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Original Research Article

Elucidation of the mechanism of action of the anti-cholecystitis effect of the Tibetan medicine "Dida" using network pharmacology

Chuan Liu[1], Fang-Fang Fan[1], Xuan-Hao Li[1], Wen-Xiang Wang[1], Ya Tu[2], Yi Zhang[1]*

[1]Ethnic Medicine Academic Heritage Innovation Research Center, Ethnic Medicine College, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, [2]Development Research Center of Traditional Chinese Medicine, China Academy of Traditional Chinese Medicine, Beijing 100700, China

For correspondence: **Email: zhangyi@cdutcm.edu.cn; **Tel:** (028)61932600*

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Abstract

Purpose: *To study the mechanism involved in the anti-cholecystitis effect the Tibetan medicine "Dida", using network pharmacology-integrated molecular docking simulations*
Methods: *In this investigation, the bioactive compounds of Dida were collected, network pharmacology methods to predict their targets, and networks were constructed through GO and KEGG pathway analyses. The potential binding between the bioactive compounds and the targets were demonstrated using molecular docking simulations.*
Results: *A total of 12 bioactive compounds and 50 key targets of Dida were identified. Two networks, namely, protein–protein interaction (PPI) network of cholecystitis targets, and compound–target–pathway network, were established. Network analysis showed that 10 targets (GAPDH, AKT1, CASP3, EGFR, TNF, MAPK3, MAPK1, HSP90AA1, STAT3, and BCL2L1) may be the therapeutic targets of Dida in cholecystitis. Analysis of the KEGG pathway indicated that the anti-cholecystitis effect of Dida may its regulation of a few crucial pathways, such as apoptosis, as well as toll-like receptor, T cell receptor, NOD-like receptor, and MAPK signaling pathways. Furthermore, molecular docking simulation revealed that CASP3, CAPDH, HSP90AA1, MAPK3, MAPK1, and STAT3 had well-characterized interactions with the corresponding compounds.*
Conclusion: *The mechanism underlying the anti-cholecystitis effect of Dida has been successfully predicted and verified using a combination of network pharmacology and molecular docking simulation. This provides a firm basis for the experimental verification of the use of Dida in the treatment of cholecystitis, and enhances its rational application in clinical practice.*

Keywords: *Tibetan medicine, Dida, Cholecystitis, Mechanism, Network pharmacology, Molecular docking simulation*

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INTRODUCTION

Cholecystitis, a common disease of the digestive system, often causes severe inflammation of the gallbladder and serious health problems [1]. Based on clinical manifestation and clinical course, cholecystitis may be acute or chronic, with cholelithiasis as one of the most common

etiologies [2]. Current treatment strategies for cholecystitis include supportive care (intravenous hydration, pain control, antibiotic administration, and correction of electrolytes); gallbladder drainage, cholecystectomy, and cholecystostomy [3]. The commonly used treatment strategies are antibiotics and cholecystectomy. When antibiotics are used for therapy, pain usually recurs and persists, and infection or sepsis is likely to occur because the gallbladder is not drained [4].

Similarly, cholecystectomy poses many risks, including organ infection, bile duct injury, and increased mortality [5].In the traditional Tibetan medical theory, cholecystitis belongs to the category of *ChiBa*, and its pathogenesis is caused by disorders in three roots and seven elements in the body and thermal evil attacking the gallbladder [6]. Traditional Tibetan medicine (TTM), known for the law of compound compatibility in treatments based on syndrome differentiation, plays a positive role in the prevention, treatment and rehabilitation of cholecystitis in China, due to its synergistic effect and minimal side effects [7].

The Tibetan medicine "*Dida*" is an original multi-base medicinal material that is often used to treat cholecystitis with a single medicine or compound prescription [8]. Drug use in major Tibetan hospitals has revealed that *Swertia chirayita* (Roxb. ex Flem.) Karst. is the most commonly used basic variety of *Dida* [9]. *Dida* contains a variety of chemical components, primarily xanthones, iridoid glycosides, flavonoids, and triterpenoids. It also has a series of pharmacological effects, such as anti-inflammatory, bacteriostatic, anti-tumor, anti-lipid, anti-liver injury, anti-oxidation, hypoglycemic and anti-mutation [10]. Although TTM has demonstrated the therapeutic effects of *Dida* on cholecystitis, the underlying molecular mechanism has not been fully elucidated.

