Discussion on the Mechanism of Lithospermumerythrorhizon in the Treatment of Cervical Cancer Based on Network Pharmacology and Molecular Docking Technology

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Objective To explore the "multi-component, multi-target, multi-pathway" mechanism of *Lithospermum erythrorhizon*against cervical cancer.Methods The active ingredients and corresponding targets were screened through TCMSP, PubChem and SwissTargetPrediction databases. The GeneCarts platform was used to collect cervical cancer-related genes, and the intersection of drug targets and cervical cancer targets was analyzed. Use STRING to analyze protein interaction network, use Cytoscape software to construct component-target and core target interaction network, perform KEGG pathway enrichment analysis on core target genes, and conduct molecular docking verification.Results After screening, 12 main active ingredients of comfrey (including Shikonin A, 1-methoxyacetylshikonin, Shikonin B, etc.) and 35 key targets related to comfrey and cervical cancer were obtained (including ESR1, SRC, MMP9, PTGS2, etc.). And these genes were mainly enriched in 39 signaling pathways such as PI3K-Akt and estrogen. Molecular docking reminder that *Lithospermum* A has a higher affinity with ESR1, and *Lithospermum* B can form a stable conformation with SRC, MMP9, and PTGS2. Conclusion *Lithospermum erythrorhizon* is a potential drug candidate for the treatment of cervical cancer. It can treat cervical cancer through multi-component, multi-target, and multi-channel action.

Key words:*Lithospermum erythrorhizon*; Network pharmacology; Molecular docking technology; Cervical cancer; Mechanism

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Cervical cancer is one of the common gynecological malignancies. The current common treatment methods include surgery, radiotherapy, chemotherapy, etc., but they all have certain limitations [1]. *Lithospermum erythrorhizon* (hereinafter referred to as *Lithospermum*)is a commonly used traditional Chinese medicine. A number of studies have shown that *Lithospermum*, especially its active substance, shikonin, has anti-tumor effects, including inhibiting the growth of breast cancer and melanoma [2].For cervical cancer, studies have shown that shikonin can inhibit the proliferation of cervical cancer cells by regulating the cell cycle [3]. Other clinical studies have shown that the mechanism of shikonin is related to its ability to block the conduction of the EMT pathway [4]. This study used network pharmacology to analyze the main active components of *Lithospermum*, and explored the molecular mechanism of *Lithospermum* in the treatment of cervical cancer based on molecular docking technology.

MATERIALS AND METHODS

EffectiveIngredients of *Lithospermum*

Enter comfrey in TCMSP[5] (https://tcmspw.com/tcmsp.php) to search for the active compounds it contains, and use oral bioavailability ≥ 30%, drug likeness ≥0.18 and the absorption of cell monolayer model ≥-0.4 for the screening conditions to obtain the effective ingredients of*Lithospermum*.

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Arrangement of Targets for the Effective Ingredients of *Lithospermum*

Combining TCMSP, PubChem (https://pubchem.ncbi.nlm.nih.gov)nd SwissTargetPredictionhttp://www.swisstargetprediction.ch) database to obtain the active ingredient target. These targets were compared with the Uniprot(https://www.uniprot.org/) human genome database, and the corresponding gene names of the targets were obtained and integrated.

Construction the Effective Component Target Network

The active ingredient names and corresponding target genes were imported into Cytoscape to construct the effective ingredient-target network of *Lithospermum*. Then use Cytoscape's Network Analyzer plug-in to analyze the drawn network diagram, so as to obtain the specific targets of the active ingredients of *Lithospermum*.

Disease Target Prediction

Use the GeneCards(https://www.genecards.org) database to search for the keyword "cervical cancer" to collect cervical cancer-related genes, and use relavance score ≥10 as the screening criteria for cervical cancer-related genes.

Obtain Core Target Genes of Drug Action Disease

Use Venny 2.1(https://bioinfogp.cnb.csic.es/tools/venny/index.html) to map between cervical cancer-related genes and comfrey target genes, and create a Venn diagram to obtain comfrey for cervical cancer Core genes.

