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

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26
27

28 **Abstract**

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

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

45

46 **1. Introduction**

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

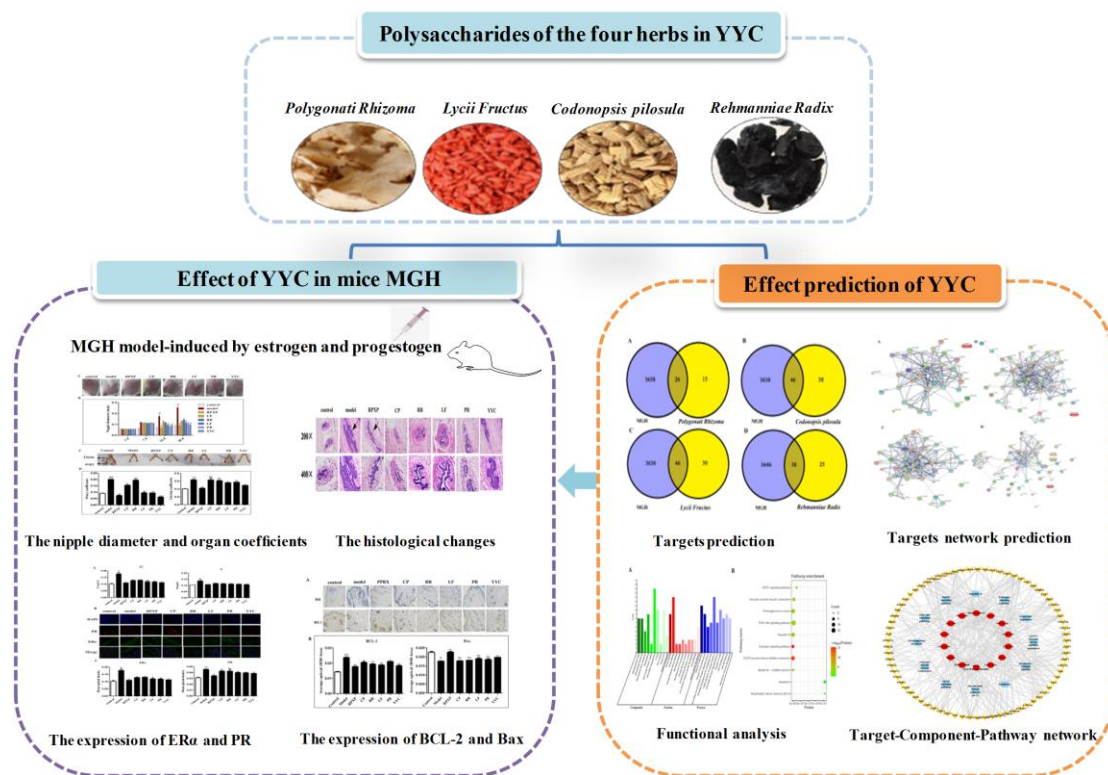
56 for treatment of MGH [4], long-term oral administration of such drugs will cause
57 hormone imbalance disorder and various medication discomforts to aggravate the
58 severity of MGH [5]. It is important for us to find new drugs with more convenient,
59 effective, and have few side effects to treat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

62 Medicine and Food Homology is regarded as a combination of food and medicine
63 functions, nutritional value, diseases prevention and treatment, and healthcare
64 activities [7]. Yiqi Yangyin complex (YYC), as a medicine and food homology from
65 TCM formula, including *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*,
66 *Rehmanniae Radix*, which are used as the common ingredients of the stew soup and
67 famous prescriptions of TCM for replenishing Qi and nourishing Yin, and called as Yi
68 Qi Yang Yin in Chinese. Polysaccharides have been regarded as the main components
69 of the stew soup or water decoction of many herbs for replenishing Qi and nourishing
70 Yin function, which play an important role in exhibiting immunomodulatory activities
71 [8]. *Polygonati Rhizoma* polysaccharide known as an important active compound, has
72 the potential as a drug or dietary adjuvant for the treatment of atherosclerosis and
73 hyperlipidemia [9], which has strong antioxidant, lipid-regulating, anti-inflammatory,
74 and endothelial function improvement effects [10, 11]. *Lycii Fructus* is traditionally
75 used in Chinese home cooking, such as tea, soups, porridge, taste sweet, and in the
76 Chinese pharmacopoeia as an aid for vision and longevity to balance the "Yin" and
77 "Yang" of the body [12]. The polysaccharide of *Lycii Fructus* partly decreased the
78 protein expression of HIF-1 α and Bax to regulate the production of inflammatory
79 factors through NF- κ B signaling pathway [13]. *Codonopsis pilosula* contained sterol,
80 triterpenes, glycoside, alkaloid, polysaccharide and other components [14], and its
81 polysaccharides had several biological activities, such as tumor growth prevention
82 [15], immune system modulation and anti-oxidant activity [16]. *Rehmanniae Radix*
83 has been traditionally known as lowering blood fever, nourish Yin and promoting the
84 body fluids, curing macula, skin rash, nosebleeds and so on. Meanwhile, *Rehmanniae*
85 *Radix* could nourish Yin and replenish blood, benefit the essence, and was mainly

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

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

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



113

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

116 **2. Materials and methods**

117 *2.1 Preparation of YYC and analysis of polysaccharides*

118 *Polygonati Rhizoma* (Sichuan, China), *Lycii Fructus* (Qinghai, China), *Codonopsis*
 119 *pilosula* (Gansu, China), and *Rehmanniae Radix* (Henan, China) were purchased from
 120 Tianfangjian (China) Pharmaceutical Co. LTD. The four raw materials, including
 121 *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, and *Rehmanniae Radix*
 122 were extracted in boiling water for 2 h and then further extracted in boiling water for
 123 1 h. All collected filtrates were processed with vacuum concentration to obtain
 124 extracts. The concentrated extracts were purified with 75% ethanol and contained
 125 30-45% solid contents. The extracts were followed by drying in a 70~100 °C oven to
 126 obtain the polysaccharides of four raw materials. Finally, the four polysaccharides
 127 were mixed to obtain YYC. The proportion of *Polygonati Rhizoma*, *Lycii Fructus*,
 128 *Codonopsis pilosula*, and *Rehmanniae Radix* in YYC were 25% (w/w), 25% (w/w),

129 25% (w/w), and 25% (w/w), respectively.