Traditional Tibetan medicine (TTM), a multi-component, multi-target, multi-pathway treatment, achieves its unique therapeutic effect by acting on the molecular biological network of the human system [11]. Thus, it is difficult to unravel the mechanism of action of TTM. However, the novel technique of network pharmacology has contributed exciting solutions and unique ideas for enhancing the scientific interpretation of the mechanisms involved in the actions of drugs. Network pharmacology integrates systems biology, molecular dynamics and other multidisciplinary techniques to construct and integrate a multi-layer network of "disease - phenotype - gene - drug", which effectively illustrates the interaction between disease and drug, and it has been widely used in research on the mechanism of Tibetan medicine [12]. Molecular docking is a method of matching a ligand (drug) to a target molecule (receptor) by producing various components in different directions. The binding capacity between the ligand and the receptor is expressed as docking score, and a large negative docking score equates to a good direction of fit [13].

In this study, the molecular mechanism underlying the use of the Tibetan medicine *Dida* in the treatment of cholecystitis was analyzed and predicted via the combination of network pharmacology method and molecular docking simulation. The flowchart details are shown in Figure 1.

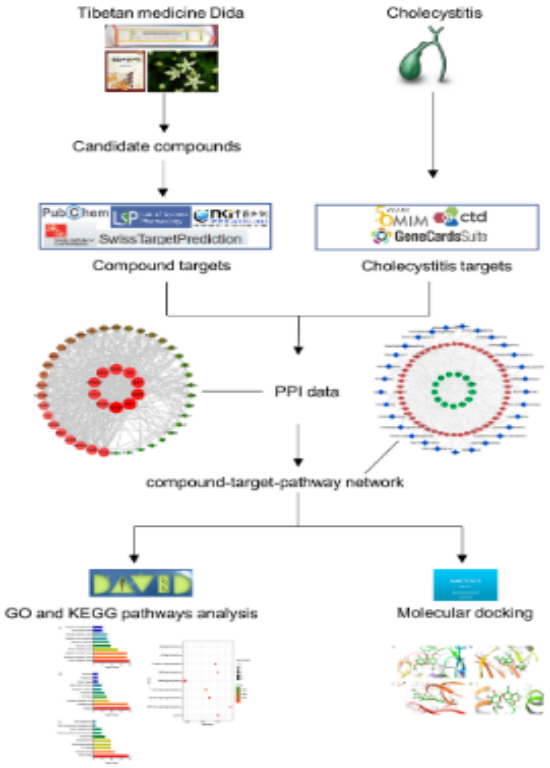

Figure 1: Flowchart for cholecystitis treatment with *Dida*

EXPERIMENTAL

Construction of database of candidate compounds

Swertia chirayita (SC) is a representative species of *Dida*. Compound data information on SC was established by screening the Traditional Chinese Medicine Systems Pharmacology Database (http://lsp.nwu.edu.cn/tcmsp.php), the TCM

Database@Taiwan (http://tcm.cmu.edu.tw/zh-tw/), and related literature [21]. When acquiring the main potential compounds, the following screening criteria were used: oral bioavailability (OB) ≥30 %, and drug-likeness (DL) ≥0.18. On the basis of the absorption, distribution, metabolism, and excretion (ADME) parameters in TCMSP and cholecystitis values obtained from previous work [9], the chemical components that met these criteria were selected as candidate compounds for further analyses.

Target prediction for bioactive compounds

The candidate compounds in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) that were converted into the standard canonical SMILES format and imported from the Swiss Target Prediction (http://www.swisstargetprediction.ch/) platform were set to "*Homo sapiens*" to predict the potential target active compounds.

