An Investigation Into the Mechanism of Li Chong Pill for Treating Endometriosis Based on Network Pharmacology and Molecular Docking Verification

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Abstract

Background and objective: Li Chong Wan (Li Chong pill, LCP) origin from *Yi Xue Zhong Zhong Can Xi Lu,* (Records of Chinese Medicine with Reference to Western Medicine), widely used in the treatment of endometriosis (EM) in China. The purpose of this study is to investigate the intrinsic mechanisms of LCP against EM and to provide new evidence for its clinical application.

Methods: Chemical compounds of LCP were screened and evaluated via retrieving public databases and literature. We also acquired their putative targets and obtained EM-related targets. The above-mentioned data were visualized as a component-target network. In addition, we use Cytoscape3.8.0 to build a protein-protein interaction network and identified hub genes and key active ingredients. Furthermore, through GO and KEGG pathway analyses, which were actualized by R3.6.1 (based on clusterProfiler, org.Hs.eg.Db, and pathview package), we obtained effective signaling pathways and biological functions. Molecular docking was used to verify binding activity between compounds and the key targets at last.

Results: Finally, a total of 122 possible active targets and 47 components were screened. Identify the core network and screen out 10 main targets; GO and KEGG enrichment analysis revealed that LCP may have functions of anti-inflammatory, anti-angiogenesis, inhibition of cell proliferation, regulation of hormone secretion, etc. The effect of LCP on EM might be achieved by PI3K/Akt signaling pathway, HIF-1 signaling pathway, estrogen signaling pathway, and VEGF signaling pathway, etc. Finally, molecular docking results demonstrated that 14 components were exhibited good binding property to the key targets of EM.

Conclusion: This research ocularly demonstrated the multi-component, multi-target, and multi-channel pharmacological effects for LCP in the treatments of EM and provides evidence for further clinical research and verification of the mechanism.

Introduction

Endometriosis is estimated to affect 10%-15% of women at childbearing age.[1] The endometriotic pathological processes involve repeated tissue injury and repair, local inflammation, angiogenesis, and neurogenesis，which result in progressive smooth muscle metaplasia and uterine fibrogenesis.[2] The preferred treatment is surgery. However, surgery carries a significant recurrence rate and cost burden. There is a critical need for safe, effective medical therapies for endometriosis patients at present, either in conjunction with or independent of surgery.[3] And an important fact is that the increased risk of deeply infiltrating endometriosis in patients is associated with surgical history.[4] It might be related to activation of the adrenergic pathway, chronic stress, and increased angiogenesis.[5] Traditional Chinese medicine has a unique view on the treatment of endometriosis. It is believed that the basic etiology of this disease is "accumulated static blood in the uterus", and blood stasis is the central link running through EM and the most basic pathological basis of the disease.[6]

Li Chong Wan (Li Chong pill, LCP) origin from *Yi Xue Zhong Zhong Can XiLu,* (Records of Chinese Medicine with Reference to Western Medicine), which is composed of Shuizhi (Leech), Huangqi (Astragalus), Sanleng (Sparganii Rhizoma), Ezhu (Curcumae Rhizoma), Danggui (Chinese Angelica), Zhimu (Anemarrhena) and Taoren (Peach kernel). It is found that these drugs have the effect of anti-inflammatory, analgesic, hemodynamic improvement, and anti-oxidative stress.[7-12] In addition to the widespread use of endometriosis, LCP is also used to treat all kinds of benign abdominal masses of women, such as ovarian cysts, old ectopic pregnancy and so on.[13, 14] The pathological mechanism of EM is complex and the symptoms are diverse, while the traditional Chinese medicine compound is

made up of many natural herbs, which has the characteristics of multi-component, multi-target, multi-function, and multi-pathway. Network pharmacology is a part of bioinformatics, which uses drug research models by constructing drug-drug, drug-target networks to find interactions between bioactive compounds and targets and between various targets,[15] and molecular docking is a method which can predict ligand-target interactions at a molecular level,[16] widely used in drug discovery. Therefore, we apply this method to explore the relationship between LCP and EM. The relevant technology route is shown in Figure 1.

Materials And Methods

Data preparation

Construction of LCP chemical ingredients database

Except for leech, all chemical ingredients of the other six drugs in LCP were coming from TCMSP (Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform, https://tcmspw.com/tcmsp.php), a website that integrates computer systems and biological sciences, with the primary objective of helping understand how drugs work in specific pathways and different cell types. It contains 499 Chinese medicines registered in the *Pharmacopoeia of the people's Republic of China*, and including 29,384 chemical ingredients, 3311 targets, and 837 related diseases, it also provides ADME-related properties data to undertake the discovery and research of drugs.[83] We used oral bioavailability (OB), drug-like properties (DL) in the TCMSP database as the index to select the target active components (OB≥30% and DL≥0.18%) referring to similar literature,[84] finally obtained the active ingredients of Huangqi (Astragalus), Sanleng (Sparganii Rhizoma), Ezhu (Curcumae Rhizoma), Danggui (Chinese Angelica), Zhimu (Anemarrhena) and Taoren (Peach kernel). Moreover, the chemical constituents of leech were screened out from exploring literature in CNKI (https://www.cnki.net/) and PubMed (https://pubmed.ncbi.nlm.nih.gov/). Then we used the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) to find canonical simplified molecular-input entry specification (SMILES) information of those constituents and used Chem Bio Draw Ultra software for drawing the 2D structures of the candidate compounds not found in PubChem. Then upload them to the Swiss ADME (http://www.swissadme.ch/), a website that can evaluate pharmacokinetics, drug-likeness, and medicinal chemistry friendliness of small molecules. An active compound can be screened out when the following conditions are met simultaneously, firstly, using the Pharmacokinetics Gatrointesinal absorption which for screening active compounds with good oral bioavailability shows "High" as a condition that drugs can be absorbed, secondly, there are 2 or more "Yes" in the 5 drug-like predictions (Lipinski, Ghose, Veber, Egan, Muegge) results.

LCP-related Target prediction

First, using TCMSP to predict protein targets of six drugs except for leeches, using UniProt KB (http://www.uniprot.org) to extract the predicted targets, convert them into symbol names, the target information was set to homo sapiens and consolidate the downloaded data for further analyses. Secondly, the results of leech active components screened by Swiss ADME were uploaded to the Swiss Target Prediction (https://www.swisstargetprediction.ch/), setting probability >0.1 (Probability for the query molecule -assumed as bioactive to have this protein as the target.) was used to screen the target of the leech, so as to establish the drug target database of LCP.

Endometriosis Related Genes Database Construction

Obtained EM-related targets through five databases which were Gene Cards, OMIM, TTD, PharmGKB, and Drug Bank. Gene Cards integrated gene databases in a variety of research areas, including genomes, transcriptomics, proteomics, which is a one-step-shop for searchable human gene annotations.[85] OMIM (Online Mendelian Inheritance in Man www. omim.org) provides concise textual information based on the peer-reviewed biomedical literature on over 15,500 genes, 26,200 allelic variants, and 7,800 genetic phenotypes.[86] TTD (Therapeutic Target Database), which enables the search of almost 900 targets, 1800 biomarkers, and 6000 drugs related to 900 diseases conditions.[87] Drug Bank is a fully curated drug and drug target database.[88] And PharmGKB today is the preeminent worldwide resource for pharmacogenomic information.[89] We searched the above-mentioned database using the keyword "endometriosis", the gene data obtained from the search were summarized and the duplicated targets were removed for subsequent analysis.

