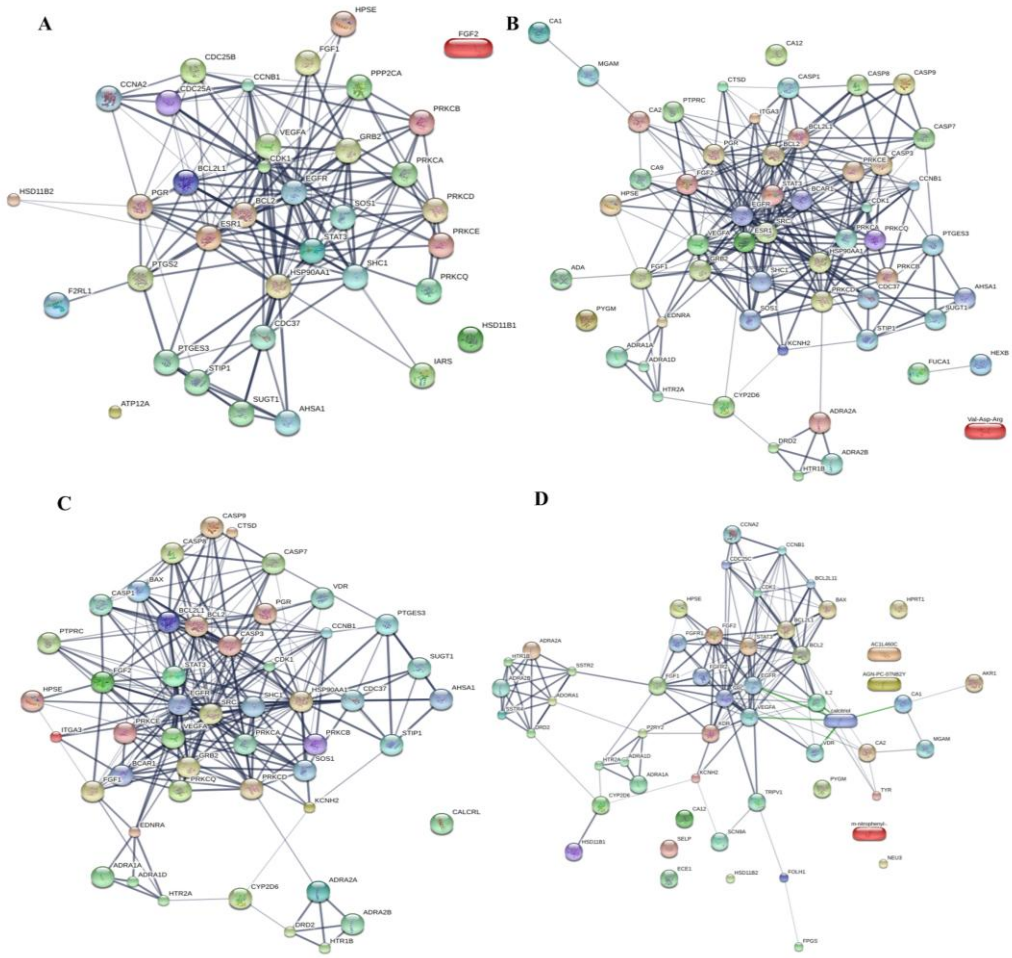


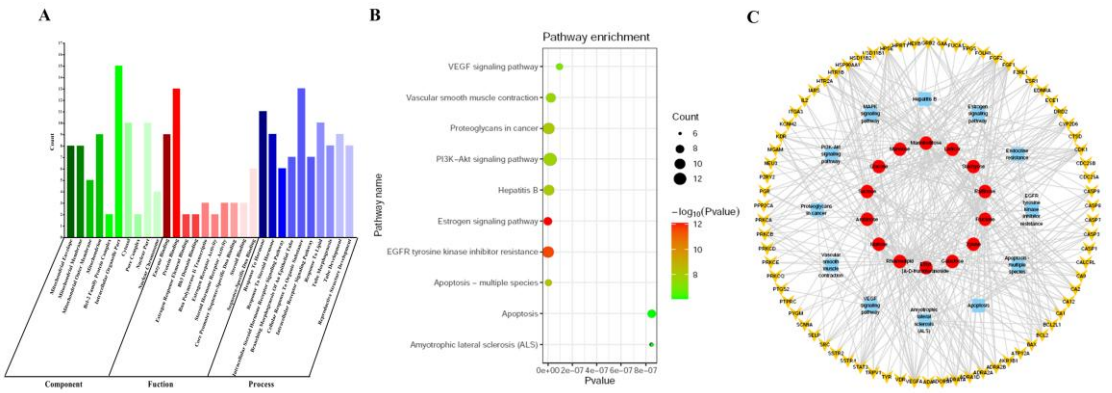
FIGURE 7 Analysis of the target network of four polysaccharides influencing MGH. A-D: *Polygonati Rhizoma* (A), *Codonopsis pilosula* (B), *Lycii Fructus* (C), *Rehmanniae Radix* (D) influencing MGH for the analysis of target network. Note: Red circle represents high combination degree with surrounding targets; blue circle represents low combination degree with surrounding targets.

3.8 The GO enrichment and KEGG signaling pathway of YYC

The targets were analyzed for GO biological functions and KEGG signaling pathway enrichments. As shown in Figure 8A, the top 10 highly enriched GO biological processes, molecular functions and cellular components were found (p-value < 0.05). The results showed that the GO functions of target protein molecules were mainly enzyme binding, hormone response process, and mitochondrial activity. The top 3 enrichments in Biological Process category were response to hormone, response to steroid hormone and intracellular steroid hormone receptor. The top 3 enrichments in

Molecular Function category were enzyme binding, protein binding, estrogen response element binding; in Cell Component category, the top 3 enrichments were mitochondrial envelope, mitochondrial membrane, mitochondrial outer membrane.

The KEGG pathway analysis was further performed to identify the signaling pathway that the predicted targets may be participated. The bubble map of KEGG pathway showed that the core targets based on top 10 enrichment pathway (Figure 8B). The top 10 involved in signaling pathways were mainly related to hormone signaling pathway, such as estrogen signaling pathway, thyroid hormone signaling pathway, apoptosis. We further constructed the target-component-pathway network diagram of action mechanism of YYC (Figure 8C). This result showed that each active compound could act on multiple targets. Notably, various targets on MGH were significantly enrichment on estrogen signaling pathway, which includes the target of ER, BCL-2, HSP90, EGFR. The KEGG pathway of polysaccharides target MGH showed that the above targets might be activated by