Construction of database of cholecystitis targets

Cholecystitis-related targets were obtained from the Comparative Toxico genomics Database (http://ctdbase.org/), GeneCards Human Gene Database (https://www.genecards.org/), and Online Mendelian Inheritance in Man (OMIM, http://www.omim.org/) using the search keyword "cholecystitis".

Protein-protein interaction data

Protein-Protein Interaction (PPI) data were extracted on the STRING database (https://string-db.org/). STRING defines a reasonable confidence range for PPI data scores (low confidence: <0.4, medium confidence: 0.4 to 0.7, and high confidence: >0.7). After the intersection of disease target and drug target was noted, cholecystitis-related targets were entered into the STRING database (the confidence score was set above 0.7) for PPI prediction. The visualization of the PPI networks was conducted on Cytoscape 3.7.0 software.

Gene ontology (GO) and pathway analyses

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses provide gene expression data and overall system visual information of core pivot genes. In the screening of potential common targets for this study, GO and KEGG pathway analyses were conducted so as to predict the mechanism of action of *Dida* in treating cholecystitis using the DAVID v6.8.

Network construction

Two networks were constructed using Cytoscape 3.7.0 software. A PPI network cholecystitis targets was constructed by connecting and analyzing the connectivity, proximity, and centrality between cholecystitis-related targets and the human proteins. A compound–target–pathway network was created by linking compounds, corresponding targets, and pathways. In a complex associative network, nodes and edges express the direction and magnitude of compounds/entities and their cross forces, respectively. A high degree value equates to a prominent node status.

Molecular docking simulation

Molecular docking simulation was performed on the selected targets and corresponding compounds using Maestro version 11.5 from the Schrodinger software suite. The lowest/minimum energy conformation was used for molecular docking via the default parameters. Docking score is the absolute value of the experimental dissociation, relative to the inhibition constant (pKd/pKi), usually ranging from 0 to 10 (low to high combination force) [13].

RESULTS

Identification of the bioactive compounds

Ten potential compounds (OB ≥ 30 %, DL ≥ 0.18) in *Dida* were identified after ADME screening. In addition to these 10 compounds, 2 compounds (i.e., gentiopicroside and swertiamarin) with low OB were classified as bioactive components and they have been shown to be effective in the treatment of cholecystitis through extensive experiments. [14]. In total, 12 active components from *Dida* were selected for further analysis. The relevant data are shown in Table 1 and Figure 2.

Targets identification and PPI network

The determination of the key targets and the analysis of PPI network involves the following three steps: In the first step, 463 potential targets were obtained through the prediction of 12 active compounds using the Swiss Target Prediction platform; Secondly, 387 cholecystitis-related targets were retrieved from GeneCards, CTD, and OMIM databases; Finally, 50 targets closely related to the prevention and treatment of cholecystitis were obtained by accurately

mapping the potential targets obtained in the previous two steps (Table 2).

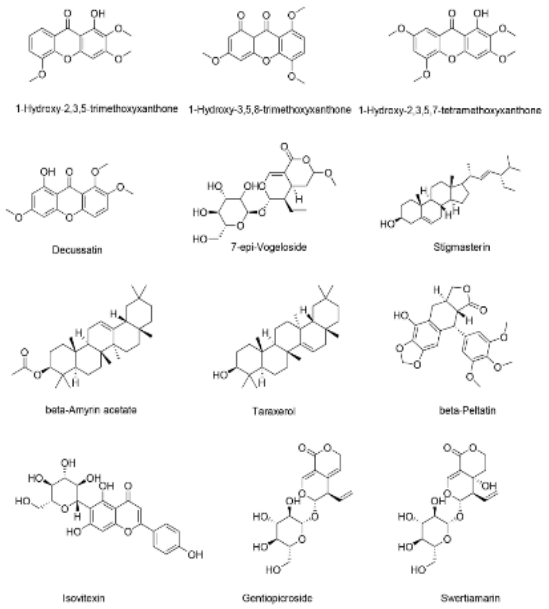

Figure 2: Structures of 12 active compounds

PPI network of target genes

The PPI relationship of 50 target genes was accurately analyzed with STRING big data tool. At the same time, the PPI relational network graph established with Cytoscape 3.7.0 contained 48 nodes and 866 edges, when the comprehensive score was set to >0.4. The nodes in the network represented the targets, the edges represented the relationship between the targets, and the free degrees indicated the target screening criteria (Figure 3). Based on the degree of freedom ranking, 10 target genes with the highest degree connectivity were identified as the hub targets, which might play a crucial role in cholecystitis progression were GAPDH, AKT1, CASP3, EGFR, TNF, MAPK3, MAPK1, HSP90AA1, STAT3, and BCL2L1.

