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ORIGINAL RESEARCH

A Network Pharmacology-Based Strategy For Predicting Active Ingredients And Potential Targets Of LiuWei DiHuang Pill In Treating Type 2 Diabetes Mellitus

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Background: Traditional Chinese medicine (TCM) formulations have proven to be advantageous in clinical treatment and prevention of disease. LiuWei DiHuang Pill (LWDH Pill) is a TCM that was employed to treat type 2 diabetes mellitus (T2DM). However, a holistic network pharmacology approach to understanding the active ingredients and the therapeutic mechanisms underlying T2DM has not been pursued.

Methods: A network pharmacology approach including drug-likeness evaluation, oral bioavailability prediction, virtual docking, and network analysis has been used to predict the active ingredients and potential targets of LWDH Pill in the treatment of type 2 diabetes.

Results: The comprehensive network pharmacology approach was successfully to identify 45 active ingredients in LWDH Pill. 45 active ingredients hit by 163 potential targets related to T2DM. Ten of the more highly predictive components (such as :quercetin, Kaempferol, Stigmasterol, beta-sitosterol, Kadsurenone, Diosgenin, hancinone C, Hederagenin, Garcinone B, Isofucosterol) are involved in anti-inflammatory, anti-oxidative stress, and the reduction of beta cell damage. LWDH Pill may play a role in the treatment of T2DM and its complications (atherosclerosis and nephropathy) through the AGE-RAGE signaling pathway, TNF signaling pathway, and NF-kappa B signaling pathway.

Conclusion: Based on a systematic network pharmacology approach, our works successfully predict the active ingredients and potential targets of LWDH Pill for application to T2DM and helps to illustrate mechanism of action on a comprehensive level. This study provides identify key genes and pathway associated with the prognosis and pathogenesis of T2DM from new insights, which also demonstrates a feasible method for the research of chemical basis and pharmacology in LWDH Pill.

Keywords: LWDH Pill, type 2 diabetes mellitus, T2DM, network pharmacology

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease, affecting about 90% of the people with diabetes. It would cause serious damages to the heart, blood vessels, eyes, kidneys, and nerves with long-time hyperglycemic status.[1–4] Single target treatment of T2DM may be effective in controlling blood glucose levels in patients, but it is not effective in treating complications. Multiple pathways of treatment are often required to achieve and maintain long-term glycemic control.[5,6] An economic cost estimate based on T2DM showed that per capita annual expenditures for treating diabetes in each

country range from $242 to $11.917, which emerge a considerable impact in terms of costs to society, health systems, individuals and employers and in terms of a reduction in the productive workforce and productivity.[7,8] Traditional Chinese medicine (TCM) herbal formula (FuFang in Chinese) was characterized by "multi-component, multichannel", which has its own advantages and peculiarity in personalized treatment and early intervention.[9]

LiuWei DiHuang Pill (LWDH Pill), first mentioned during the Song Dynasty (AD 1119) writen by Qian Yi in his book "Pediatric Medicinals and Patterns", is a traditional prescription for the treatment of many types of diseases in China clinically,such as asthenia of renal yin.[10] It is described as good effect in T2DM with less toxicity and side effects, consisting six herbs: *Rehmanniae radix* preparata (Shu Di Huang: SDH), *Cornus officinalis* Sieb. (Shan Zhu Yu: SZY), *Paeonia suffruticosa* Andr. (Mu Dan Pi: MDP), *Dioscorea opposite* Thunb (Shan Yao: SY), *Poria cocos* (Schw.) Wolf (Fu Lin: FL), and *Alisma orientale* (Sam.) Juzep (Ze Xie: ZX), in the ratio of 8:4:4:3:3:3, respectively.[11] Researches had been demonstrated that LWDH, as a safe and effective formula, were employed to improve T2DM and its complications, including diabetic nephropathy, diabetic encephalopathy, and diabetic muscle atrophy.[11–13] Most of these correlations between LWDH and the corresponding treatment are derived from practical experience and long-term therapeutic observations. Thus, it is necessary to investigate scientific and technologic approaches to extend the understanding of the synergistic effects of LWDH Pill chemical compounds in treating T2DM.[14,15]

Recently, network pharmacology (NP) was proposed as a promising approach to discover TCM from a systems perspective and at the molecular level.[16,17] Zeng et al, illuminated the molecular synergy of YHD for HER2-positive breast cancer (such as: reduces TGF-β1 secretion, regulating IGF-1receptors, down-regulating the expression of EGFR, et al.), by a network pharmacology approach.[18] NP considers the contribution of each ingredient in TCMs to adequately explain the effects generated by an entire formula.[19,20] Lee et al predicted 21 bioactive compounds and 57 genes linked to hyperlipidemia and atherosclerosis in Yijin-Tang by Network analysis.[21] Pang et al, built constituent-target network, constituent-target-target network and target-biological pathway network indicate CDK5, MAOB, 5-HTR1A, GSK3-β, and found that COMT were important nodes for Naodesheng (NDS) formula in the treatment of Alzheimer's disease (AD).[22] These previous studies suggested that NP will be a good predictive tool for exploring the chemical compositions of LWDH and its relationships with T2DM. Our study is the first to identify potential bioactive compounds in LWDH Pill and elucidate its mechanisms in T2DM treatment by using the NP approach.