130 2.2 Determination of sugar and protein content

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

136 2.3 Animal experiments

137 Eight-week-old female KunMing mice weighing 18-20 g (license number:
138 SCXK2018-0002) were commercially obtained from the Experimental Animal Centre
139 of Guangdong Province. The mice were housed at a controlled room (23±1°C,
140 humidity 60±5%, 12 h day/light). They were acclimated under climate-controlled
141 conditions for 7 days before the experiments began. Mice were randomly divided into
142 seven groups with six mice in each group, including a control group (without
143 treatment), model group (0.5 mg/kg/d estrogen for first 25 days and 5 mg/kg/d
144 progestogen for last days), positive group (250 mg/kg/d, Rupixiao Pian, RPXP),
145 *Codonopsis pilosula* group (67 mg/kg/d, CP), *Rehmanniae Radix* group (67 mg/kg/d,
146 RR), *Polygonati Rhizoma* group (67 mg/kg/d, PR), *Lycii Fructus* group (67 mg/kg/d,
147 LF) and YYC group (*Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*, and
148 *Rehmanniae Radix*, at a ratio of 0.25:0.25:0.25:0.25, 67 mg/kg/d). The dose of
149 polysaccharides were used according to the guidelines of the Chinese Pharmacopoeia
150 (2015). Mice except for control group were injected with estrogen (0.5 mg/kg/d) into
151 the muscle of hind leg for consecutive 25 days, and followed with progestogen (5
152 mg/kg/d) for another 5 days [28]. For positive group, CP group, RP group, PR group
153 and YYC group, mice were treated with once daily intragastric administration before
154 intramuscular injection for 30 days. For control group and model group, mice were
155 intragastrically administered with equal volume of saline. Mice were sacrificed 24 h
156 after the last polysaccharides administration. The nipple height was firstly detected,

157 and mice were weighed. The blood were collected from eyeball extraction, and then
158 the mice were sacrificed. The mammary glands were immediately removed and fixed
159 4% paraformaldehyde. The blood was centrifuged at 3000 rpm for 15 min to separate
160 the serum without hemolysis, and then stored at -80°C. This study was approved by
161 the Animal Care and Use Committee of Guangdong University of Technology.

162 *2.4 Determination of nipple diameter and organ coefficients*

163 The diameter of the mice's nipple was measured on 1, 7, 15, 30 day (d). The ovary
164 and uterus were collected and weighted at the end of this experiment. The uterus and
165 ovary index were calculated by uterus or ovary weight divided by body weight [29,
166 30].

167 *2.5 Biochemical analysis and enzyme-linked immunosorbent assay (ELISA)*

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

172 *2.6 Histological analysis*

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

179 *2.7 Immunofluorescence assays*

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

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

189 *2.8 Immunohistochemistry assay*

190 In situ expression of Bcl-2 and Bax in mammary gland was performed as follows.
191 Paraffin-embedded sections (4 µm) were dewaxed in xylene, sequentially rehydrated
192 in alcohol and incubated in 3% H₂O₂ for 20 min. The sections were heated twice in a
193 microwave oven for 5 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval and
194 followed by overnight incubation at 4°C with the primary antibodies Bcl-2 (1:50) and
195 Bax (1:50) (Danvers, MA, United States) [30]. The sections were washed and
196 incubated with the HRP conjugated secondary antibody for 30 min at 37°C. After
197 staining with DAB, the tissue slides were counterstained with hematoxylin,
198 dehydrated with a graded ethanol series, and sealed with neutral gum in the end. The
199 sections were captured by microscope (Olympus, Tokyo, Japan). The mean integrated
200 optical density (IOD) of these areas was measured by software Image J.

201 *2.9 Database construction*

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

212 2.10 Target protein-protein interaction (PPI) network construction

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

223 2.11 Go gene enrichment analysis and KEGG pathway

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

229 2.12 Statistical analysis

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

234 3. Results

235 3.1 The physicochemical properties of YYC

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

239 22.31% (Table 1). The protein content of *Polygonati Rhizoma*, *Lycii Fructus*,
 240 *Codonopsis pilosula*, *Rehmanniae Radix* and YYC are respectively 4.28%, 17.67%,
 241 22.19%, 10.68%, 9.72% (Table 1). The components of *Polygonati Rhizoma* with
 242 molecular weight less than 5×10^3 Da had 92% of the peak area. The components of
 243 *Lycii Fructus* with molecular weight between 5×10^3 Da and 4.8×10^4 Da had 92% of
 244 the peak area. The components of *Rehmanniae Radix* with molecular weight less than
 245 5×10^3 Da had 84% of the peak area. The components of *Codonopsis pilosula* with
 246 molecular weight between 5×10^3 Da and 3×10^5 Da had 80% of the peak area. The
 247 components of YYC with molecular weight between 5×10^3 Da and 2×10^4 Da had
 248 70% of peak area by HPGPC (Table 1, supplementary material Fig. S1).

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

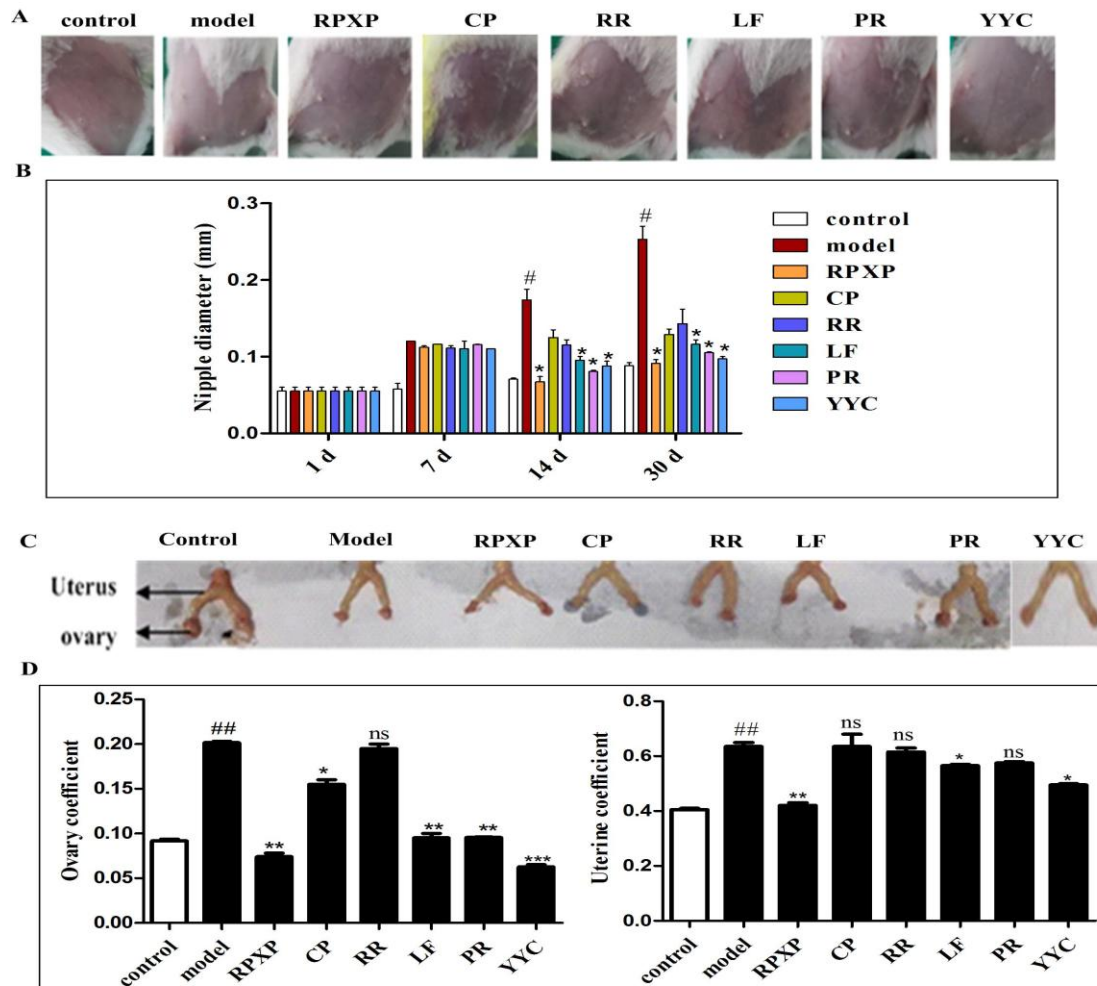
Samples	Total sugar %	Protein %	Molecular Weights (Da)
<i>Polygonati Rhizoma</i>	22.9%	4.28	$< 5 \times 10^3$ Da
<i>Lycii Fructus</i>	16.21%	17.67	5×10^3 Da - 4.8×10^4 Da
<i>Codonopsis pilosula</i>	19.2%	22.19	5×10^3 Da - 3×10^5 Da
<i>Rehmanniae Radix</i>	18.4%	10.68	$< 5 \times 10^3$ Da
YYC	22.31%	9.72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