GO and KEGG pathway analyses

To investigate the potential molecular mechanisms involved in the anti-cholecystitis effect of *Dida*, DAVID database was used for GO and KEGG enrichment analysis of 50 candidate targets. The GO evaluation results were illustrated using biological process (BP), cell component (CC), and molecular function (MF) terms. A preliminary GO analysis revealed the top 10 enriched conditions in the BP (e.g., the primary metabolic process, regulation of biological process, regulation of cellular process, and cell differentiation), CC (e.g., organelle, intracellular organelle lumen, and nucleoplasm), and MF (e.g., protein binding, nucleotide binding, and hydrolase activity) categories (Figure 4). Subsequently, a total of 29 pathways were obtained ($p < 0.05$) and 9 major pathways associated with cholecystitis were identified (Figure 5). Finally, through the review of relevant literature, it was found that apoptosis, NOD-like receptor signaling pathway, toll-like receptor signaling pathway, T cell receptor signaling pathway, MAPK signaling pathway and other signaling pathways may interact with each other and result in an anti-cholecystitis effect.

Table 1: Bioactive compounds of Tibetan medicine "*Dida*"

Code	PubChem CID	Compound	Molecular formula	OB (%)	DL
H1	5318372	1-hydroxy-2,3,5-trimethoxyxanthone	C16H14O6	101.06	0.3
H2	5378285	1-hydroxy-3,5,8-trimethoxyxanthone	C16H14O6	99.75	0.3
H3	85544105	1-hydroxy-2,3,5,7-tetramethoxyxanthone	C17H16O7	97.52	0.37
H4	5378284	decussatin	C16H14O6	77.13	0.3
H5	275299001	7-epi-vogeloside	C17H24O10	46.13	0.58
H6	5280794	stigmasterin	C29H48O	43.83	0.76
H7	92156	beta-amyrin acetate	C32H52O2	42.06	0.74
H8	92097	taraxerol	C30H50O	38.4	0.77
H9	92122	beta-peltatin	C22H22O8	36.91	0.75
H10	162350	isovitexin	C21H20O10	31.29	0.72
H11	88708	gentiopicroside	C16H20O9	22.98	0.39
H12	442435	swertiamarin	C16H22O10	21.9	0.42

OB represents oral bioavailability; DL represents drug-likeness

Table 2: Target information on Tibetan medicine "Dida" in the treatment of cholecystitis

S/no.	Gene name	PDB ID	S/no..	Name	PDB ID
1	GAPDH	2VYN	26	CHEK1	2HOG
2	AKT1	4EKL	27	HSPA5	3FE1
3	CASP3	3GJQ	28	MMP1	3SHI
4	EGFR	3W2S	29	ABCB1	6GDI
5	TNF	1TNR	30	BCL2	5JSN
6	MAPK3	2ZOQ	31	NOS2	4NOS
7	MAPK1	3O71	32	ELANE	1C6W
8	HSP90AA1	3O0I	33	NQO1	2F1O
9	STAT3	3CWG	34	MMP14	5OM3
10	BCL2L1	3PL7	35	PLK1	2RKU
11	MMP9	5TH6	36	HK1	5BWJ
12	PTGS2	5F19	37	G6PD	6E07
13	CASP8	2LR8	38	CASP6	3P45
14	MAPK14	5ETI	39	CFTR	1Q3H
15	MCL1	5LOF	40	CYP2C9	1OG2
16	MDM2	4JRG	41	GUSB	3USB
17	IL2	1T1B	42	ABCC1	4C3Z
18	PARP1	5XSR	43	HK2	2GRY
19	MAP2K1	3DV3	44	FGFR1	4UWY
20	VCAM1	5OEO	45	PREP	2FGE
21	CDK2	1PYE	46	TYMP	1OO9
22	MMP3	4G9L	47	EPHX1	3KKA
23	F2	2KXD	48	CES2	3K9B
24	CTSB	3AI8	49	SOAT1	2KA6
25	HSPA8	4FL9	50	PI4KB	2N73