Construction of an LCP-compound-target-EM Network

We map the Venn between gene and drug targets via the "venn" R package disease. And the LCP-compound-target-EM network was constructed to clarify the relationship between active compounds in LCP and potential targets. This network was constructed and visualized using Cytoscape 3.8.0 software.

Protein-protein Interaction Network Between Targets

Since protein-protein interactions (PPI) are associated with the development of disease states,[90] we introduce potential targets into the STRING database (https://string-db.org/) for PPI analysis. STRING is an online PPI analysis database that aims to collect, score and integrate all publicly available sources of PPI information, and construct a visualized interaction network of genome-wide datasets.[91] The species was limited to "Homo sapiens", and the minimum required interaction score was set to be high confidence (0.900). We used the STRING database to explore the protein interaction relationship between LCP and endometriosis and map the protein interaction network.

GO and KEGG enrichment analysis

To explore the enrichment in gene function of interaction proteins obtained from the above analysis, we used R3.6.1 (based on "clusterProfiler""org.Hs.eg. db" and" pathview" package) to analyze the biological enrichment of GO and KEGG.

Verification through Molecular Docking

The Protein Data Bank (PDB), the single global repository of experimentally determined 3D structures of biological macromolecules and their complexes, was established in 1971, becoming the first open-access digital resource in the biological sciences. The PDB archive currently houses 130,000 entries (May 2017).[92] Therefore, we searched the PDB database for the 3D structures of the significant potential targets of the protein-protein interaction network, apply PyMOL2.4.0 to modify the target protein, including removal of ligands and water. Select the active ingredients whose degree>5 in the LCP-compound-target-EM network, use the PubChem to download the 2D structure of those components, and use the ChemDraw to draw things that are not found. Apply the MM2 in the Chem3D to optimize the energy of molecular configuration. Finally, we used AutoDock Tools1.5.6 to optimize the structure of protein molecules and ligands. Upload the results to CB-Dock (http://cao.labshare.cn/cb-dock/), a website that automatically determines the active pocket and sets the active site size and center coordinates. AutoDock vina software is used to operate molecular docking, which can also calculate the best binding energy of the optimal docking mode.

Result

Active compounds of the LCP

A total of 98 components were screened from LCP. Among the active ingredients screened from the TCMSP, we selected 68 ingredients according to OB and DL (see Additional file 1), including Huangqi (astragalus, HQ)20 species, Taoren (Peach kernel TR) 23 species, Zhimu (anemarrhena, ZM)15 species, Ezhu (Curcumae Rhizoma, EZ) 3 species, Sanleng (Rhizoma sparganii, SL) 5 species, Danggui (Chinese angelica, DG) 2species. In addition, we upload 50 (see Additional file 2) compounds of leech obtained from document retrieval.[17-21] According to the two conditions, we get 30 active ingredients of leech in the end (see Additional file 3).

Putative targets of LCP

According to the above active ingredients,881 potential targets were to predicted by using TCMSP and Swiss Target Prediction, the targets included 209 from HQ, 59 from TR,85 from ZM, 9 from EZ, 54 from SL,29from DG, 436 from SZ (see Additional file 4), gathering them up and removed the duplicate targets, the final number was 380.

EM related targets

Endometriosis-related genes, obtained from five databases previously described, we get 90 genes from the Drug Bank, 1009 genes from the GeneCards, two genes from the OMIM, 86 genes from the PharmGKB, 15 genes from the TTD, a total of 1115 targets were obtained after sorting out the screening data (Figure 2).

Construction of LCP-compound-target-EM Network

From the Venn chart, we found there were 122 overlapping targets between drugs and disease potential targets (Figure 3). Use Cytoscape3.8.0 to map the LCP-compound-target-EM network (Figure 4), there are 168 nodes (Except overlapping targets, 45 nodes are drug constituents, including 12 from TR, 10 from SZ,7 from ZM, 11 from HQ, 1 from SL, and A1, B1, C1, D1, E1 means the ingredient comes from a variety of drugs, see Table 1) and edge 298 in the network It can be seen that the effects of traditional Chinese medicines are widely distributed, each ingredient in LCP can take part in the treatment of diseases through a synergistic action.

PPI Network Analysis and Hub Genes Investigation

122 potential targets were uploaded to the STRING database. High confidence will improve the accuracy of the results so we set the minimum required interaction score to "highest confidence" (0.900). There were 110 proteins in the network, which contained 371 protein interaction relationships (Figure 5), and then filtered with application CytoNCA[22], a Cytoscape plugin for centrality analysis and evaluation of protein interaction networks (Figure 6a), based on the median values for betweenness, closeness, degree, eigenvector, LAC and network, which were39.31546869, 0.1244298725, 6, 0.0291560585, 2.5333333335, 3.4583333335, identified 28 highly connected node (Figure 6b), then performed second screening, which was 9.1005245735,0.586956522,9,0.1712328495, 4.211111111,5.154761905. We gradually obtained the core network of hub genes, finally identified 10 highly connected nodes significant endometriosis-related targets (Figure 6c), which are MAPK1, FOS, JAK2, EGFR, RELA, SRC, MAPK8, PIK3CA, MAPK14, and ESR1. (Figure 6d)

GO and KEGG enrichment analysis

The Gene Ontology (GO) can describe how genes act in biological systems and produce a dynamic, controlled vocabulary that can be applied to all eukaryotes,[23] which concludes three parts, biological progress (BP), cellular compound (CC), and molecular function (MF) to describe the biochemical activity of a gene product. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for systematic analysis of gene functions in terms of the networks of genes and molecules, which can provide most of the known metabolic pathways and some of the known regulatory pathways.[24] We used R packets to perform GO and KEGG enrichment analysis of 122 intersected genes, the detailed results are listed in additional files (see Additional file 5 and Additional file 6), the top 10 GO enrichment results of each project are shown in figure 7a/b. At the top of the BP group was "response to nutrient levels", "cellular response to chemical stress", "reproductive structure development", "reproductive system development", "response to oxygen levels" and "response to oxidative stress", etc. The MF group mainly included "steroid binding", "protein tyrosine kinase activity", "nuclear receptor activity" and "ligand-activated transcription factor activity", etc. And in the CC group, the GO terms were mainly involved "membrane raft", "membrane microdomain", "membrane region", "organelle outer membrane", "outer membrane mitochondrial outer membrane" and so on, which reflected the abnormality of multiple biological processes involved in endometriosis, and indicates that LCP may play a therapeutic role by improving the above biological pathways.

The KEGG pathway enrichment shows the top 30 contains, (Figure 8 a/b.) such as PI3K-Akt signaling pathway, EGFR tyrosine kinase inhibitor resistance, Th17 cell differentiation, relaxin signaling pathway, HIF-1 signaling pathway, Ovarian steroidogenesis, PD-L1 expression, and PD-1 checkpoint pathway in cancer and so on. The results of the first 30 of the GO and KEGG enrichment analysis were made into bubble charts and histograms, and produced a pathway map that the gene most enriched.