polysaccharides intervention, and then influenced EGFR tyrosine kinase inhibitor resistance signaling pathway, apoptosis-multiple species signaling pathway, hepatitis B signaling pathway, proteoglycans in cancer signaling pathway, PI3K-Akt signaling pathway. The induction of estrogen signaling pathway and apoptosis play an important role in the treatment of MGH with drugs (Supplementary Figure 2). Therefore, the two significant enrichment pathways were in accordance with the animal experimental verification.



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426 FIGURE 8 GO and KEGG enrichment analysis of YYC. (A) The GO enrichment analysis of
427 potential targets including molecular function, cellular component, biological process. (B) The
428 enrichment analysis of KEGG signaling pathways. The size of point indicates the number of genes
429 in the pathway and the color of point corresponds to *p*-value ranges. (C) The network diagram of
430 YYC with target-compound-pathway analysis.

431 **4. Discussion**

432 The sex hormones were mainly secreted from mammary gland, and its imbalanced
433 secretion inducing endocrine disorders was considered as main cause to lead to MGH
434 [33, 34]. MGH, a common disease in middle-age women has severe cancerous
435 tendency to cause higher risk of mammary gland cancer [35]. At present, the surgery
436 and medication were referred as the main treatment of MGH, whereas its adverse side
437 effects severely impact quality of life [36]. Hence, it is crucial to find few side effects
438 drugs improvement of estrogen-induced endocrine disorders to prevent the prevalence
439 and progression of MGH. As an alternative program to traditional therapeutic
440 interventions, Chinese herbal products are getting more and more attention to deal
441 with estrogen-related health issues. YYC, a part of medicine and food homology
442 formula, was traditionally used for Chinese home cooking to exert the function of
443 Reinforcing Qi and Nourishing Yin. However, the molecular mechanism of YYC for
444 MGH has been yet unclear. Thus, in our study, the underlying biological mechanism
445 of YYC against MGH was explored.