Construct a Protein Interaction Network of Key Targets

Introduce the core target gene of *Lithospermum* for the treatment of cervical cancer into STRING(https://string-db.org), select human(Homo sapiens) as the research species, and obtain the protein interaction network (PPI). Use Cytoscape software to visualize the network, export the core gene sub-network, and obtain the protein interaction network of the key target protein of the core gene.

KEGG Pathway Analysis

Use R software to analyze the KEGG pathway of the core target genes (set $P<0.05$), and get the main pathways involved in the target protein.

Molecular Docking

Download the 2D structure of the active ingredient and the 3D structure of the required protein through the PDB database(http://www1.rcsb.org/). Use Chem3D software to draw the component 3D structure and minimize the energy, save it as mol2 format, and use AutoDock tools software to convert mol2 format to pdbqt format. Dehydrate the protein and its related compounds by pymol, then use AutoDock Tools software to hydrogenate the protein and save it in pdbqt format. Use Vina for molecular docking, and finally visualize the docking file.

RESULT

Lithospermum Active Ingredients and Corresponding Targets

Through screening, 12 main active ingredients of comfrey (including Lithospermidin A, 1-methoxyacetylshikonin, and Lithospermidin B) were obtained, as shown in Table 1. Then, the main active ingredient targets were obtained from the database and gene annotation was performed, and a total of 1079 targets were obtained. After deleting invalid targets and duplicate targets, 144 targets (mainly including AR, ESR1, PGR and other gene targets) were obtained.

Table 1
Main active ingredients of *Lithospermidin*

ID	OB (%)	DL	Caco-2	Name
MOL-007728	75.08	0.38	-0.13	Lithospermidin A
MOL-007714	73.09	0.29	0.24	1-methoxyacetylshikonin
MOL-007722	64.79	0.2	0.06	Isoarnebin 4
MOL-007716	62.39	0.27	0.3	Acetylshikonin
MOL-007734	61.8	0.24	0.92	5-[(E)-5-(3-furyl)-2-methyl-pent-2-enyl]-2,3-dimethoxy-p-benzoquinone
MOL-007736	60.48	0.39	-0.16	Lithospermidin B

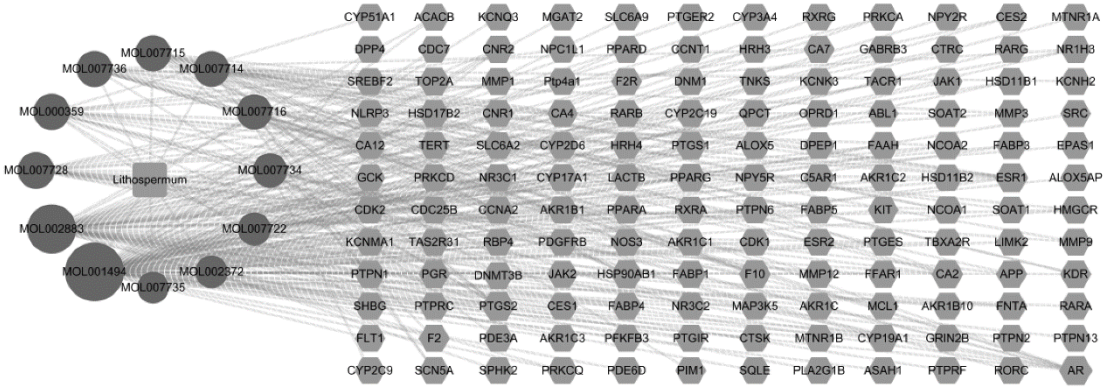
MOL-007715	54.64	0.29	0.25	[(1R)-1-(5,8-dihydroxy-1,4-dioxo-2-naphthyl)-4-methyl-pent-3-enyl] propanoate
MOL-001494	42	0.19	1.46	Mandenol
MOL-000359	36.91	0.75	1.32	Sitosterol
MOL-002372	33.55	0.42	2.07	(6Z,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene
MOL-002883	32.4	0.19	1.4	Ethyl oleate (NF)
MOL-007735	30.12	0.2	0.83	Des-O-methyllasiodiplodin

Note:OB: oral bioavailability, DL:drug likeness, Caco-2:cell monolayer model.