Verification of Molecular Docking

We screened 14 key components according to the LCP-compound-target-EM network(degree>5) as small-molecule drug ligands, which were docked with the 10 key targets identified in the previous PPI network (Table 2), then we obtained 140 sets of results(Table 3). The binding energy<0, indicating that the ligand molecules can bind spontaneously to the receptor proteins, however, there was no clear definition of the criteria for free energy screening after searching the literature．This paper take the binding energy<-5.0 kal˙mol-1 as a standard to judge the binding property is good, and the smaller the binding energy, the better running of docking. We found that the main way of molecules docking were hydrogen bonding and π-π stacking, The minimum binding energy is -10.5 kal˙mol-1 (Figure 9), and the binding energy<-5.0kal˙mol-1accounts for 96.43% of the total, and the binding energy>-5.0 kal˙mol^{-1}was abandoned. It can fully explain that the pivotal components of LCP have strong binding force with their key targets.

Table 1 Description of nodes in the LCP-compound-target-EM Network (Figure 3)

Node Name	Source	Compound
A1	Zhimu,Danggui,Sanleng	Stigmasterol
B1	Taoren,Danggui,Sanleng	Beta-sitosterol
C1	Huangqi, Sanleng	Formononetin
D1	Huangqi, Zhimu	Kaempferol
E1	Huangqi,Taoren,Sanleng,Ezhu	Hederagenin
HQ1	Huangqi (astragalus,HQ)	Bifendate
HQ2	Huangqi (astragalus,HQ)	Jaranol
HQ3	Huangqi (astragalus,HQ)	Calycosin
HQ4	Huangqi (astragalus,HQ)	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol
HQ5	Huangqi (astragalus,HQ)	FA
HQ6	Huangqi (astragalus,HQ)	Quercetin
HQ7	Huangqi (astragalus,HQ)	Isorhamnetin
HQ8	Huangqi (astragalus,HQ)	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol
HQ9	Huangqi (astragalus,HQ)	Mairin
HQ10	Huangqi (astragalus, HQ)	7-O-methylisomucronulatol
HQ11	Huangqi (astragalus,HQ)	3,9-di-O-methylnissolin
SL1	Sanleng (leech, SZ)	trans-gondoic acid
SZ1	Sanleng (leech, SZ)	gamma-aminobutyric acid
SZ2	Sanleng (leech, SZ)	L-tryptophan
SZ3	Sanleng (leech, SZ)	phenylalanine
SZ4	Sanleng (leech, SZ)	hexadecyl ethers of glycerol
SZ5	Sanleng (leech, SZ)	methyl 14-methylpentadecanoate
SZ6	Sanleng (leech, SZ)	L-tyrosine
SZ7	Sanleng (leech, SZ)	methyl (Z)-11-hexadecenoate
SZ8	Sanleng (leech, SZ)	hirudinoidine a
SZ9	Sanleng (leech, SZ)	palmitic acid
SZ10	Sanleng (leech, SZ)	glycerol
TR1	Taoren (peachkernel, TR)	Gibberellin A44
TR2	Taoren (peach kernel, TR)	3-O-p-coumaroylquinic acid

TR3	Taoren (peach kernel, TR)	2,3-didehydro GA70
TR4	Taoren (peach kernel, TR)	2,3-didehydro GA77
TR5	Taoren (peach kernel, TR)	GA120
TR6	Taoren (peach kernel, TR)	GA63
TR7	Taoren (peach kernel, TR)	4a-formyl-7alpha-hydroxy-1-methyl-8-methylidene-4aalpha,4bbeta-gibbane-1alpha,10beta-dicarboxylic acid
TR8	Taoren (peach kernel, TR)	Sitosterol alpha1
TR9	Taoren (peach kernel, TR)	GA60
TR10	Taoren (peach kernel, TR)	GA121-isolactone
TR11	Taoren (peach kernel, TR)	GA77
TR12	Taoren (peach kernel, TR)	campesterol
ZM1	Zhimu (anemarrhena, ZM)	Timosaponin B III_qt
ZM2	Zhimu (anemarrhena, ZM)	Anemarsaponin F_qt
ZM3	Zhimu (anemarrhena, ZM)	Hippeastrine
ZM4	Zhimu (anemarrhena, ZM)	Anemarsaponin C_qt
ZM5	Zhimu (anemarrhena, ZM)	coumaroyltyramine
ZM6	Zhimu (anemarrhena, ZM)	Anhydroicaritin
ZM7	Zhimu (anemarrhena, ZM)	diosgenin

Table 2 The PDB ID corresponding to each core target in the PPI network

Target	PDB ID
JAK2	3UGC
MAPK1	6SLG
FOS	1AO2
EGFR	2MOB
RELA	3QXY
SRC	2H8H
MAPK8	2NO3
PIK3CA	6PYS
MAPK14	2LGC
ESR1	2BJ4

Table 3 Docking scores of the active ingredients of LCP with their potential targets

Compound	3UGC	6SLG	1AO2	2MOB	3QXY	2H8H	2NO3	6PYS	2LGC	2BJ4
Quercetin	-7.8	-7.9	-8.4	-6.5	-9.2	-9.2	-9	-8.6	-8.6	-8.1
Hirudinoidine a	-7.2	-7	-7.6	-6	-7.4	-7.3	-7.6	-7.5	-7.5	-6.2
Methyl (Z)-11-hexadecenoate	-6.4	-	-5.5	-	-6.5	-6.1	-5.6	-5.9	-7.1	-6.2
Methyl 14-methylpentadecanoate	-6.3	-	-5.9	-	-6.5	-5.6	-5.5	-6.1	-7.1	-5.9
Kaempferol	-9	-7.9	-8.1	-6.3	-9.4	-9.1	-8.9	-8.3	-8.5	-8.3
Isorhamnetin	-8.8	-7.9	-9.8	-6.3	-9.2	-9.4	-8.7	-8.5	-8.4	-8.2
Beta-sitosterol	-8.9	-9.2	-8.1	-7	-8.2	-9.5	-9	-8.4	-9	-7.8
Stigmasterol	-9.5	-9.8	-8.2	-6.8	-9.1	-9.1	-9.3	-9	-8.3	-7.2
7-O-methylisomucronulatol	-7.5	-7	-7.4	-6.6	-9	-8.4	-7.5	-7.1	-8.2	-7
Anhydroicaritin	-8.4	-8.2	-8.7	-6.3	-9.3	-9.4	-8.8	-9.5	-7.8	-8.3
Formononetin	-8.8	-7.8	-7.9	-6.5	-9.2	-9.2	-8.7	-9	-9	-8.6
Calycosin	-8.3	-7.9	-7.7	-6.4	-9.3	-9.1	-8.7	-9.3	-8.9	-8.2
Diosgenin	-9.6	-9.4	-9.2	-7.2	-9.1	-10.4	-9.2	-9.8	-10.5	-8.1
phenylalanine	-6.4	-5.5	-6.2	-	-6.3	-6.4	-5.9	-6.6	-6.5	-6.2

Discussion

Abnormally located endometrium can grow and shed during the menstrual cycle, causing painful menstrual cramps or heavy menstrual periods. The development of inflammation, cysts, scar tissue, and adhesions leading to intestinal problems, chronic pelvic inflammatory disease, and infertility.[25] The problems caused by EM have brought great pressure to women's psychology and life, research on the disease has become a hot area in gynecology. Modern medicine suggests that the occurrence of EM possibly related to1) Retrograde menstruation 2) Coelomic metaplasia and 3) Vascular and lymphatic metastatic spread.[26] Besides, studies have shown that the growth of ectopic endometrial tissue leads to the recruitment of a large number of immune cells and the abnormal function of almost all immune cells, which increase pro-inflammatory cytokines, growth factors, and acceleration of angiogenesis. These mechanisms aggravate the inflammatory response of local tissues.[27] In a review in 2019 that proposed a concept named "endometriosis life",[2] the literature suggests that doctors should first consider addressing pelvic pain or providing assisted reproduction technologies rather than surgery immediately in cases of EM. Traditional Chinese medicine also has made a lot of research and clinical observation in the treatment of it, based on the complex mechanism of EM, it can provide multi-target treatment and new ideas for clinical treatment of this disease.