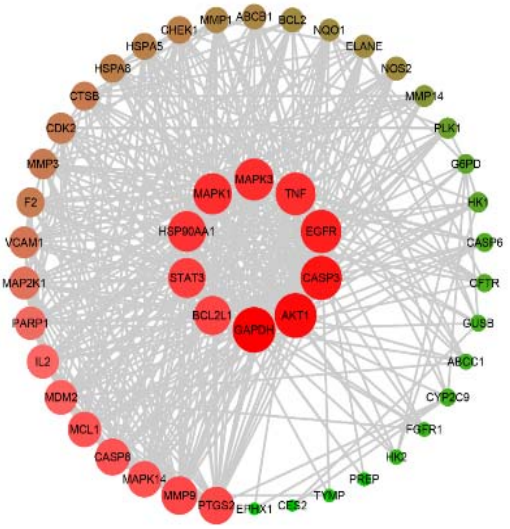

Figure 3: PPI networks of *Dida* for the treatment of cholecystitis. Each node represents the relevant gene, size and the color of the node represent the value of the free degree. The node size is proportional to the target degree in the network

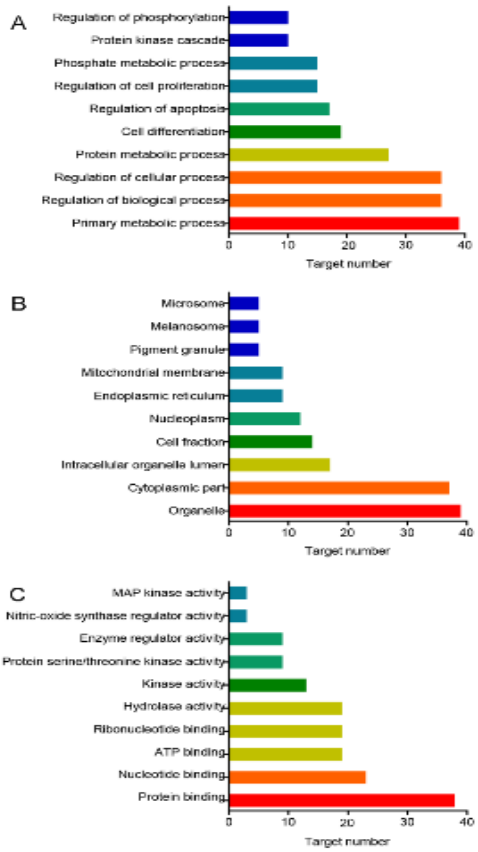

Figure 4: Results of Gene Ontology enrichment analysis. A: biological process (BP). B: cell component (CC) and C: molecular function (MF); $p < 0.05$

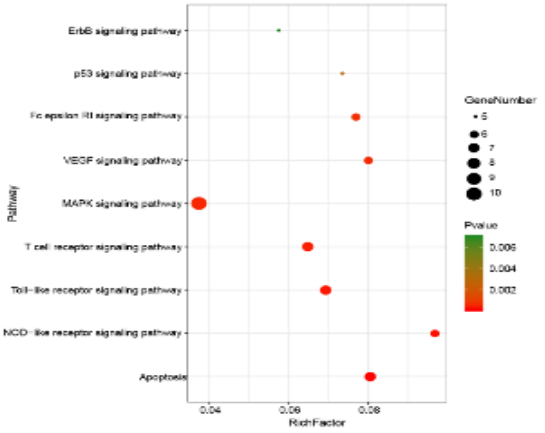

Figure 5: KEGG pathway enrichment analysis for the 50 targets. The y-axis reveals significantly enriched KEGG pathways of the target genes, and the x-axis demonstrates the rich factor (FDR < 0.01). The size of the dot represents the level of rich factor. Rich factor stands for the ratio of the number of target genes belonging to a pathway to the number of all the annotated genes located in the pathway