Construction and Analysis of Effective Ingredient-Target Network

Import the effective ingredients and key targets of comfrey into Cytoscape software to obtain a network diagram of "effective ingredients-targets", as shown in Figure 1. In the figure, the square on the left represents *Lithospermidin*, the circle represents the active ingredient contained in *Lithospermidin*, and the hexagonal area on the right represents the target of the active ingredient. The figure contains a total of 158 nodes, and 259 edges are formed between the nodes, showing the effect of comfrey on the treatment of cervical cancer with multiple components and multiple targets.

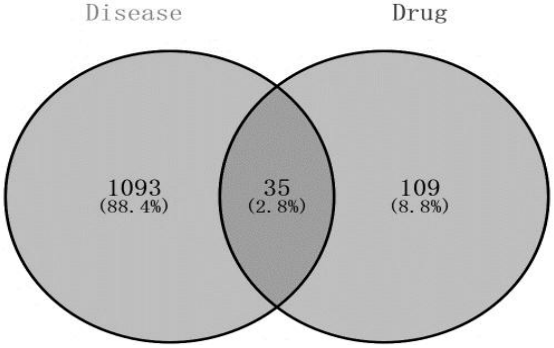
Figure 1
Network diagram of the target points of *Lithospermidin* active ingredients



Collection of Genes Related to the Treatment of Cervical Cancer by *Lithospermidin*

Searching for "cervical cancer" targets in the GeneCarts database, screening targets with a correlation score greater than 10, and finally screening out a total of 1128 cervical cancer-related targets. The active ingredient targets of *Lithospermidin* and cervical cancer disease targets were introduced into the Venny2.1 platform, and the intersection of *Lithospermidin* targets and cervical cancer-related targets was obtained, as shown in Figure 2. It can be seen from Figure 2 that there are 35 intersecting targets. These 35 targets are the key targets forcervical cancer by the active ingredients of *Lithospermidin* (including ESR1, TERT, KIT,etc.).

Figure 2
The intersection of *Lithospermidin* targets and cervical cancer related targets



PPI Network Drawing and Analysis

Use Cytoscape software to draw the total PPI network of comfrey treatment of cervical cancer interaction, as shown in Figure 3. Figure 3 includes 35 nodes and 175 edges. Select the top 20 targets in the ranks and define them as "core targets" (See Table 2 for the specific information of each target)

Figure 3
The interaction network of *Lithospermidin* on cervical cancer target protein