251 3.2 YYC improved the nipple diameter and organ coefficients of mice with MGH

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

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

261 endocrine status of the ovary, while the uterus index and ovary index reflect the
 262 changes of the uterus and ovary [4]. Compared with the control group, ovary and
 263 uterine coefficient were significantly increased in model group. Compared with the
 264 model group, the ovary index was markedly inhibited in RPXP ($p < 0.01$), CP (p
 265 < 0.05), LF ($p < 0.01$), PR ($p < 0.01$) and YYC group ($p < 0.001$), and uterine coefficient
 266 was significantly reduced in RPXP ($p < 0.01$), LF ($p < 0.05$) and YYC group ($p < 0.05$)
 267 (Figure 2C and Figure 2D). Taken together, this result suggested that YYC
 268 significantly down-regulated the ovary index and uterine coefficient to relieve the
 269 symptom of mice with MGH.



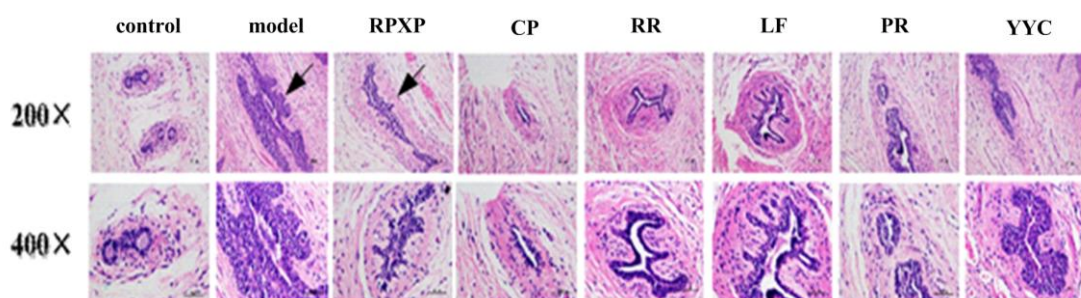
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271 FIGURE 2 Effects of polysaccharides on the nipple diameters in MGH. (A) The pathological
 272 features of mammary gland tissues in different group. (B) Diameter of nipples in different group.
 273 (C) The pathological features of uterus and ovary.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

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

279 3.3 Effect of YYC on the histological changes in mice with MGH

280 In order to verify whether the YYC could alleviate MGH, HE staining was used to
281 detect the pathological changes of mammary gland tissue. As shown in Figure 3,
282 compared with the control group, there was obvious proliferative lesions in mammary
283 epithelial cell tissue, including lobules hyperplasia, increase of count of acini and
284 ducts, and irregular arrangement and obvious expansion of duct lumen in model group.
285 Compared with the model group, administration of PRPX and YYC for consecutive
286 30 days significantly inhibited the typical histological patterns, whereas treatment of
287 CP, RR and PR were also capable to decrease the area of proliferative lesions and
288 counts of mammary acini and ducts in different degrees (Figure 3). This result showed
289 that YYC had therapeutic efficacy on mice with MGH.



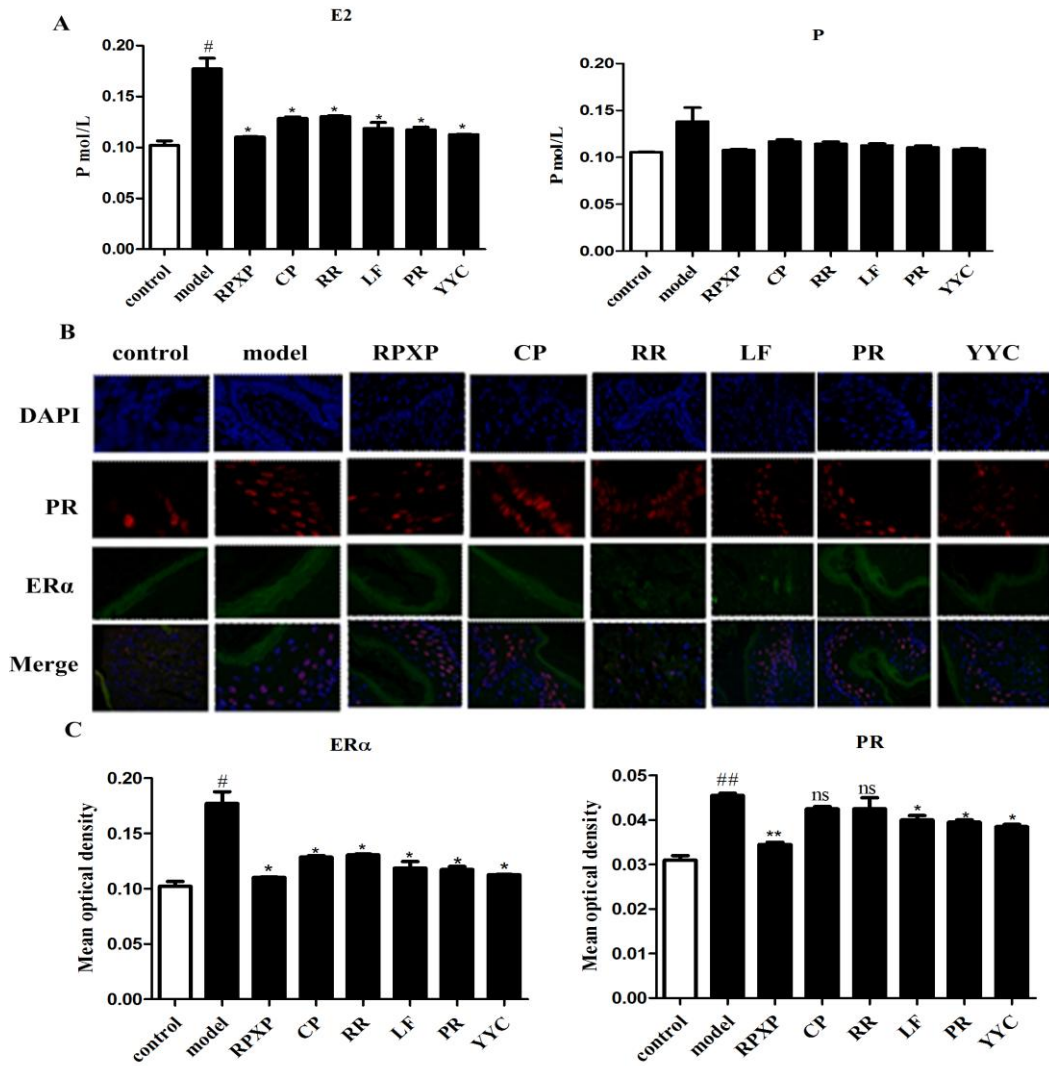
290
291 FIGURE 3 Analysis of histopathological for mammary gland tissue with polysaccharides. Control
292 group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP), *Codonopsis*
293 *pilosula* (CP), *Rehmanniae Radix* (RR), *Polygonati Rhizoma* (PR), *Lycii Fructus* (LF), Yiqi
294 Yangyin Complex (YYC). (Arrow indicated hyperplasia of ductal epithelial cells.)