LCP is one of the classical prescriptions of traditional Chinese medicine for EM, modern medicine made a more careful study of drugs it contains. In this research, diosgenin, kaempferol, quercetin, stigmasterol, isorhamnetin, beta-sitosterol, formononetin, anhydroicaritin, calycosin, hirudinoidine a, and 7-O-methylisomucronulatol were the compounds that degree>5 of the LCP-compound-target-EM network and performed well in subsequent molecular docking. As we can see from the molecular docking results, the best performing component is diosgenin, which from

the Zhimu, it's a steroid sapogenin found in several plants which possesses a wide range of biological activities.[28] First of all, it has good anti-inflammatory activity,[29] there were some researches demonstrated that diosgenin significantly inhibited leukocyte migration and adhesion which was partly linked with the downregulation of TNF-α-induced expression of ICAM-1 via inhibiting NF-κB p65 activation. It also can inhibit the up-regulation of adhesion molecules induced by TNF-α, through the inhibition of MAPK/Akt and NF-κB signaling pathways and ROS production.[30, 31] An experiment indicated that diosgenin may be an effective NADPH oxidase inhibitor that can restrain superoxide anion production to play an anti-inflammatory role which is associated with inhibition of the cPLA2, PAK1/2, Akt, p38MAPK, ERK1/2, and JNK signaling pathway.[28] Secondly, ectopic endometrial lesions not only induce a local inflammatory response but also accompanied by abnormal coagulation function,[32] meanwhile, oxidative stress is involved in the pathogenesis of EM through various mechanisms, which is one of the probable mechanisms leading to carcinogenesis and infertility of patients.[33] Experiments prove diosgenin has anti-thrombosis properties,[34] the cytoprotective effect on vascular endothelial cells.[30] It also can modulate antioxidant defense and prevent endothelial apoptosis under oxidative stress,[35] At last, an experimental result showed that diosgenin can increase mechanical and thermal nociceptive thresholds and lowered pain scores in diabetic rats through lowering oxidative stress and inflammation and improving antioxidant defense system.[36] We can speculate that diosgenin can alleviate the pain caused by EM, in the same way, which needs deep and extensive clinical trials to prove. Of all the docking results, the binding energies of diosgenin act on MAPK14, SRC, and PIK3CA were -10.5 kal˙mol-1, -10.4kal˙mol-1, and -9.8kal˙mol-1 respectively. The first two have been shown to be associated with inflammatory disease and angiogenesis.[37, 38] PIK3CA is one of the most common mutation genes found in tumor types in recent years.[39] It is reported that mutations in this gene involve the occurrence of ovarian cancer in EM.[40] Hence, in addition to the above analysis, diosgenin may play a therapeutic role mainly through these three targets. In summary, we can see that diosgenin can affect EM in various aspects. Besides, quercetin is one of the important ingredients too, it has anti-proliferative and anti-inflammatory effects on endometriosis auto-implanted mouse models, this ingredient not only can induce ectopic endometrial atrophy[41] but also inhibited the proliferation and induced the cell cycle arrest in VK2/E6E7 and End1/E6E7 cells. It also can induce cell apoptosis by DNA fragmentation, reactive oxygen species production, and loss of mitochondrial membrane potential.[42] The main components selected in LCP also include kaempferol, which can decrease cell trauma rely on reducing the inflammatory mechanism caused by oxidative stress, and could have anti-inflammatory effects with inhibiting the expression of MAPK signaling pathway and inhibited some inflammatory mediators.[43] Isorhamnetin, too, has distinct anti-inflammatory properties,[44] it can inhibit the inflammation, proliferation, and migration of BEAS-2B cells by regulating the MAPK and NF-κB signaling pathways and is a drug candidate for asthma.[45] According to the above analysis. Active compounds of LCP may play a major role in the treatment of EM with the function of anti-inflammatory, anti-oxidative stress, anti-angiogenesis, smoothing of pain, and promoting ectopic tissue atrophy.

We screened out 10 major potential targets after Cytoscape3.8.0 analysis to unraveling the complex molecular relationships, emphasizing the regulatory role of LCP in endometriosis through this part of the target. RELA is one of the core targets selected by the PPI. Although there are many dimer forms of NF-κB, the main form is still p50and p65/RELA subunits, encoded by the NFKB1 and RELA genes.[46] NF-κB is a major transcription factor in apoptosis and cell growth processes that regulates the expression of genes and molecules, a study showed that inhibition of NF-κB/relA activity in ovarian cancer cells can suppress angiogenesis and progressive growth.[47] In endometriosis, stimulation of pro-inflammatory cytokines such as IL-6, Tumor necrosis factor α (TNFα), and IL-1β induce nuclear factor-κB (NF-κB) activation, which in turn, stimulates the production of a wide spectrum of pro-inflammatory cytokines, resulting in constant NF-κB activation and a cascade of downstream changes in the cells.[46] Furthermore,

the results of molecular docking showed that RELA had good binding activities to each active ingredient, it is not difficult to conjecture that LCP may achieve the purpose of anti-inflammatory by inhibiting the NF-κB signaling pathway. Epidermal growth factor (EGF) is immunolocalized in both glandular epithelia and stroma of endometriotic tissue. Compared to women without this disease, a marked increase in EGF is detected in women with endometriosis.[48, 49] EGF increased the expression of endometriosis-associated hyaluronan and its synthase HAS2, both of which mediated EGF-induced stromal cell migration and invasion in women with EM.[50] Dysregulation of Matrix metalloproteinases (MMPs) is related to endometriosis risk,[51] EGF receptor (EGFR) inhibitor treatments regressed endometriotic lesions, and decreased MMP-7 activities in a mouse model of endometriosis.[52] And the good molecular docking results suggested that the active components of LCP can inhibit the proliferation of ectopic tissue by depressing the activity of EGFR, to achieve the therapeutic effect. Another core target we have identified is FOS, FOS is a nuclear phosphoprotein, encoded by mature m RNA from gene transcription of c-fos.[53] EM is an estrogen-dependent gynecological disease,[54] C-fos gene expression presented a significant correlation with the circulating estradiol concentrations,[55] and there was an experiment further demonstrated that 17β-E2-induced human endometrial stromal cell (HESC) invasion is dependent on c-fos mediated MMP-9 expression.[56] Therefore, it is speculated that LCP can achieve the purpose of treatment by inhibiting c-FOS expression. Moreover, the ratio of ESR1 to ESR2 of ectopic lesions in patients with EM was lower than eutopic endometrium, excessive levels of ESR2 mediates an estrogen-driven inflammatory process and prostaglandin formation.[57] Our molecular docking results showed that each active ingredient had a degree of regulatory effect on the estrogen receptor 1 (ERS1) gene.