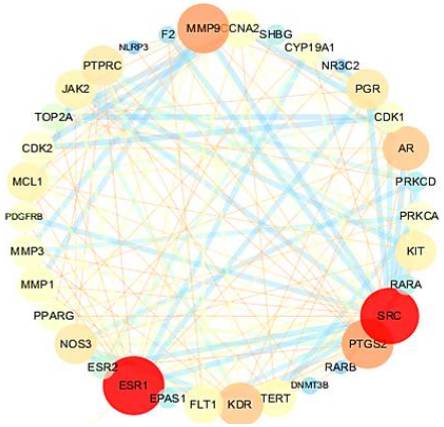


Table 2
"Core target" information

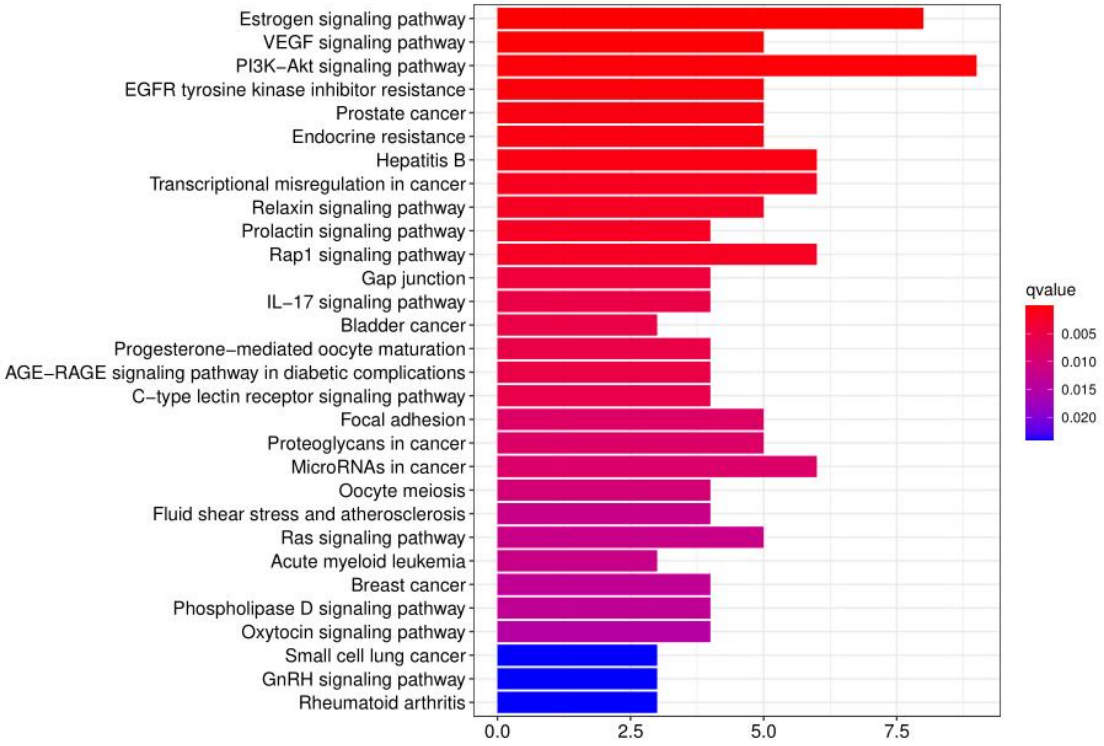
Sequence	Degree	Gene name	Protein name
1	27	ESR1	Estrogen receptor alpha
2	25	SRC	Tyrosine-protein kinase SRC
3	20	MMP9	Matrix metalloproteinase 9
4	20	PTGS2	Cyclooxygenase-2
5	16	AR	Androgen Receptor
6	16	KDR	Vascular endothelial growth factor receptor 2
7	13	NOS3	Nitric-oxide synthase, endothelial
8	13	PGR	Progesterone receptor
9	13	PTPRC	Leukocyte common antigen
10	12	JAK2	Tyrosine-protein kinase JAK2
11	12	KIT	Stem cell growth factor receptor
12	12	MCL1	Induced myeloid leukemia cell differentiation protein Mcl-1
13	11	FLT1	Vascular endothelial growth factor receptor 1
14	11	TERT	Telomerase reverse transcriptase
15	10	CCNA2	Cyclin-A2
16	10	MMP1	Matrix metalloproteinase 1
17	9	CDK2	Cell division protein kinase 2
18	9	MMP3	Matrix metalloproteinase 3
19	8	CDK1	Cyclin-dependent kinase 1
20	8	CYP19A1	Cytochrome P450 19A1

KEGG Pathway Analysis

KEGG pathway annotation analysis results show that the 35 potential targets of comfrey against cervical cancer are mainly related to 39 related information pathways. The reddish the rectangle in the figure, the greater the significance; the longer the rectangle, the greater the number of genes enriched in this pathway. The analysis of the top 20 enriched pathways revealed that the targets are mainly enriched in PI3K-Akt, estrogen, VEGF and EGFR tyrosine kinase inhibitor resistance pathways, as shown in Figure 4. The PI3K-Akt pathway contains key genes: KIT, FLT1, PDGFRB, CDK2, MCL1, KDR, JAK2, PRKCA, NOS3. The Estrogen pathway contains key genes: ESR1, SRC, RARA, MMP9, ESR2, PGR, PRKCD, NOS3. The VEGF pathway contains key genes: SRC, PTGS2,

KDR, PRKCA, and NOS3.

Figure 4
KEGG Pathway Analysis



Results of Molecular Docking

The five active ingredients (*Lithospermidin* A, *Lithospermidin* B, mandenol, ethyl oleate, and sitosterol) with the highest degree value in the "drug component-target" analysis were selected to be associated with the four core targets related to cervical cancer (ESR1, SRC, MMP9, and PTGS2) for molecular docking, the binding energy of docking is shown in Table 3. It can be seen that *Lithospermidin* A can stably bind to ESR1, and *Lithospermidin* B can stably bind to SRC, MMP9 and PTGS2.