295 3.4 YYC modulated the serum biochemical parameters and protein expression of ER α 296 and PR

297 Estrogen receptors play a critical role in regulating cell proliferation and

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

304 To investigate the effect of YYC on Estrogen receptors, the levels of ER α and PR
305 were detected by immunofluorescence assay. The fluorescence intensity of ER α ($p <$
306 0.05) and PR ($p < 0.01$) in model group were significantly elevated in comparison
307 with control group (Figure 4B and Figure 4C). Compared with model group, the
308 fluorescence intensity of ER α was markedly inhibited in all group ($p < 0.05$), while
309 the fluorescence intensity of P was significantly decreased in RPXP ($p < 0.01$), LF (p
310 < 0.05), PR ($p < 0.05$) and YYC ($p < 0.05$) group (Figure 4B and Figure 4C). This
311 result illustrated that LF, PR and YYC could regulate the estrogen receptors on the
312 mice with MGH-induced by estrogen and progestogen.



313

314 FIGURE 4 Effect of polysaccharides on sex hormones levels and the expression of ERα and PR in

315 mice's mammary gland tissue. (A) Sex hormones level for E2 and P in serum. (B)

316 Immunofluorescence analysis for ERα and PR in mammary gland tissue. Red signals represents

317 PR expression, green signals represents ERα expression, blue signals represents nuclei. (C) The

318 quantification of immunofluorescence signals in different groups. Data are expressed as the mean

319 ± SEM. Control group (Control), Model group (Model), Positive group (Rupixiao Pian, RPXP),

320 *Codonopsis pilosula* group (CP), *Rehmanniae Radix* group (RR), *Polygonati Rhizoma* group (PR),

321 *Lycii Fructus* group (LF), Yiqi Yangyin Complex group (YYC). Data are expressed as the mean±

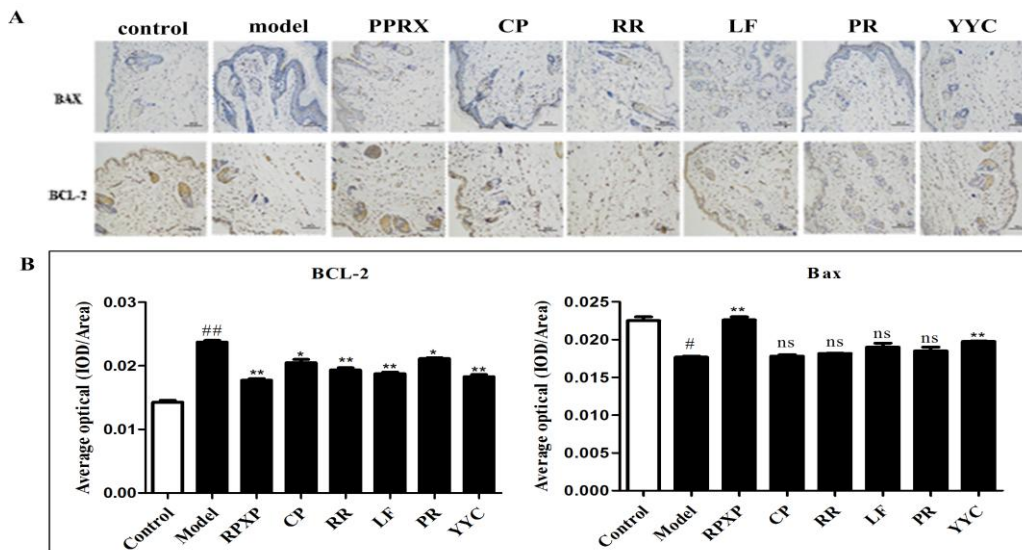
322 SEM. # represents MGH model group vs control group ([#] $p < 0.05$, ^{##} $p < 0.01$); * indicated

323 significant difference in polysaccharide treatment group vs MGH model group. (* $p < 0.05$, ** $p <$

324 0.01), ns indicated no significant difference.

325 3.5 Effect of YYC on expression of BCL-2 and Bax in mice with MGH

326 Apoptosis plays an important role in maintaining tissue homeostasis and cancer
 327 prevention [32]. In order to verify the effect of YYC on apoptosis in MGH, the
 328 apoptotic protein Bax and anti-apoptotic protein BCL-2 were detected by
 329 immunohistochemical assay. Compared with the control group, the levels of BCL-2 (p
 330 < 0.01) were significantly up-regulated, whereas Bax expression ($p < 0.05$) were
 331 markedly down-regulated in model group. Meanwhile, we found that the level of
 332 BCL-2 was significantly down-regulated in RPXP ($p < 0.01$), CP ($p < 0.05$), LF ($p <$
 333 0.01), PR ($p < 0.05$), RR ($p < 0.01$) and YYC group ($p < 0.01$) in comparison with
 334 model group, while Bax expression was up-regulated in RPXP ($p < 0.01$), and YYC
 335 group ($p < 0.01$) (Figure 5A and Figure 5B). This result suggested that YYC might be
 336 involved in regulating the symptom of mice with MGH via apoptosis.