Network construction and analysis

A compound-target-pathway Network of *Dida* for the treatment of cholecystitis was constructed (Figure 6). The network was composed of 91 nodes and 728 edges, showing the correlation and intersection among 12 chemical components, 50 targets and 29 pathways. The mechanism underlying the anti-cholecystitis effect of Tibetan medicine *Dida* was initially revealed through the analysis of the network.

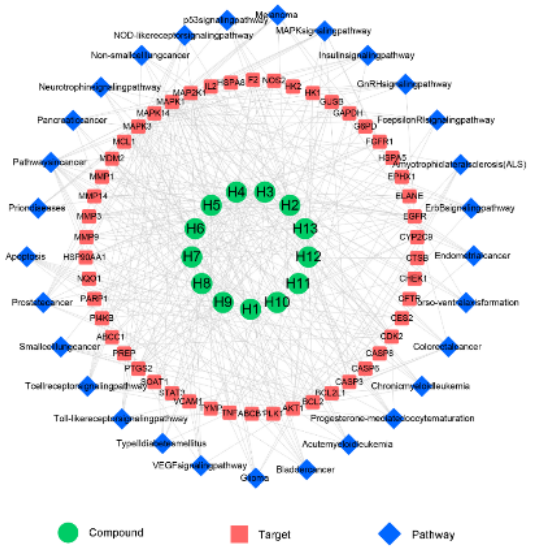

Figure 6: Compound-target-pathway Network (C-T-P). The network consisted of 91 nodes and 728 edges, including 12 active compounds, 50 potential protein targets, and 29 pathway interactions. The corresponding relationship is as follows: Green circles correspond to compound; Red rectangles correspond to potential target; Blue diamonds correspond to pathway

Molecular docking analysis

The three-dimensional structures of 10 core targets related to cholecystitis were obtained from the PDB database. The 10 targets and 12 compounds were inputted into Maestro11.5 (Schrodinger Suites) for molecular docking verification. The top 20 docking scores obtained from the combination of six targets (GAPDH, GASP3, MAPK3, MAPK1, HSP90AA1, and STAT3) and seven compounds (1-hydroxy-2,3,5-trimethoxyxanthone, 1-hydroxy-3,5,8-trimethoxy-xanthone, 1-hydroxy-2,3,5,7-tetramethoxy-xanthone, decussatin, 7-epi-vogeloside, isovitexin, and swertiamarin) were higher than 5.5, indicating that these targets and compounds had excellent binding capabilities Finally, the detailed molecular docking scores of the target-compound are shown in Table 3 and Figure 7.

Table 3: Docking information of 6 targets with corresponding compounds

Number	Target	Compound	Docking score
1	MAPK3	H1	6.487
		H2	6.503
		H3	5.821
		H4	6.350
		H5	6.598
		H10	6.546
2	MAPK1	H1	5.691
		H2	5.872
		H3	6.010
		H4	5.691
		H10	8.948
3	GAPDH	H2	6.239
		H5	5.981
		H10	5.567
4	HSP90AA1	H4	6.078
		H10	5.629
		H12	6.247
5	STAT3	H10	7.020
		H12	5.748
6	CASP3	H10	5.730

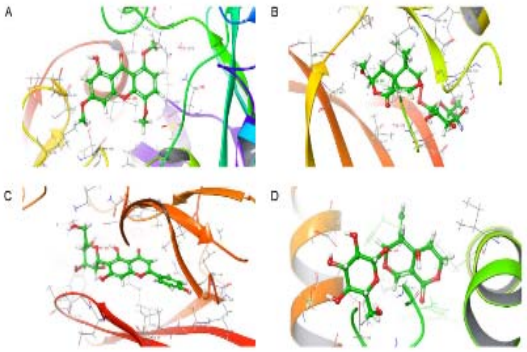

Figure 7: Conformations of some important compounds and targets. A: GAPDH with 1-hydroxy-3,5,8-trimethoxyxanthone, B: MAPK3 with 7-epi-vogeloside, C: MAPK1 with isovitexin, D: HSP90AA1 with swertiamarin