446 To study the molecular mechanism for YYC on MGH in more detail, a mouse
447 model of MGH was successfully constructed. Compared with model group, the level

of E2 could be significantly reduced in YYC group in mice, but the level of P has no significant difference with control group. This result suggested that the absolute or relative increase of E2 or P deficiency could lead to imbalance of E2/P ratio to cause excessive proliferation of mammary glandular parenchyma. ER and PR could specially bind to E2 and P. ER is a protein molecule with two subtypes of ERα and ERβ, which combines with E2 to form a hormone receptor complex [37]. PR as the regulatory protein of E2 is accompanied by ER. E2 could promote expression of ER and PR in breast epithelial cells, and increase the sensitivity of mammary gland tissue to hormones-induced MGH [38]. Meanwhile, the proliferating cells promote the synthesis of ER and PR through the hormone receptor system, forming a pathological proliferation cycle. Here, our animal study showed that the polysaccharides *from Polygonati Rhizoma*, *Lycii Fructus* and YYC significantly improved the expression of ERα and inhibited production of PR by immunohistochemical assay in MGH mice model, indicating that the polysaccharides *from Polygonati Rhizoma and Lycii Fructus* in YYC played an important role in relieving MGH. Previous study showed that *Polygonati Rhizoma* polysaccharide has potential anti-inflammatory effect on 12-O-tetradecanoylphorbol-acetate (TPA)-induced inflammatory in mice [39]. *Lycii Fructus* inhibits growth of ER^+ human breast cancer cells (MCF-7 cell line) by altering E2 metabolism [40]. Interestingly, KEGG pathway analyses by network pharmacology showed that two targets ER and PR were directly involved in the enrichment on Estrogen signaling pathway. This pathway could transmit the signal to specific functional modules, such as apoptosis, carcinogenesis, cell proliferation, differentiation/development and inflammation, which are mainly initiated through estrogen or estrogen chemicals binding to ERs [41]. ERs will transducer signals by PI3K, MAPK/ERK and NF-κB signaling pathway [42], and then influence each other by crosstalk or bypassing at the intracellular level, or deliver signals to different cells or tissues by the secretion of hormones and growth factors to cause completely different types of functional outcome [41]. Taken together, these results suggested that YYC may not only alleviate the symptoms of MGH, but also further inhibit MGH development to carcinogenesis by crosstalk of various pathways.

Apoptosis is the primary mode of cell death involved in development and homeostasis [43]. BCL-2 is a well-known inhibitor of apoptosis to induce tumorigenesis [44], while Bax is a pro-apoptotic protein. Both of them are generally considered as important molecular proteins for apoptosis [45]. BCL-2 could block MOMP to antagonize Bax and then prevent apoptosis [46]. It has been reported that Tongru Sanjie decoction could regulate the expression of BCL-2 to inhibit MGH, thereby blocking malignant transformation of mammary gland. Also, the excessive expression of BCL-2 in many B-cell malignancies could induce its growth and proliferation [47]. Apoptosis could cause diseases, such as hyperplasia in the peripheral lymphoid organs, which accelerates autoimmune disease and tumorigenesis [48]. Here, we analysed apoptosis by immunohistochemical assay and found that the polysaccharides *from Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, *Rehmanniae Radix* and YYC significantly inhibited the level of anti-apoptotic factor BCL-2, whereas only YYC markedly elevated the pro-apoptotic factor Bax expression. *Lycii Fructus* contains three bioactive compounds, including carotenoids, phenolics, and polysaccharides, and the polysaccharide is as the most important and highest component [49]. *Lycii Fructus* polysaccharides could up-regulate anti-apoptotic protein BCL-2 in lens epithelial cells and increase ratio of BCL-2 to BAX (pro-apoptotic protein) in the lens mainly by its anti-oxidative effects [50]. *Polygonati Rhizoma* consists of saponins and polysaccharides and exerts its pharmacological activity, such as immune promotion, antiaging, antifatigue, blood glucose regulation and lipid regulation [51]. *Codonopsis pilosula* contains polysaccharides, sesquiterpenes, saponins, and phytosterols [52], and the polysaccharides are active compounds to exert multiple functions, including antitumor, antimicrobial, antioxidant, and immunoenhancing properties [53, 54]. The main components of *Rehmanniae Radix* are polysaccharides, triterpenoid saponins, iridoids, ionones, phenylglycol glycosides, phenolic acids, and lignans [55], and the polysaccharides are major active components to exhibit anticancer, anti-aging, antioxidant, and immunomodulatory activities [56, 57]. The above literature suggested that the main anti-hyperplasia potential may be attributed to the high content of polysaccharides in