By exploring the results of KEGG enrichment, we conclude that the signaling pathway related to endometriosis involves cell proliferation and apoptosis, immune response, sex hormone regulation, angiogenesis and pain regulation, etc. A large number of genes were associated with PI3K/Akt signaling pathways (Figure 10). Research shows that this pathway is significantly expressed in endometriosis.[58] PI3Ks is involved in regulating different cellular functions including inflammation, auto-immune disorders, cell apoptosis, and cancer progression,[59-63] phosphoinositol-dependent protein kinase 1 (PDK1) can be activated by the phosphoinositide-3,4,5- triphosphate (PIP3) which generated from PI3K，those changes can activate the serine/threonine kinase Akt by phosphorylating its kinase domain,[64] a study shows that inhibition of the PI3K/Akt/mTOR signaling pathway attenuates the inflammatory response in rats with osteoarthritis.[65] Active Akt also resulting in activation of the NF-κB,[66] the central mediator of the inflammatory process and innate immunity.[67] Except in regulating inflammatory responses, this signaling pathway may play an important role in autophagy. The combination of Akt inhibitor and chloroquine can significantly reduce the growth and regeneration of endometriosis stromal cells by inducing autophagy,[68] certainly, the theoretical basis for this experiment is that PI3K/Akt signaling pathways play an important role in the disease, in fact, there is also surmise that the pathway is involved in progesterone antagonism.[58] Moreover, the inhibition of the PI3K/Akt/mTOR signaling pathway may relieve endometriosis-associated sciatic nerve pain in a rat model of sciatic endometriosis.

IL-6 is a major pro-inflammatory cytokine that is synthesized as a 26 kDa protein. In canonical IL-6 signaling, IL-6 first binds to the membrane-bound IL-6 receptor (mIL-6R) then gp130 dimerization results in the activation of tyrosine kinase Janus kinases 1,2 (JAK1,2).[69] The transcription factor, STAT3 is localized in the cytoplasm until activated by phosphorylation.[70, 71] STAT3 is one of the major signal transducers of JAKs and plays a pivotal role in regulating many cellular functions such as cell differentiation and proliferation.[72] Phosphorylated STAT3 is highly expressed in the endometrium of patients with endometriosis. Elevated pSTAT3 induces and stabilizes HIF1A within the eutopic endometrium of women with endometriosis.[73] HIF1A has previously been shown to up-regulate many of the aberrant

proteins and factors associated with endometriosis, including increased cell proliferation and viability,[74] aberrant estrogen receptor-beta (ERβ) expression,[75, 76] and increase vascular endothelial growth factor (VEGF).[77] A study proved silencing HIF1A (siRNA) suppressed the expression of hypoxia-induced VEGF in endometrial cells.[78]

It can be seen from the above analysis that the molecular mechanism of LCP in the treatment of EM is mainly focused on regulating the inflammatory response, and the latest research results also show that inflammation plays an important role in this disease.[79] In summary, through KEGG enrichment analysis, we sorted out the possible molecular mechanisms of LCP in the treatment of EM into four categories.1) Control inflammatory response. For example, this may reduce the reactivity of inflammatory factors by regulating TNF and HIF-1 signaling pathways and reduce adhesion, infiltration, growth, and shedding of ectopic focus. Also, ectopic tissue growth may be inhibited by regulating PD-L1expression and PD-1checkpoint pathway in cancer improving the immunity of the local tissue microenvironment.[80] 2) Regulated hormone secretion. It may regulate estrogen signaling pathways to control EM progression by regulating HIF-1signalingpathways.3) Inhibit cell proliferation and angiogenesis and growth. The progression of EM can be controlled by regulating VEGF signaling pathways or PI3K/Akt signaling pathways. 4) Regulation of neurotrophin signaling pathway relieve pelvic pain. In addition, by reading the literature, we found that the molecular biological characteristics of the eutopic endometrium play an important role in the development of EM. However, proteins post-translational modifications (PTMs) like include phosphorylation, acetylation, ubiquitination, and glycosylation, etc may be involved in the pathogenesis of EM, which leads to enhance the facility of adhesion, invasion, and angiogenesis in ectopic endometrium, making it more susceptible to get the disease.[81] The molecular biological characteristics of the eutopic endometrium play an important role in determining the biological behavior of the ectopic endometrium, which is the classic "eutopic endometrium determinism" proposed by Lang Jing-he.[82] It can categorize complex pathogenesis into monistic cognition. Based on this theory, the purpose of our research looks forward to finding key targets through network pharmacology and molecular docking will further promote the study of this disease.

Conclusion

We performed a network pharmacological analysis and molecular docking validation to discuss the therapeutic method of EM and explored the active components and mechanism of LCP. And the results fully show that the main active molecules in the treatment of EM and relief related symptoms in terms of anti-inflammation, promoting apoptosis, inhibiting angiogenesis, and relieving pain via the PI3K-Akt signaling pathway, HIF-1signaling pathway, VEGF signaling pathway, estrogen signaling pathway, neurotrophin signaling, and the regulation of related protein such as RELA、EGFR、FOS, etc. However, there are still some limitations that should be noted. Firstly, the target research beyond molecular docking verification is not clear, in the next place, there is a lack of further experimental verification. It is hoped that the following clinical trials and basic research can further verify the molecular mechanism of LCP.

Abbreviations

EM: Endometriosis; LCP: Li Chong Pill; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform

Declarations

Ethics approval and informed consent

Not applicable

Consent for publication

Not applicable.

Data Availability

The data used to support the result of this study can be obtained from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, Have agreed on the journal to which the article will be submitted, and agree to be accountable for all aspects of the work.