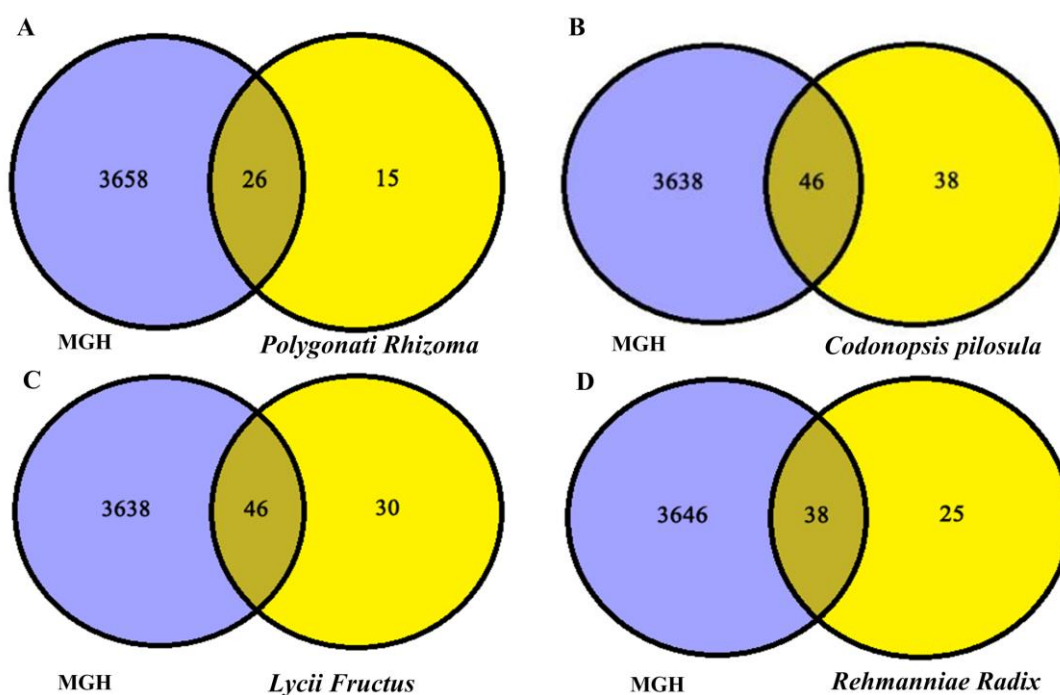


337

338 FIGURE 5 Effects of polysaccharides on level of BCL-2 and Bax in mammary gland with MGH.
 339 (A) Immunohistochemical analysis of BCL-2 and Bax in different group. (B) The quantification of
 340 immunohistochemistry signals in different groups. Control group (Control), Model group (Model),
 341 Positive group (Rupixiao Pian, RPXP), *Codonopsis pilosula* group (CP), *Rehmanniae Radix* group
 342 (RR), *Polygonati Rhizoma* group (PR), *Lycii Fructus* group (LF), Yiqi Yangyin Complex group
 343 (YYC). Data are expressed as the mean \pm SEM. [#] represents MGH model group vs control group ([#]
 344 $p < 0.05$, ^{##} $p < 0.01$); * indicated significant difference in polysaccharide group vs MGH model
 345 group (* $p < 0.05$, ** $p < 0.01$). ns indicated no significant difference.

346 3.6 The targets of four polysaccharides affecting MGH were predicted by network
347 pharmacology

348 A total of 3684 targets for MGH were firstly predicted by the GeneCards server.
349 Meanwhile, the targets of four polysaccharides were predicted by
350 Swiss-target-prediction server. After the duplicate genes have been removed, the
351 targets for *Polygonati Rhizoma* polysaccharide, *Codonopsis pilosula* polysaccharide,
352 *Lycii Fructus* polysaccharide, *Rehmanniae Radix* polysaccharide are respectively 41,
353 76, 63, 84. Finally, the Venn diagram was constructed by predicted targets for MGH
354 and targets of four polysaccharides. The targets of affecting MGH for *Polygonati*
355 *Rhizoma* polysaccharide, *Codonopsis pilosula* polysaccharide, *Lycii Fructus*
356 polysaccharide, *Rehmanniae Radix* polysaccharide were 26, 46, 38, 46 (Figure 6).



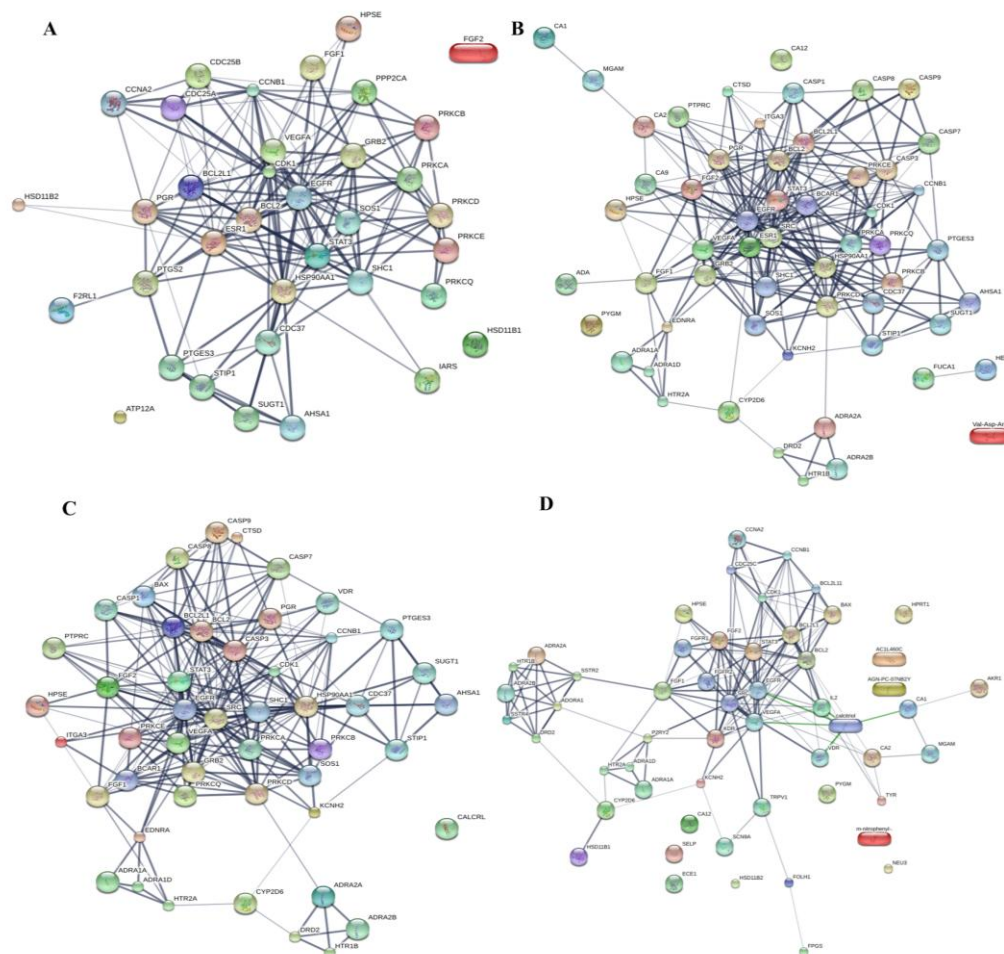
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358 FIGURE 6 The targets of affecting MGH for four polysaccharides. A-D: Venn diagram of the
359 candidate targets in four polysaccharides (*Polygonati Rhizoma* (A), *Codonopsis pilosula* (B), *Lycii*
360 *Fructus* (C), *Rehmanniae Radix* (D)) and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