YYC, and the synergistic effect of YYC ingridents probably own to their different polysaccharides interacted to different targets with a synergistic way, and reduce the side effects or enhance the pharmacological potency. Here, it is boldly speculated, although the four polysaccharides alone could not completely affect the expression of related apoptotic factors, the combination of four polysaccharides in equal proportion (YYC) might exhibit synergy effect on MGH by inducing apoptosis. In addition, YYC used for this study has strict quality control to test the polysaccharides content and molecular weight distribution from the herbal sources, extraction, and final product for assuring the stability of every batches, referenced by the standard of the YYC related product successfully launched in market by Infinitus (China) Co., LTD. GO and KEGG pathway analyses showed that YYC could be significantly enriched on estrogen signaling pathway and apoptosis pathway. Interestingly, BCL-2 was as common target between estrogen signaling pathway and apoptosis pathway by network pharmacology, indicating that crosstalk of different pathways regulate MGH development. Hence, the above result demonstrated that polysaccharides from YYC may activate multiple mechanisms of action to regulate MGH.

To further enrich animal experimental results, the potential targets of the four single polysaccharides on MGH were predicted by network pharmacology. The results showed that the direct targets of *Polygonati Rhizoma* polysaccharide, *Codonopsis pilosula* polysaccharide, *Lycii Fructus* polysaccharide and *Polygonati Rhizoma* polysaccharide affecting on MGH were respectively 16, 21, 23, and 12. To further elucidate the relevant targets of four single polysaccharides, the relevant targets were chosen for analysis. Here, ER, PR, STAT3 and BCL-2 were as our interesting targets for four single polysaccharides. Estrogens are sex steroid hormones, which could regulate menstrual cycle and reproduction, cholesterol mobilization, development of mammary gland and sexual organs, and control of inflammation [58]. Estradiol promotes epithelial cell proliferation in the uterine endometrium and mammary glands starting in puberty [59]. The increase of E2 and persistent lack of P promoted the mammary gland excessive hyperplasia and incomplete repairment [60]. The predicted targets ER and PR were in accordance with the animal experimental results. STAT3

could interact with polypeptide receptor to regulate extracellular signals [61]. BCL-2 as an anti-apoptotic protein is the key regulator in intrinsic apoptosis pathway [62]. It has been reported that the post-treatment of curcumin has an effect against myocardial ischemia and reperfusion to activate JAK2/STAT3 pathway by down-regulation of Caspase3 and up-regulation of BCL-2 [63, 64]. STAT3 activated BCL-2 to inhibit autophagy, or inhibition of STAT3 could cause autophagy [65]. Autophagy has been function as a tumor suppressive mechanism to remove or mitigate harmful stimuli, including oxidative stress, inflammation [61]. Inhibition of autophagy resulted in an accumulation of toxic proteins and mitochondrial dysfunction to trigger apoptosis [66]. These results demonstrated that YYC played an important role in regulating MGH by apoptosis pathway. This is consistent with our animal experimental results. Taken together, our results provides preliminary evidence that YYC may induce apoptosis and Estrogen signaling pathway, which further verified the synergetic effects of YYC on MGH (Figure 9). However, there are some limitations, which only verified the key molecules significantly enrichment on estrogen signaling pathway and apoptosis, but several targets have not been yet detected in this study. Additionally, although potential active compounds were predicted by network pharmacology, and our animal experimental results displayed the combination of four polysaccharides in equal proportion (YYC) may had better synergistic effect than polysaccharide alone on MGH, the composition and mechanism of these active ingredients are still unclear. In future study, we will pay more attention to the composition of the active components of polysaccharides in YYC and crosstalk of multi-pathway.