Table 3
The docking situation of the main active components of *Lithospermidin* and related targets

Compound	CAS number	Relative molecular weight	Binding energy/(kJ/mol)			
			ESR 1	SRC	MMP 9	PTGS2
Mandenol	544-35-4	308.56	-4.1	-4.2	-6.6	-5.8
Ethyl oleate (NF)	111-62-6	310.58	-4.6	-3.2	-6.5	-5.6
Lithospermidin A	83415-78-5	388.45	-9.0	-6.2	-7.4	-7.8
Sitosterol	83-46-5	414.79	-7.0	-6.1	-8.4	-6.3
Lithospermidin B	---	388.45	-6.7	-6.4	-8.7	-8.1

DISCUSSION

Cervical cancer is one of the cancers that have long plagued women's physical and mental health. At present, a consensus has been reached on the multidisciplinary treatment of cervical cancer. In clinical practice, a combination of multiple drugs is often used, including radiotherapy and chemotherapy drugs and Chinese herbal medicines. *Lithospermidin* contains many active substances such as naphthoquinones, monoterpene phenols and benzoquinones, which have anti-inflammatory, anti-viral, anti-bacterial, anti-tumor and immune-regulating effects. Shikonin, the active ingredient of *Lithospermidin*, has been shown to

inhibit the growth of breast cancer cells [2].

This study found that the active ingredients of *Lithospermidin* and cervical cancerrelated genes have 35 common targets, including ESR1, SRC, MMP9, PTGS2 and so on. Among them, ESR1 can mediate the invasion and metastasis of cervical cancer, and inhibiting the expression of ESR1 will increase the invasiveness of cervical cancer cells [6]. The activity of Src family kinases in cervical cancer cells is increased, and is related to the high aggressiveness of tumors [7].In addition, studies have shown that a variety of proteins regulate the proliferation, invasion and metastasis of cervical cancer cells by mediating Src tyrosine phosphorylation, and treatment with Src kinase inhibitors can block their cancer-promoting effects to a certain extent [8]. Clinical studies have shown that the expression of MMP9 protein in patients with early cervical cancer is significantly related to the metastasis of pelvic lymph nodes and para-aortic lymph nodes [9]. PTGS2 encodes prostaglandin-endoperoxidase 2, which is a necessary enzyme in the biosynthesis of prostaglandin, and has the dual functions of dioxygenase and peroxidase. Studies have shown that it may be a predictor of poor prognosis and resistance to radiotherapy and chemotherapy in patients with cervical cancer [10].

Enrichment analysis of KEGG metabolic pathway indicated that estrogen, PI3K-Akt and VEGF pathway may be the main pathways for comfrey. Estrogen mainly acts by binding to the estrogen receptor. After estrogen binds to the estrogen receptor or G protein-coupled receptor on the plasma membrane of the cell, it activates the PI3K/Akt signaling pathway [11]. PI3K/Akt pathway accelerate the occurrence and development of tumors, and mainly regulates the biological processes of tumor cell proliferation, apoptosis, and migration [12]. VEGF is the most important tissue factor that promotes vascular cell differentiation and angiogenesis. It is closely related to the density of blood vessels in tumors, invasiveness, and patient prognosis [13]. At present, a variety of inhibitors against VEGF and its ligands have been developed, such as bevacizumab, ramucirumab, sorafenib, etc., and have been clinically applied to the treatment of various solid tumors including cervical cancer [14]. The above results indicate that *Lithospermidin* may inhibit the proliferation of cervical cancer cells and promote their apoptosis by regulating PI3K/Akt, VEGF and other signal pathways. The important active components of *Lithospermidin* are molecularly docked with the key target proteins of cervical cancer, and the results indicate that the active components can be stably docked with multiple targets.

In summary, this study explored the possible mechanism of *Lithospermidin* in the treatment of cervical cancer, and found 12 main active ingredients of comfrey. These active ingredients participate in the regulation of multiple related pathways, forming a multi-component, multi-target, and multi-channel synergistic network, thereby directly or indirectly exerting its anti-cervical cancer effect. The research results provide a theoretical basis for in-depth elucidation of the effective substances and mechanism of action of *Lithospermidin* against cervical cancer.

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