362 3.7 Network construction and analysis of target protein-protein interaction

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

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



385

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

391 3.8 The GO enrichment and KEGG signaling pathway of YYC

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

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

402 The KEGG pathway analysis was further performed to identify the signaling
403 pathway that the predicted targets may be participated. The bubble map of KEGG
404 pathway showed that the core targets based on top 10 enrichment pathway (Figure
405 8B). The top 10 involved in signaling pathways were mainly related to hormone
406 signaling pathway, such as estrogen signaling pathway, thyroid hormone signaling
407 pathway, apoptosis. We further constructed the target-component-pathway network
408 diagram of action mechanism of YYC (Figure 8C). This result showed that each
409 active compound could act on multiple targets. Notably, various targets on MGH were
410 significantly enrichment on estrogen signaling pathway, which includes the target of
411 ER, BCL-2, HSP90, EGFR. The KEGG pathway of polysaccharides target MGH
412 showed that the above targets might be activated by polysaccharides intervention, and
413 then influenced EGFR tyrosine kinase inhibitor resistance signaling pathway,
414 apoptosis-multiple species signaling pathway, hepatitis B signaling pathway,
415 proteoglycans in cancer signaling pathway, PI3K-Akt signaling pathway. The
416 induction of estrogen signaling pathway and apoptosis play an important role in the
417 treatment of MGH with drugs (Supplementary Figure 2). Therefore, the two
418 significant enrichment pathways were in accordance with the animal experimental
419 verification.

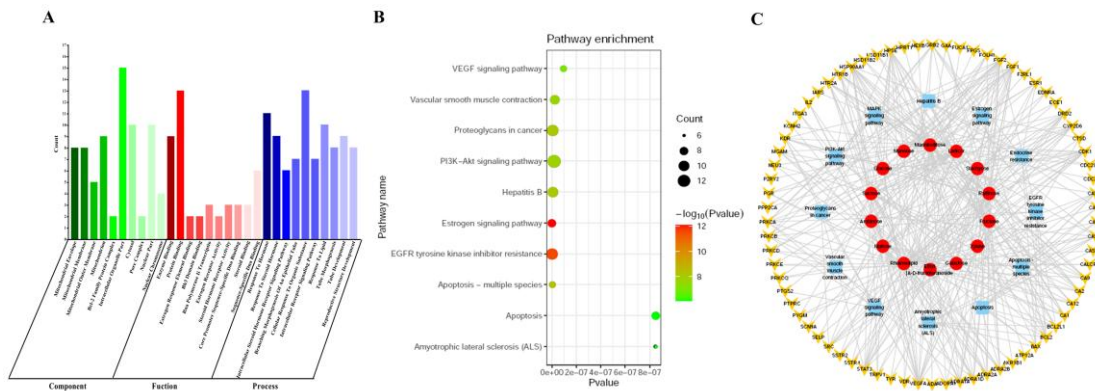
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426 FIGURE 8 GO and KEGG enrichment analysis of YYC. (A) The GO enrichment analysis of
 427 potential targets including molecular function, cellular component, biological process. (B) The
 428 enrichment analysis of KEGG signaling pathways. The size 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

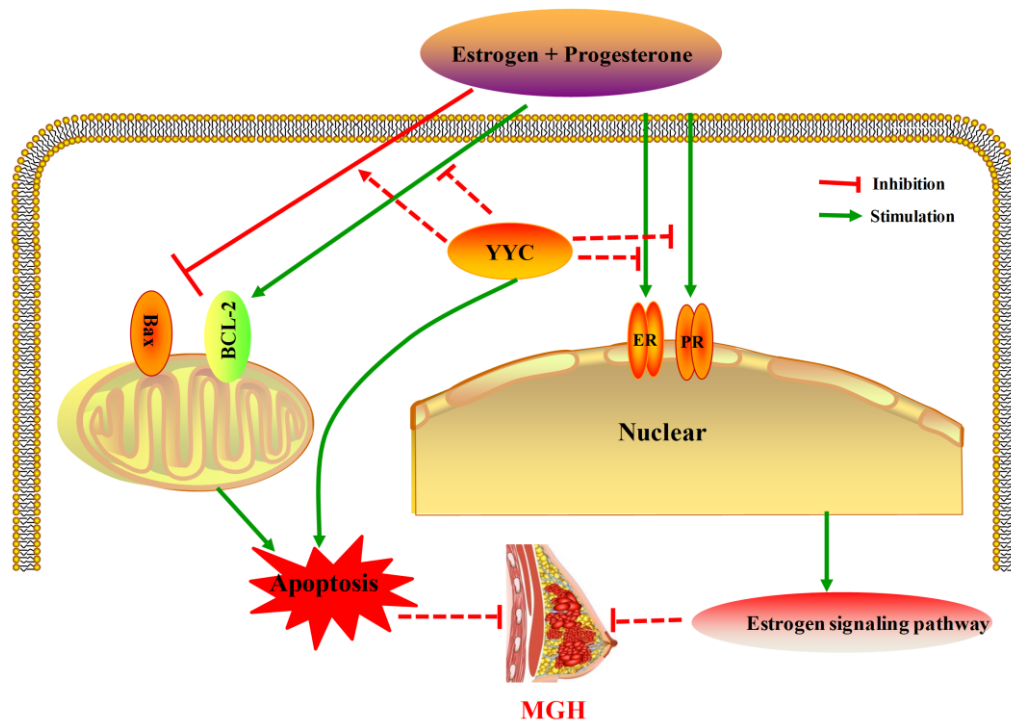
448 of E2 could be significantly reduced in YYC group in mice, but the level of P has no
449 significant difference with control group. This result suggested that the absolute or
450 relative increase of E2 or P deficiency could lead to imbalance of E2/P ratio to cause
451 excessive proliferation of mammary glandular parenchyma. ER and PR could
452 specially bind to E2 and P. ER is a protein molecule with two subtypes of ER α and
453 ER β , which combines with E2 to form a hormone receptor complex [37]. PR as the
454 regulatory protein of E2 is accompanied by ER. E2 could promote expression of ER
455 and PR in breast epithelial cells, and increase the sensitivity of mammary gland tissue
456 to hormones-induced MGH [38]. Meanwhile, the proliferating cells promote the
457 synthesis of ER and PR through the hormone receptor system, forming a pathological
458 proliferation cycle. Here, our animal study showed that the polysaccharides *from*
459 *Polygonati Rhizoma*, *Lycii Fructus* and YYC significantly improved the expression of
460 ER α and inhibited production of PR by immunohistochemical assay in MGH mice
461 model, indicating that the polysaccharides *from Polygonati Rhizoma and Lycii*
462 *Fructus* in YYC played an important role in relieving MGH. Previous study showed
463 that *Polygonati Rhizoma* polysaccharide has potential anti-inflammatory effect on
464 12-O-tetradecanoylphorbol-acetate (TPA)-induced inflammatory in mice [39]. *Lycii*
465 *Fructus* inhibits growth of ER⁺ human breast cancer cells (MCF-7 cell line) by
466 altering E2 metabolism [40]. Interestingly, KEGG pathway analyses by network
467 pharmacology showed that two targets ER and PR were directly involved in the
468 enrichment on Estrogen signaling pathway. This pathway could transmit the signal to
469 specific functional modules, such as apoptosis, carcinogenesis, cell proliferation,
470 differentiation/development and inflammation, which are mainly initiated through
471 estrogen or estrogen chemicals binding to ERs [41]. ERs will transducer signals by
472 PI3K, MAPK/ERK and NF- κ B signaling pathway [42], and then influence each other
473 by crosstalk or bypassing at the intracellular level, or deliver signals to different cells
474 or tissues by the secretion of hormones and growth factors to cause completely
475 different types of functional outcome [41]. Taken together, these results suggested that
476 YYC may not only alleviate the symptoms of MGH, but also further inhibit MGH
477 development to carcinogenesis by crosstalk of various pathways.