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Figures
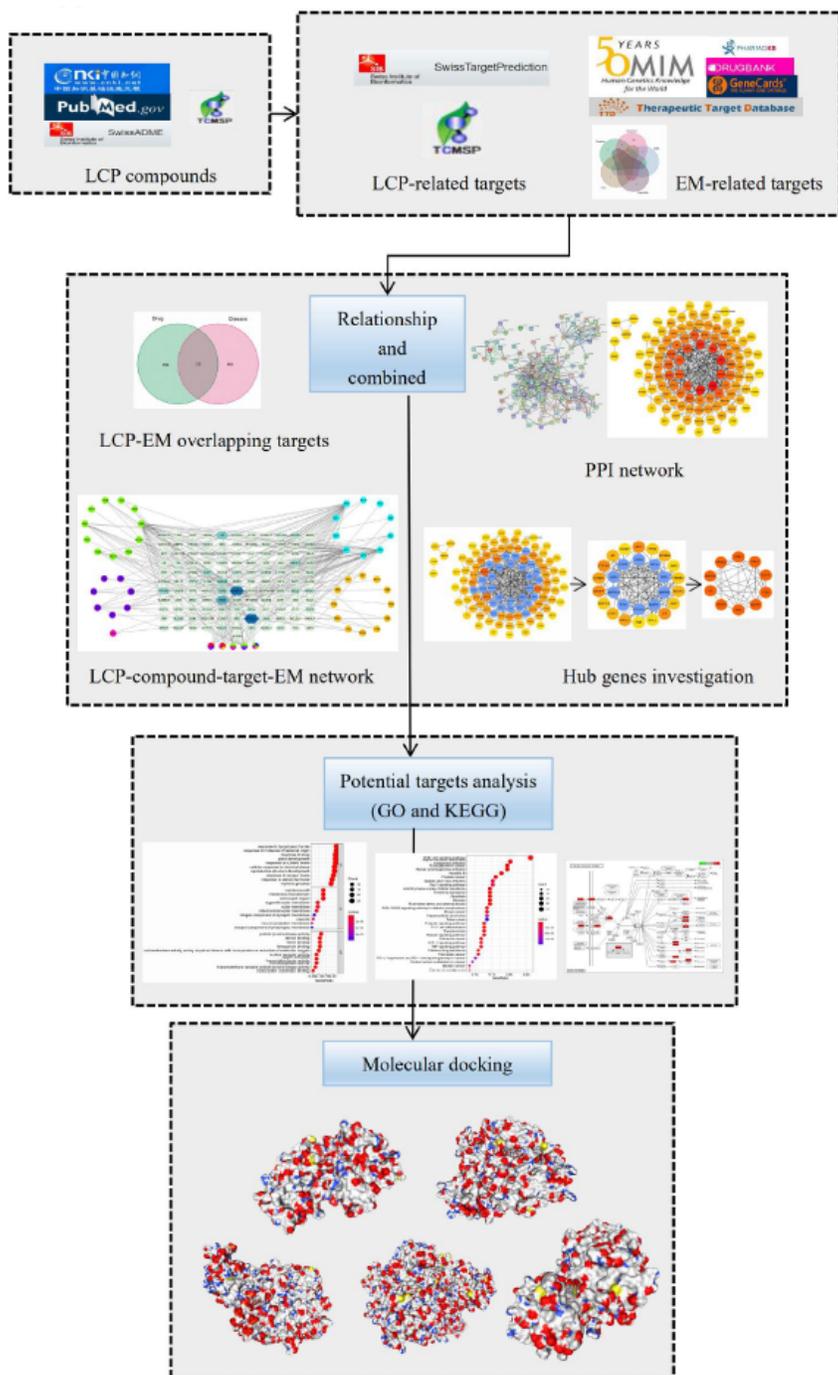
Figure 1

Technology route for exploring molecular mechanisms of LCP against EM based on network pharmacology and molecular docking
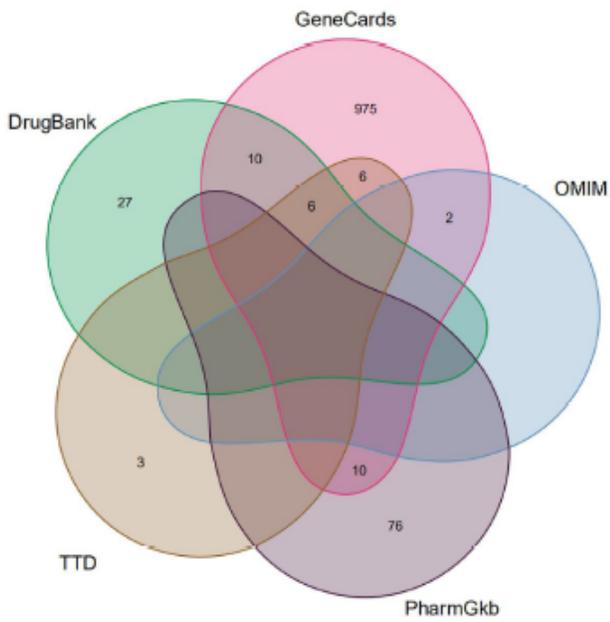
Figure 2

Venn diagram. Showing the EM-related targets of the overlapped and the specific genes among the five databases (OMIM, DrugBank, TTD, GeneCards, and PharmGKB).
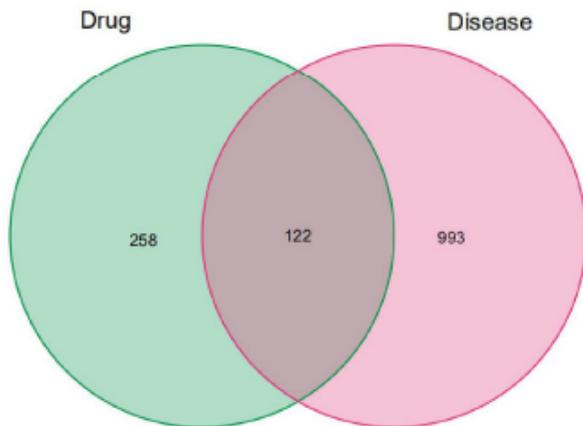
Figure 3

Venn diagram, 122 overlapping targets between LCP-related targets and EM-related targets.
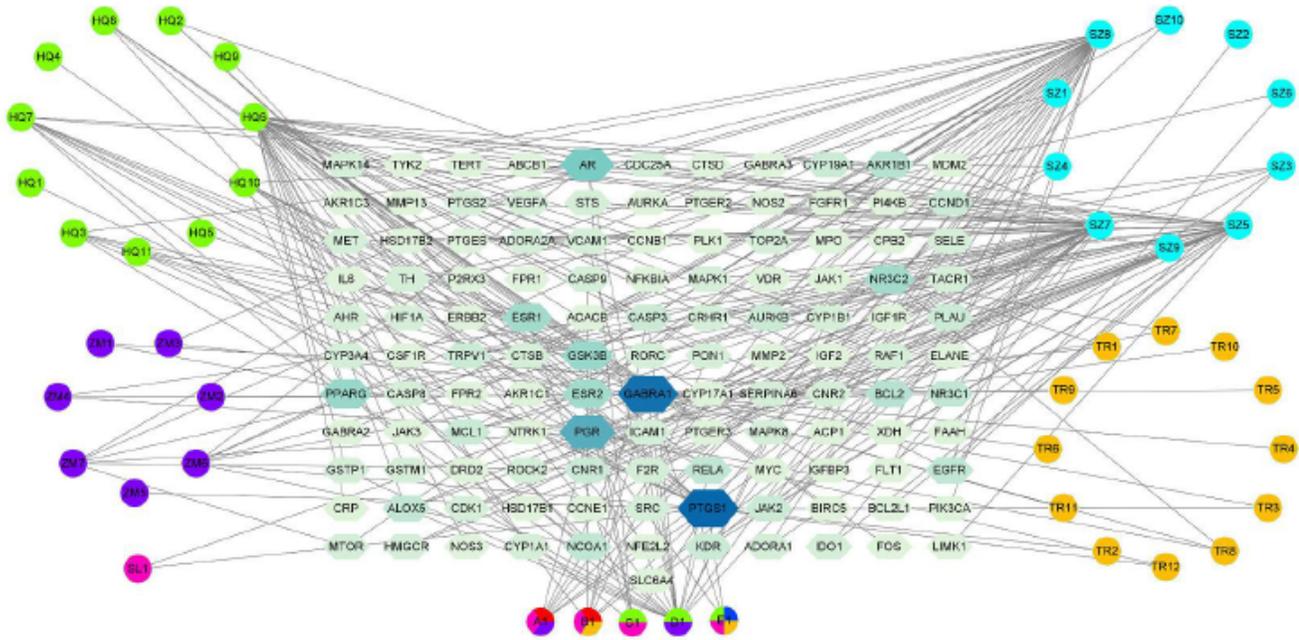
Figure 4

LCP-compound-target-EM Network (The blue pentagons represent 122 overlapping targets, different color means different herbs. Green represents Huangqi, blue represents Shuizhi, orange represents Taoren, red represents Danggui, pink represents Sanleng, indigo represents Zhimu). The edge between two nodes represented the interaction, the size and the gradation in color of each target node indicates the degree of connections.