DISCUSSION

Cholecystitis is a common surgical disease that was first discovered in humans 3500 years ago [26]. Although surgical and drug treatments are crucial, their clinical effect is not ideal for the treatment of cholecystitis. Therefore, it is

necessary to develop treatment methods for cholecystitis from the mechanism of the disease and integrate different medical systems from a comprehensive perspective.[3]. Traditional Tibetan medicine (TTM) focuses on the stability of the entire body by balancing the three humors (nyes pa), namely, “wind” (rlung), “bile” (mkhris pa), and “phlegm” (*bad kan*) in the treatment of complex cholecystitis [15]. *Dida*, the preferred Tibetan medicinefor treatment of cholecystitis, has a definite curative effect and it is widely used and recognized. It can be traced back to Tibetan medicine books such as Yue Wang’s Classical Medicinal Book (*Somaratsa* in Tibetan), "the four medical tantras"(*rGyud-bzhi* in Tibetan) and Jing Zhu Materia Medica (*Shel Gong Shel Phreng* in Tibetan) [6,8]. However, the development of TTMs was restricted by the unclear qualitative and quantitative characteristics of drug components and the complex target and mechanism of action. Network pharmacology has provided an innovative method for investigating the pharmacological mechanisms of TTMs.

In this study, the pharmacologic mechanism involved in the use of Tibetan medicine *Dida* for treatment of cholecystitis was preliminarily obtained through the organic combination of network pharmacology and molecular docking simulation. The results revealed that 12 representative compounds and 50 targets involving 29 pathways were associated with the anti-cholecystitis effect of *Dida*. Through pathway enrichment, it was determined that the mechanism could involve mutual antagonism and mutual synergism of multiple pathways, such as apoptosis, NOD-like receptor signaling pathway, toll-like receptor signaling pathway, T cell receptor signaling pathway, and MAPK signaling pathway. In addition, the degree of cross-comparison between genes was high according to the PPI system analysis of cholecystitis targets, and GAPDH, AKT1, CASP3, EGFR, TNF, MAPK3, MAPK1, HSP90AA1, STAT3, and BCL2L1 were identified as the central genes.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an energy metabolism-related enzyme. Several studies have indicated that GAPDH regulates the immune function and exert anti-inflammatory effects. It has been reported that the anti-inflammatory effect of GADPH was regulated by downregulating aerobic glycolysis in activated myeloid cells and lymphocytes [16]. Protein kinase (AKT1) AKT1, a member of the Akt family, is expressed in macrophages which participate in various biological processes, such as metabolism, proliferation, and cell survival. Protein kinase (AKT1) AKT1 activation leads to the production of large amounts of pro-inflammatory cytokines which play a crucial role in inflammatory responses [17]. Caspase-3(CASP3), a key enzyme in the process of apoptosis, belongs to the caspase family, which activation and inflammation occur simultaneously and in different tissues [18].

Epidermal growth factor receptor (EGFR) is considered to be a key pathway for GPCRs signaling. Thus, there may be a mechanism linking chronic inflammation to a variety of diseases, especially cancer. Studies have shown that EGFR expression is increased in chronic cholecystitis and gallbladder carcinoma, suggesting that inflammation has a certain relationship with EGFR expression [19]. Tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine and an apoptosis-inducing factor, can be used as a drug for the treatment of inflammatory diseases via the pathogenesis of various inflammatory diseases of the liver and gallbladder. The increase in supersaturated bile and hydrophobic bile acids in guinea pigs with gallstone disease enhanced the release of TNF-α and trigger the TNF-α/Caspase8/ Caspase3 cascade to induce apoptosis [20].