478 Apoptosis is the primary mode of cell death involved in development and
479 homeostasis [43]. BCL-2 is a well-known inhibitor of apoptosis to induce
480 tumorigenesis [44], while Bax is a pro-apoptotic protein. Both of them are generally
481 considered as important molecular proteins for apoptosis [45]. BCL-2 could block
482 MOMP to antagonize Bax and then prevent apoptosis [46]. It has been reported that
483 Tongru Sanjie decoction could regulate the expression of BCL-2 to inhibit MGH,
484 thereby blocking malignant transformation of mammary gland. Also, the excessive
485 expression of BCL-2 in many B-cell malignancies could induce its growth and
486 proliferation [47]. Apoptosis could cause diseases, such as hyperplasia in the
487 peripheral lymphoid organs, which accelerates autoimmune disease and tumorigenesis
488 [48]. Here, we analysed apoptosis by immunohistochemical assay and found that the
489 polysaccharides from *Polygonati Rhizoma*, *Lycii Fructus*, *Codonopsis pilosula*,
490 *Rehmanniae Radix* and YYC significantly inhibited the level of anti-apoptotic factor
491 BCL-2, whereas only YYC markedly elevated the pro-apoptotic factor Bax expression.
492 *Lycii Fructus* contains three bioactive compounds, including carotenoids, phenolics,
493 and polysaccharides, and the polysaccharide is as the most important and highest
494 component [49]. *Lycii Fructus* polysaccharides could up-regulate anti-apoptotic
495 protein BCL-2 in lens epithelial cells and increase ratio of BCL-2 to BAX
496 (pro-apoptotic protein) in the lens mainly by its anti-oxidative effects [50]. *Polygonati*
497 *Rhizoma* consists of saponins and polysaccharides and exerts its pharmacological
498 activity, such as immune promotion, antiaging, antifatigue, blood glucose regulation
499 and lipid regulation [51]. *Codonopsis pilosula* contains polysaccharides,
500 sesquiterpenes, saponins, and phytosterols [52], and the polysaccharides are active
501 compounds to exert multiple functions, including antitumor, antimicrobial,
502 antioxidant, and immunoenhancing properties [53, 54]. The main components of
503 *Rehmanniae Radix* are polysaccharides, triterpenoid saponins, iridoids, ionones,
504 phenylglycol glycosides, phenolic acids, and lignans [55], and the polysaccharides are
505 major active components to exhibit anticancer, anti-aging, antioxidant, and
506 immunomodulatory activities [56, 57]. The above literature suggested that the main
507 anti-hyperplasia potential may be attributed to the high content of polysaccharides in

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

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

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



560

561 FIGURE 9 The schema of synergistic mechanism of YYC on MGH

562 5. Conclusion

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

574

575 Authors' contributions

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

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

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587 **Availability of data and materials**