Figure 5

PPI network diagram from STRING protein database.
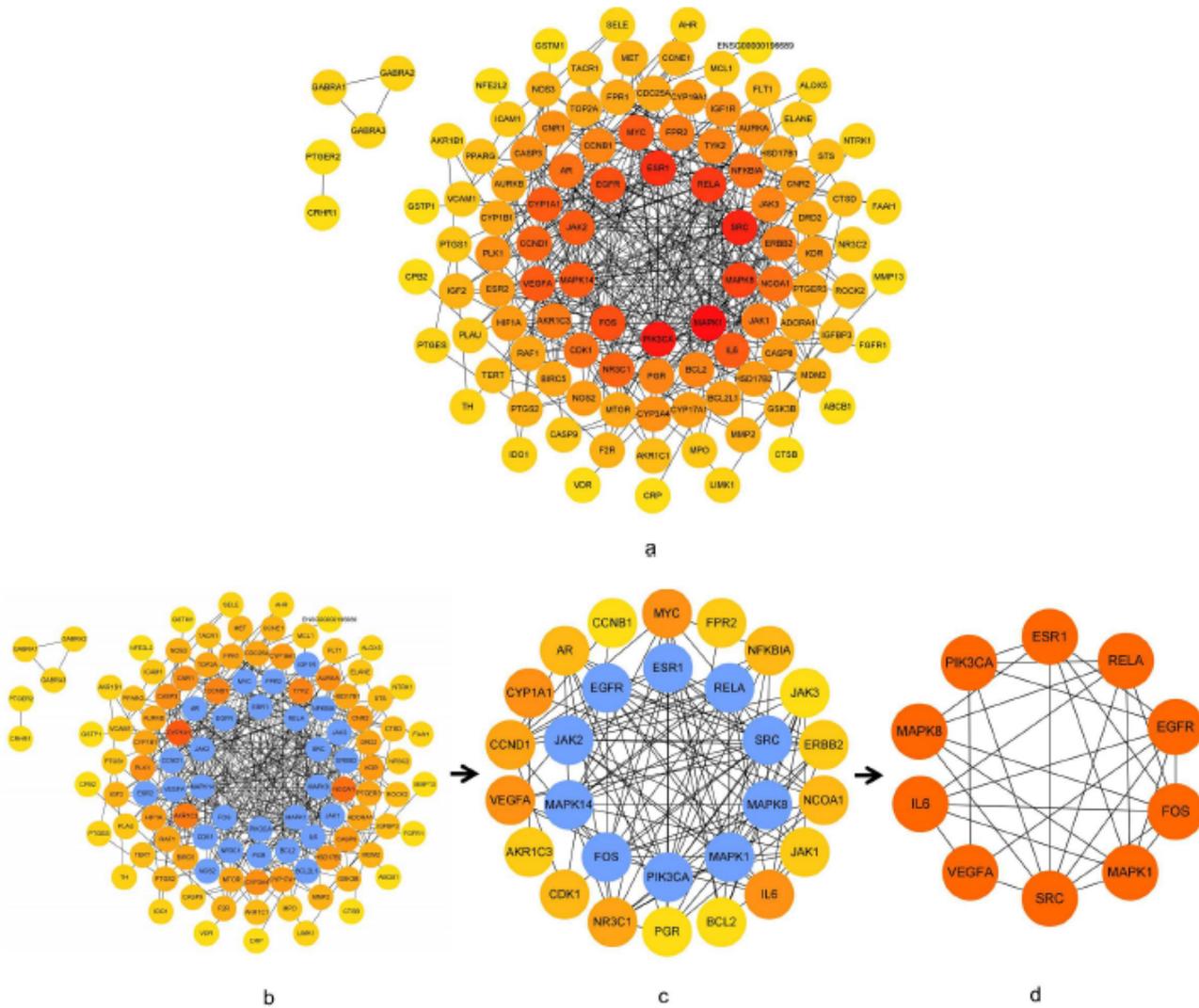
Figure 6

(a) PPI network related to potential targets. (b/c) Module analysis of the network for the identification of potential targets,the orange color indicates primary proteins, and the blue color indicates the next group proteins screened by betweeness, closeness, degree, eigenvector, LAC and network. (d) 10 highly connected nodes significant endometriosis-related targets.
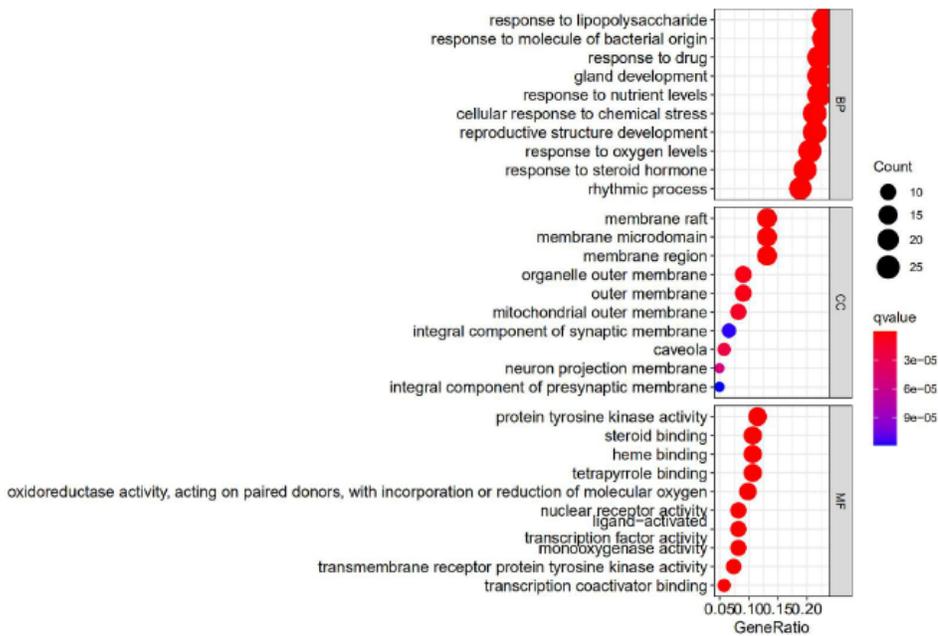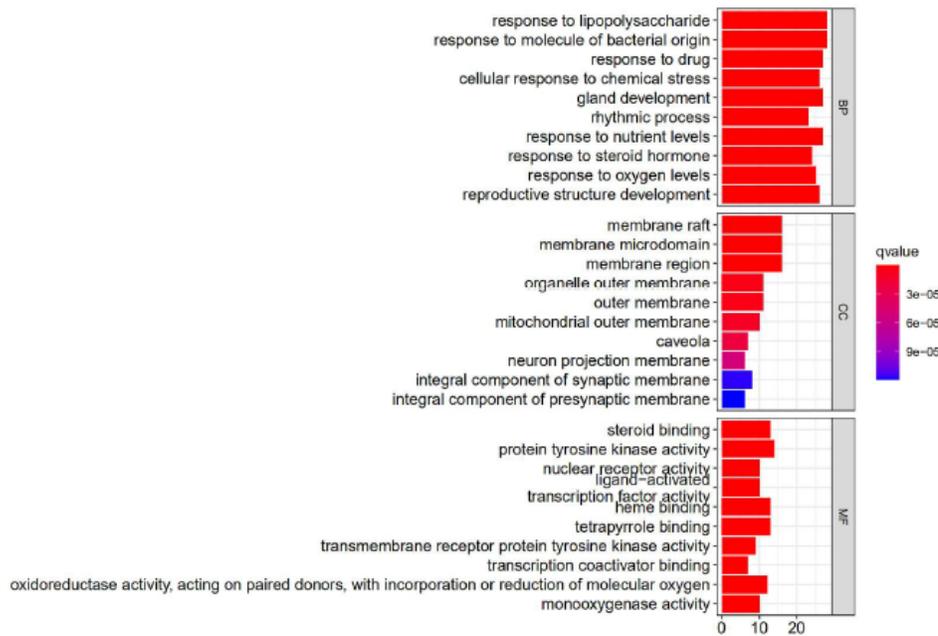
Figure 7