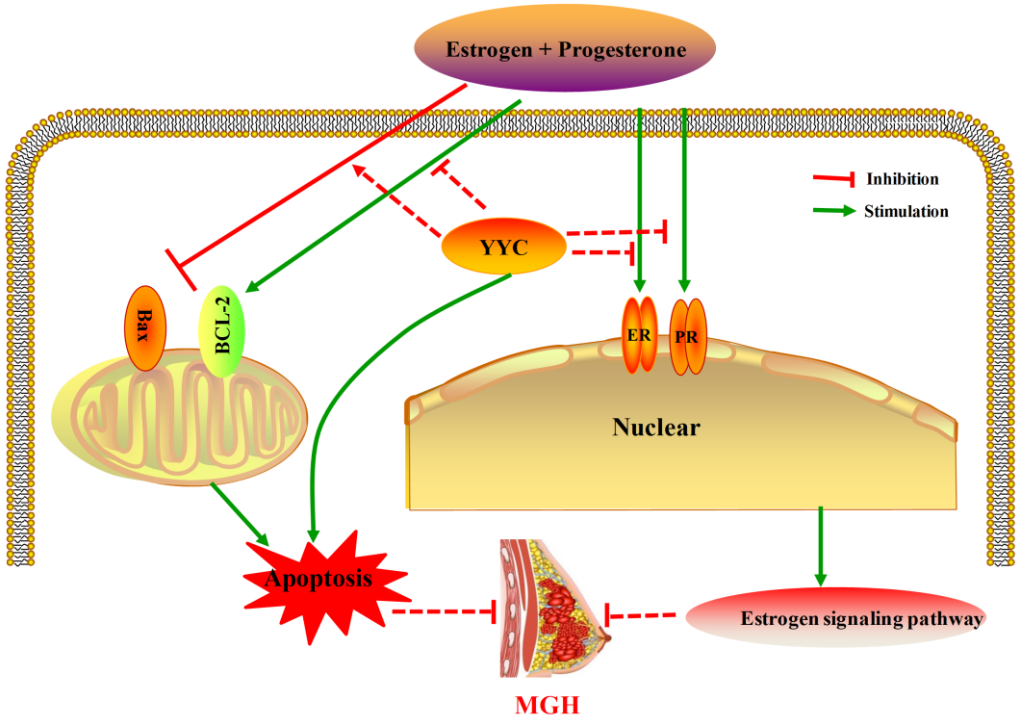


FIGURE 9 The schema of synergistic mechanism of YYC on MGH

5. Conclusion

In our current study, YYC-induced apoptosis in mice MGH model may occur by activating apoptosis, increasing expression of Bax and inhibiting expression of BCL-2. Also, YYC induced Estrogen signaling pathway in mice MGH model by effecting production of ER and PR. In addition, the potential targets and mechanisms of YYC were predicted via network pharmacology. This result showed that the top 10 related pathways were enriched in KEGG database, significant enrichment on Estrogen signaling pathway and apoptosis pathway in accordance with animal experimental results. Taken together, our results suggested that the key targets may provide new ideas for future drug development on MGH. Nevertheless, the target through which the component of YYC is involved in activating the crosstalk of Estrogen signaling pathway and apoptosis remains to be further explored.

Authors' contributions

ZY and DZ conceived the experiments and organized the manuscript. QX wrote the

manuscripts. QX and WB performed the experiments and analyzed the data. YZ revised the manuscript. YF performed bioinformatics analysis. WY, ZY, YY, TJ, JZ analyzed the data, LL, ZL contributed reagents and materials. All authors read and approved the final manuscript.

Acknowledgments

The current research work was supported financially by National Natural Science Foundation of China (21272043), Guangdong Provincial Department of Science and Technology (2015KQNCX024). National Science Foundation of China (NSFC) (22178070), Project of Rejuvenation of science and technology for Mongolia (2021CG0029).