Mitogen-activated protein kinases (MAP kinases) are highly conserved serine/threonine protein kinases that function in a variety of essential cellular processes, including proliferation, differentiation, motility, apoptosis, and survival. One of the MAP kinases, MAPK1, similar to MAPK3, is a major member of the MAPK family and plays a crucial role in the MAPK/ERK cascade. Previous reports have confirmed that ERK/MAPK pathway is activated in gallbladder cancer, resulting in promotion cell proliferation and invasion. [21]. Heat shock protein HSP 90-alpha 1(HSP90AA1), a significant class of heat shock proteins, belongs to the HSP90 family. Extracellular HSP90AA1 induces inflammation by activating NF-kB and STAT3 transcriptional programs (including pro-inflammatory cytokines IL-6 and IL-8) [22].

Signal transducer and activator of transcription 3 (STAT3), a transcription factor involved in immune and inflammatory responses, is rapidly activated by various cytokines, growth factors, and hepatocyte growth factors [23]. The Bcl-2-like protein 1(BCL2L1), a member of the family of Bcl-2 proteins, is a crucial apoptosis-regulating gene that participates in the regulation of mitochondrial apoptosis pathways by controlling the release of pro-apoptotic factors in mitochondria [23]. In this network, a high degree of target involved various inflammatory processes, among which was cholecystitis. However, only a few studies on cholecystitis are

available, and other unreported targets need to be confirmed via further experiments.

In the compound–target–pathway network, the enrichment of 29 pathways was associated with *Dida* in the treatment of cholecystitis, and also with the classical inflammatory pathways, such as apoptosis, NOD-like receptor signaling pathway, toll-like receptor signaling pathway, T cell receptor signaling pathway, and MAPK signaling pathway. Apoptosis was activated in gangrenous cholecystitis cells, leading to the inhibition of cell viability. These results indicate that. the mechanism involved in the use of *Dida* for cholecystitis may improve cell survival by regulating the apoptosis pathway [24]. The NOD-like receptors and toll-like receptors (TLRs), which are the members of pattern recognition receptors (PRRs), have been associated with human diseases such as infections, cancer, and inflammatory disorders [25]. The MAPK signaling pathway has a hand-in-hand relationship with the occurrence of various underlying diseases, such as inflammation and tumors, and it is fundamental in the pathogenesis of gallbladder cancer and cholecystitis [21]. In summary, the above classical inflammatory pathways are predictive pathway for Dida intervention in cholecystitis, and require further cellular experiments and animal validation.

In addition, molecular docking simulation, a ligand- and structure-based target tool, has been far developed in silicon target prediction, which is a promising approach for determination of the target of a specific hypothetical ligand. [13]. In this study, molecular docking results indicated that seven compounds (1 - hydroxy - 2, 3, 5-3 armour oxygen radicals flavonoids, 1 - hydroxy - 3, 5, 8 - yellow ketone, 1 - hydroxy - 3 armour oxygen radicals, 2, 3, 5, 7-4 methoxy flavone, decussatin, 7 - the epi - vogeloside, isovitexin, swertiamarin) and six targets (i.e., GAPDH, GASP3, MAPK3, MAPK1, HSP90AA1, STAT3) has the strong abilities and high docking scores. These compounds may be promising inhibitors for the treatment of cholecystitis in the future, a finding that is highly consistent with the network pharmacologic analysis.

CONCLUSION

In this study, network pharmacology method has been successfully used to investigate the complex network relationships among multiple components, multiple targets and multiple pathways, and to predict the mechanism underlying the anti-cholecystitis effect of *Dida*. Molecular docking supported the application of bioactive compounds in *Dida* in the treatment of cholecystitis. However, the results of these *in silico* methods need to be verified via *in vivo* and *in vitro* experiments.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yi Zhang and Ya Tu proposed the idea and designed the study. Chuan Liu performed the study and wrote the manuscript. Chuan Liu, Fang-Fang Fan and Xuan-Hao Li participated in the data analysis. Fang-Fang Fan, Xuan-Hao Li and Wen-Xiang Wang improved the manuscript. All authors read and approved the final manuscript.

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