The results of Go (a/b) enrichment analysis.Picture (a) is bubble chart while the (b) is histogram. The abscissa and the circle size represent the enriched gene ratio, the gradation of color means enriched adjust P-value.
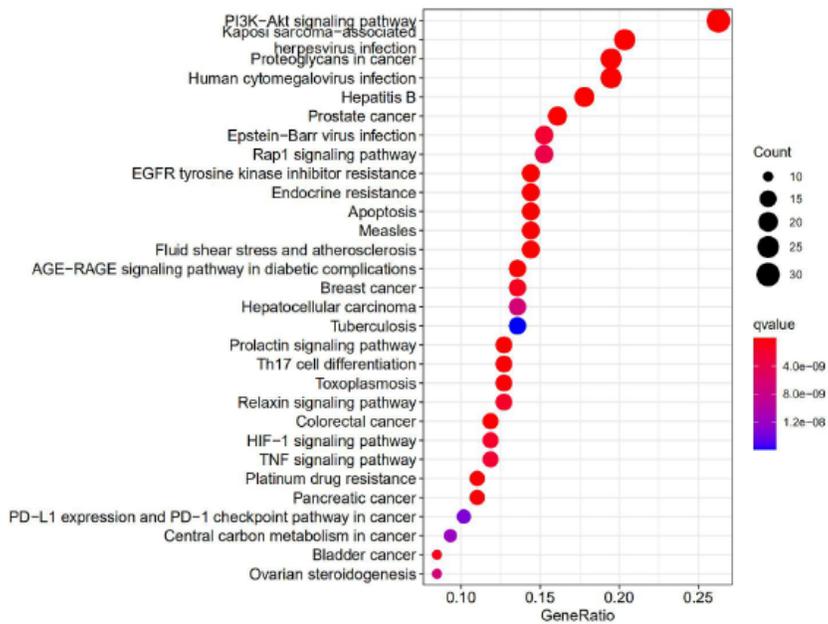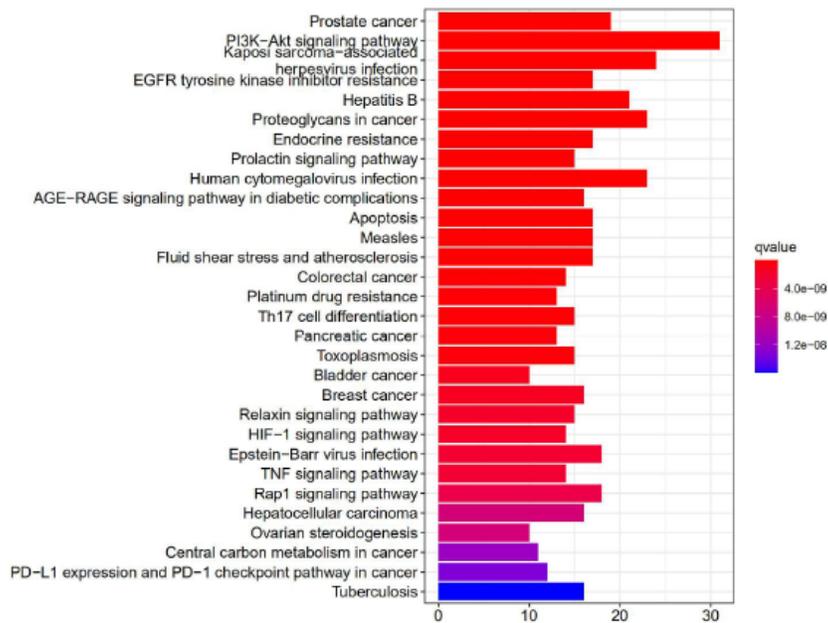
Figure 8

The results of KEGG (a/b) enrichment analysis.Picture (a) is bubble chart while the (b) is histogram. The abscissa and the circle size represent the enriched gene ratio, the gradation of color means enriched adjust P-value.
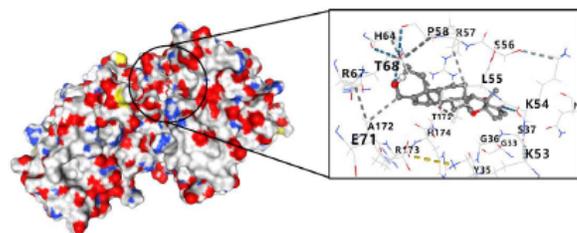
a. Diosgenin act on MAPK14
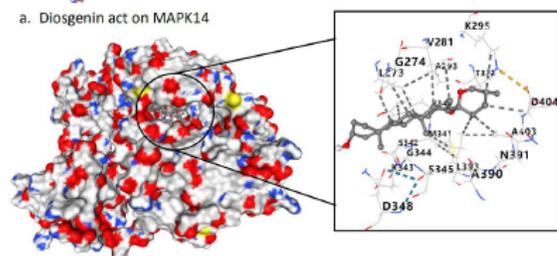
b. Diosgenin act on SRC
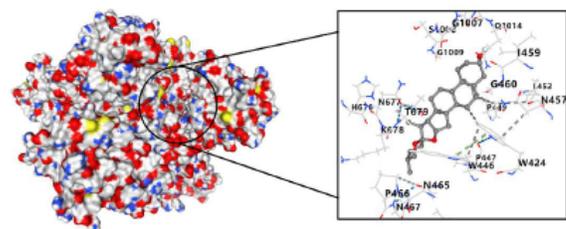
c. Diosgenin act on PIK3CA
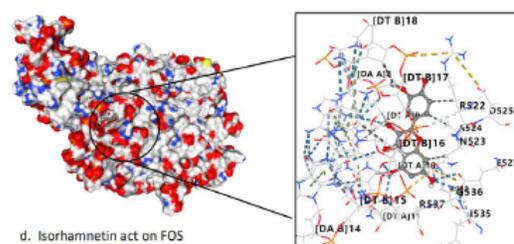
d. Isorhamnetin act on FOS
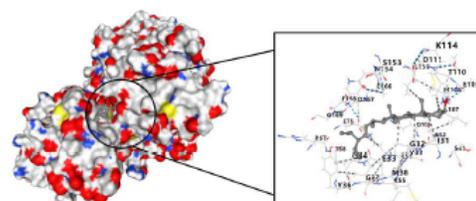
e. Isorhamnetin act on FOS

Figure 9

Detailed target-compound interactions with the five highest molecular docking affinity.

Figure 10

KEGG analysis pathway map (PI3K/Akt signaling pathway) of potential targets.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.xls
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- Additionalfile4.xls
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