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

590 **Consent for publication**

591 Not applicable

592 **Conflicts of interest**

593 The authors declare that they have no conflicts of interests.

594 **Ethical statement**

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

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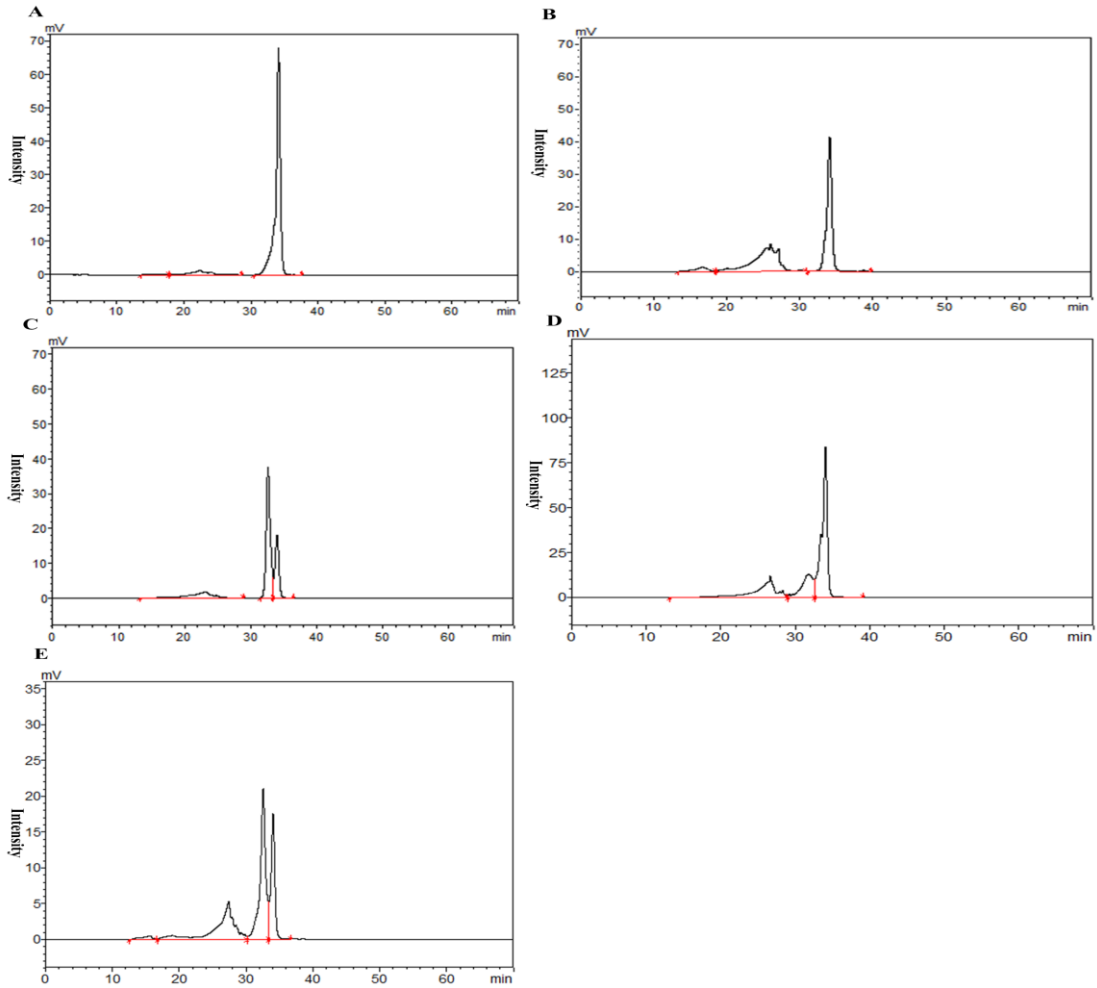
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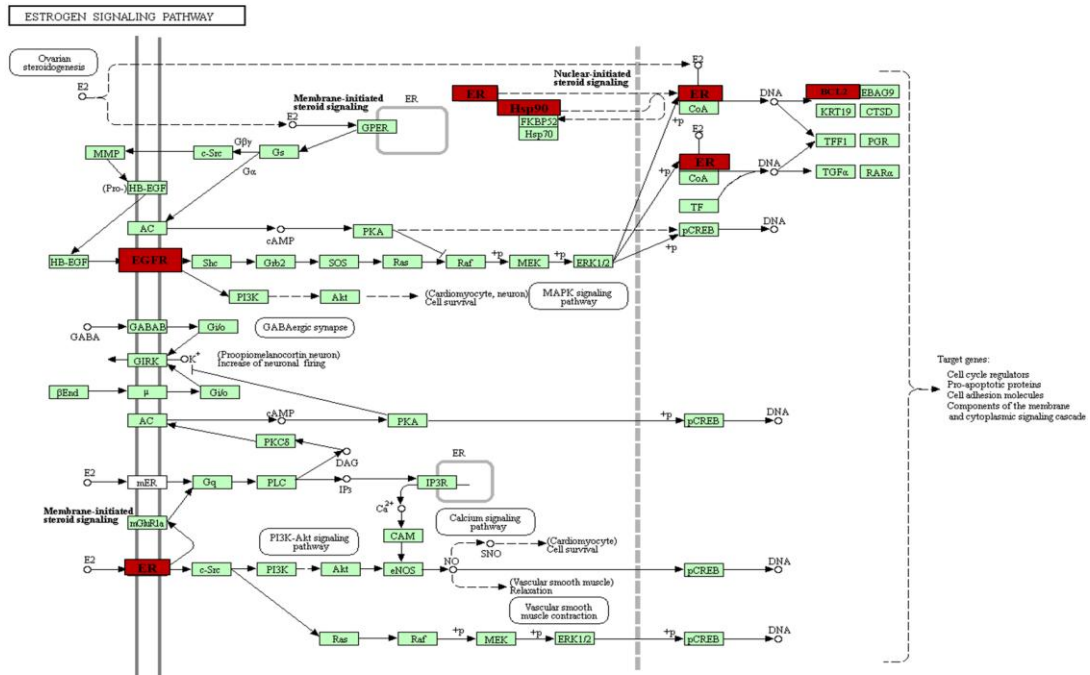
603 **Supplementary FIGURE 1** The spectra of four polysaccharides and YYC were
604 performed by high-performance gel permeation chromatography (HPGPC). *Polygonati*
605 *Rhizoma* (A), *Lycii Fructus* (B), *Rehmanniae Radix* (C), *Codonopsis pilosula* (D), YYC (E)



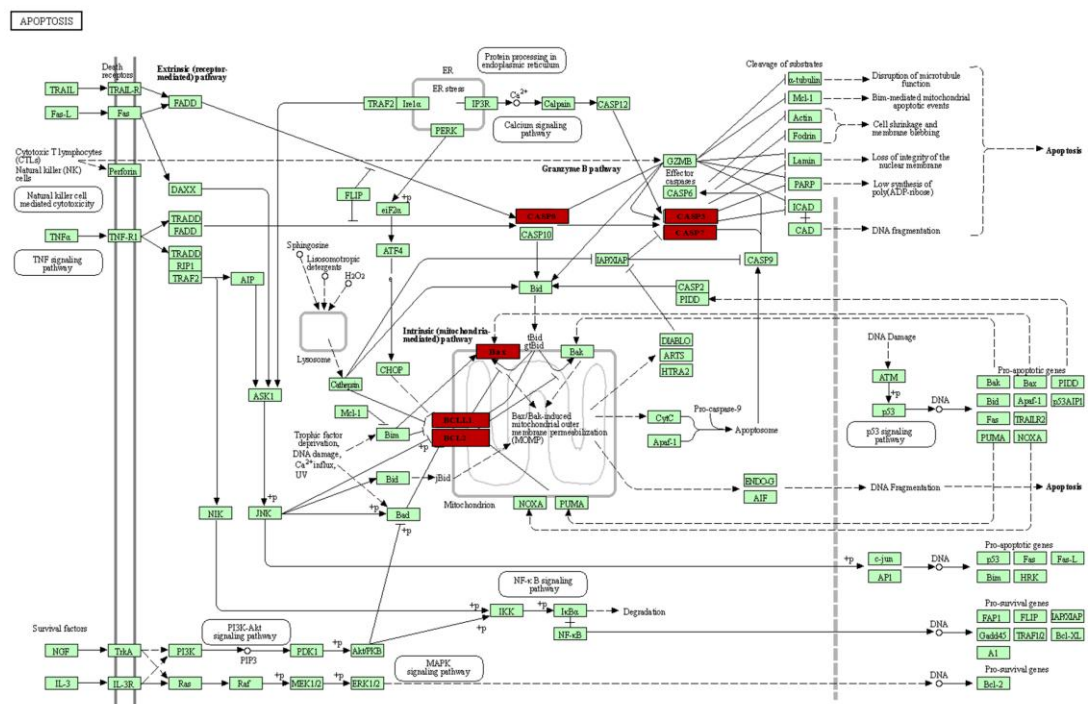
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618 **Supplemental FIGURE 2** The KEGG pathway suggested that various targets of MGH were
 619 associated with the activity of polysaccharides. (A) The targets enriched in estrogen signaling
 620 pathway; (B) The targets enriched in apoptosis signaling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

A



B



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