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Consent for publication

Not applicable

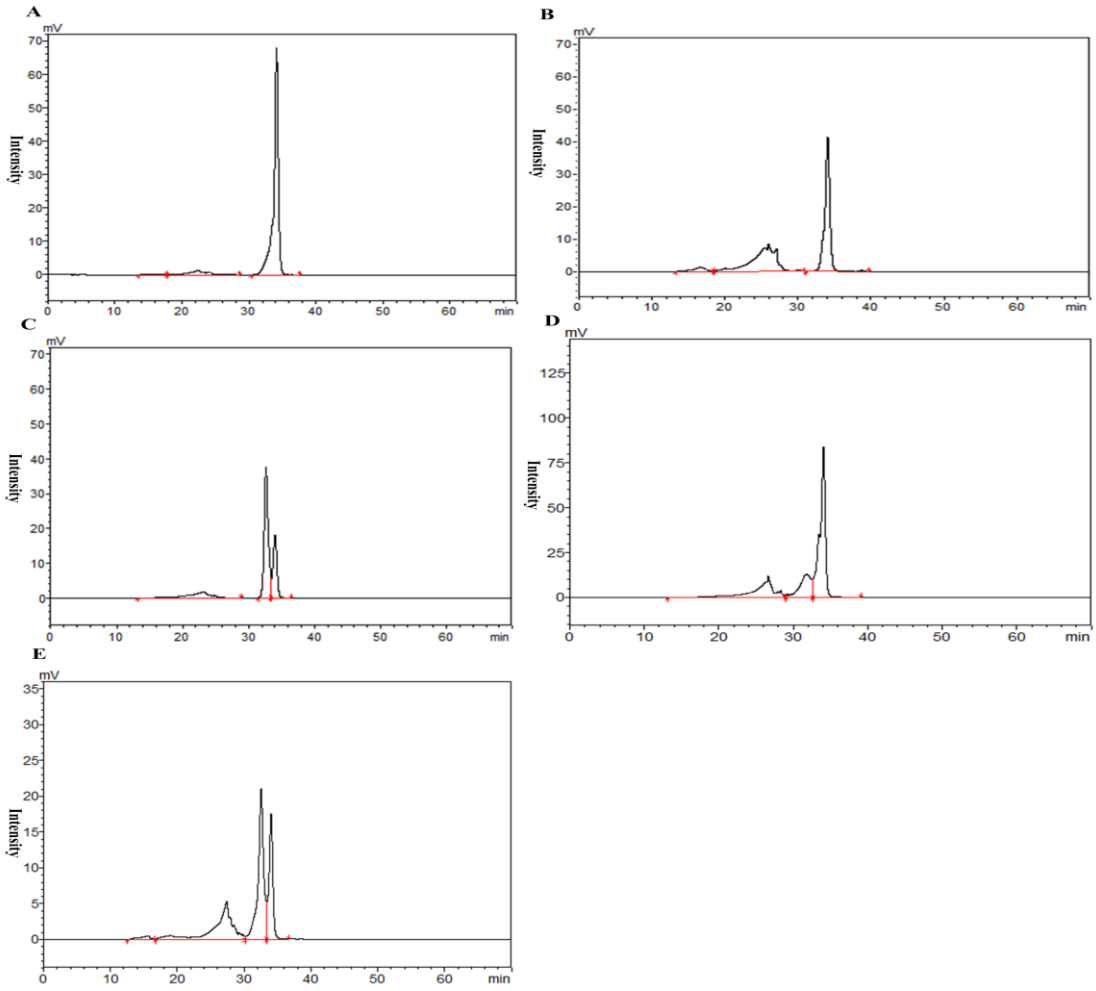
Conflicts of interest

The authors declare that they have no conflicts of interests.

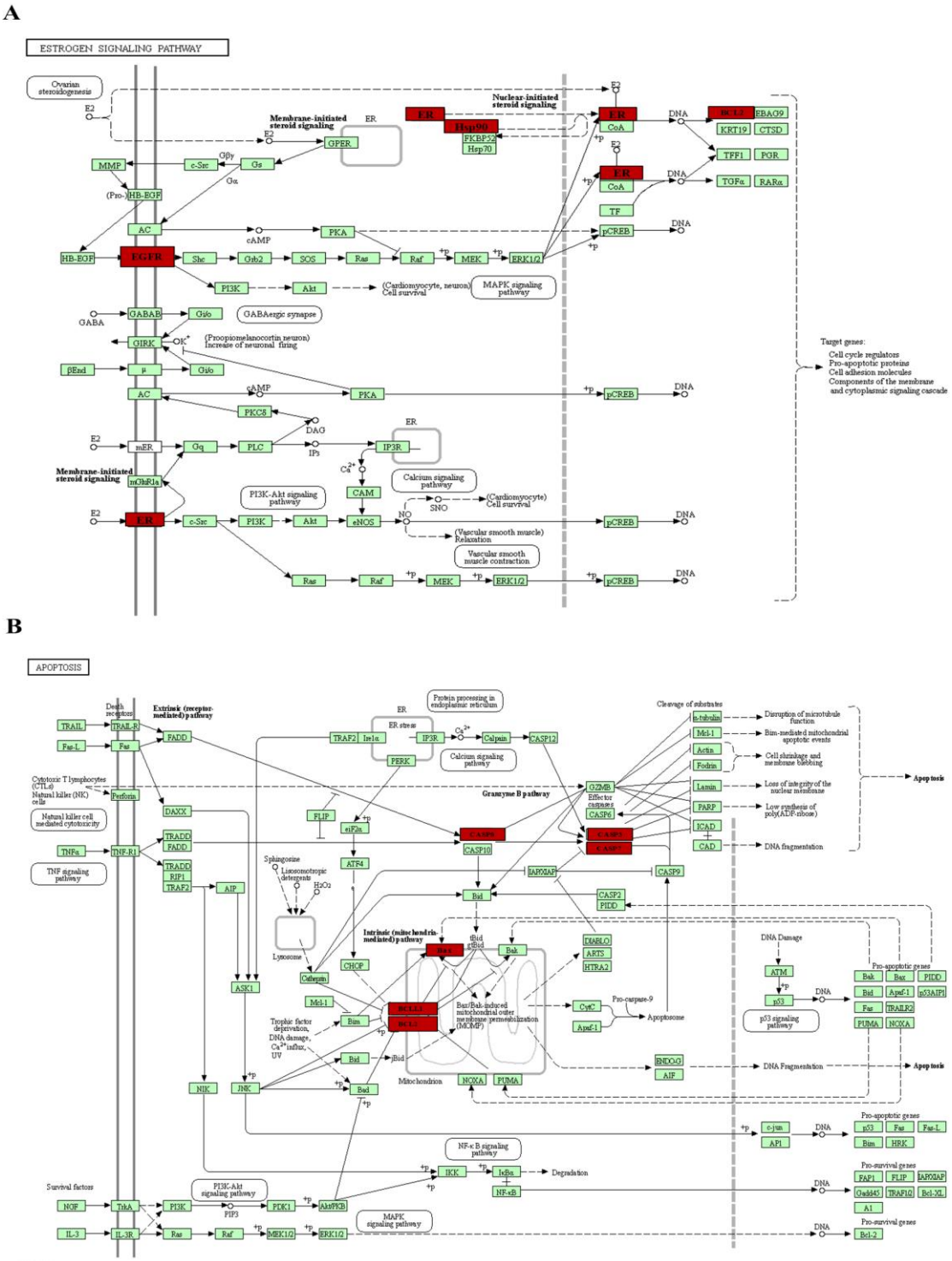
Ethical statement

The study was approved by the Animal Care and Use Committee of Guangdong University of Technology.

Supplementary FIGURE 1 The spectra of four polysaccharides and YYC were performed by high-performance gel permeation chromatography (HPGPC). *Polygonati Rhizoma* (A), *Lycii Fructus* (B), *Rehmanniae Radix* (C), *Codonopsis pilosula* (D), YYC (E)



Supplemental FIGURE 2 The KEGG pathway suggested that various targets of MGH were associated with the activity of polysaccharides. (A) The targets enriched in estrogen signaling pathway; (B) The targets enriched in apoptosis signaling pathway. The red nodes represented the most significant targets of the polysaccharides's activity.



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