Materials And Methods

Ingredients Database Construction And ADME Screening

All candidate compounds of these six Chinese medicinal herbs in LWDH were collected from the three following databases: (1) Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (http://lsp.nwu.edu.cn/tcmsp.php, Ver.2.3).[23] A total of 500 Chinese herbal medicines and 30,069 ingredients from the Chinese Pharmacopoeia (2010 edition) were registered in the TCMSP database.[24] (2) Bioinformatics Analysis Tool for Molecular mechanism of Traditional Chinese Medicine (BATMAN) (http://bionet.ncpsb.org/batman-tcm/, updated January 2016), which consists of 46,914 formulas, 8159 herbs, and 25,210 ingredients through literature mining and database integration.[25] (3) TCM Database@Taiwan (http://tcm.cmu.edu.tw/). A total of 351 compounds were identified in LWDH, including 77 in SDH, 231 in SZY, 59 in MDP, 75 in SY, 65 in FL, and 45 in ZX. Details of the 351 compounds are shown in Table S1.

Prior to target prediction, absorption, distribution, metabolism, and excretion (ADME) was employed to select bioactive components that contribute to its therapeutic effects, while those with poor pharmacological properties and bad drug ability compounds were removed.[26] In order to obtain compounds with higher oral absorption, utilization, and biological properties for further analysis, we require that the candidate components meet two of the following parameters (1) Oral bioavailability (OB) ≥ 30%, (2) Drug-likeness (DL) ≥ 0.18.

The related genes of LWDH PILL bioactive components were obtained from TCMSP and DrugBank (https://www.drugbank.ca/, ver. 5.1.2) databases under the condition of Homo sapiens. UniProt KB (http://www.uniprot.org/), which prevents, for instance, over-annotation of similar proteins like paralogs and putative products of pseudogenes, was employed to standardize gene names and organisms.[27] After that, these revised targets will lead to STRING (https://string-db.org/, ver. 11.0) predicting the associated protein–protein

interaction (PPI) to obtain target genes directly and indirectly related to T2DM.

Predicting Putative Targets Of T2DM

The T2DM-related target proteins were screened from two sources: (1) GeneCards (https://www.genecards.org/, ver. 4.9.0) database; it is an online catalog of human genes and genetic diseases, which enables effectively navigate gene–disease linkages.[28] 2. OMIM (http://www.omim.org/, updated February 19, 2019) database possesses more than 15,500 gene entries and focuses on explaining gene–phenotype relationships.[29] Only "Homo sapiens" proteins linked to T2DM were selected, and it should be considered whether there was a protein–ligand complex, crystal structure determination method, resolution, and so on. We got 7822 genes totally, and details are described in Table S2.

Protein–Protein Interaction (PPI)

The retrieved LWDH PILL active ingredient targets are correlated with T2DM targets by the STRING (https://string-db.org/, ver. 11.0) database.[30] The condition was limited to "Homo sapiens". In this study, a high confidence score with correlation degree ≥ 0.700, as the cut-off value, was set to obtain a network.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis For Targets

KEGG pathway analysis was performed to further probe the vital biological process of achieved targets and to validate the reliability of the integrated results.[31–33] Finally, KEGG (http://www.genome.ad.jp/kegg/, updated February 1, 2019) terms with p-value ≤ 0.05 were selected for further research.

Network Construction And Analysis

Network construction was performed by the network visualization software Cytoscape (ver. 3.5.0) as follows: (1) Compound–compound target network; (2) Compound–T2DM PPI network; (3) LWDH PILL–T2DM network. Moreover, four topological properties of the hub network, including "Degree" and "Betweenness Centrality", were calculated to screen the LWDH Pill candidate targets with topological importance.[34,35]

To obtain the links between network clusters and picked out hub genes with high degree of connectivity, "Molecular Complex Detection" (MCODE: a plug-in of Cytoscape) was employed to investigate node composition.[36] Degree cutoff=2, Node Score Cutoff=0.2, and K-Core=2 were selected as the advanced options.[37,38]

Validation Of Compound–Target Interaction

Molecular Operating Environment (MOE 2015.10 software) was employed to validate the compound–target association in our study.[26,39] The target proteins in all networks were obtained 3D structures from the RCSB PDB database (http://www.rcsb.org/). The proteins and ligands were prepared in the MOE tool prior to performing the docking process. The crystal structure of the target protein is pretreated, including removal of water molecules, Protonate 3D hydrogenation, correction of protein structure, energy optimization and retention of the active region of the target.[40] The structure of ligands needs to meet to low-energy conformations. MOE-dock tool was used to semi-flexibly couple the active ingredient to the target protein. The placement function and scoring function use Triangle Matcher and London dG, respectively, and the rest use default parameters.

Results And Discussion

Compound–Compound Target Network Analysis

There are 229 nodes (185 compound target nodes and 45 compound nodes) and 418 edges composed compound–compound target network (Figure 1). Details of the 45 potentially bioactive ingredients and 185 target genes are provided in Table 1. In this network, we can find that some special compounds, interacting with multiple targets, participated in the regulation of multiple targets (central nodes, for instance, Quercetin, Kaempferol, Stigmasterol, Beta-Sitosterol, Kadsurenone, Diosgenin, Hancinone C, Hederagenin, Garcinone B, Isofucosterol).

This suggests that LWDH Pill compounds may act synergistically on these targets, exerting pharmacological effects in T2DM and other diseases.

Compound–T2DM PPI Network Analysis

Compound–T2DM PPI Network

A total of 174 overlapping genes were obtained by looking for the intersection of the above compound target and the 7822 T2DM targets. The compound–disease co-targets information was exhibited in Figure S1 and Table S3. Import compound–disease co-targets information into STRING,

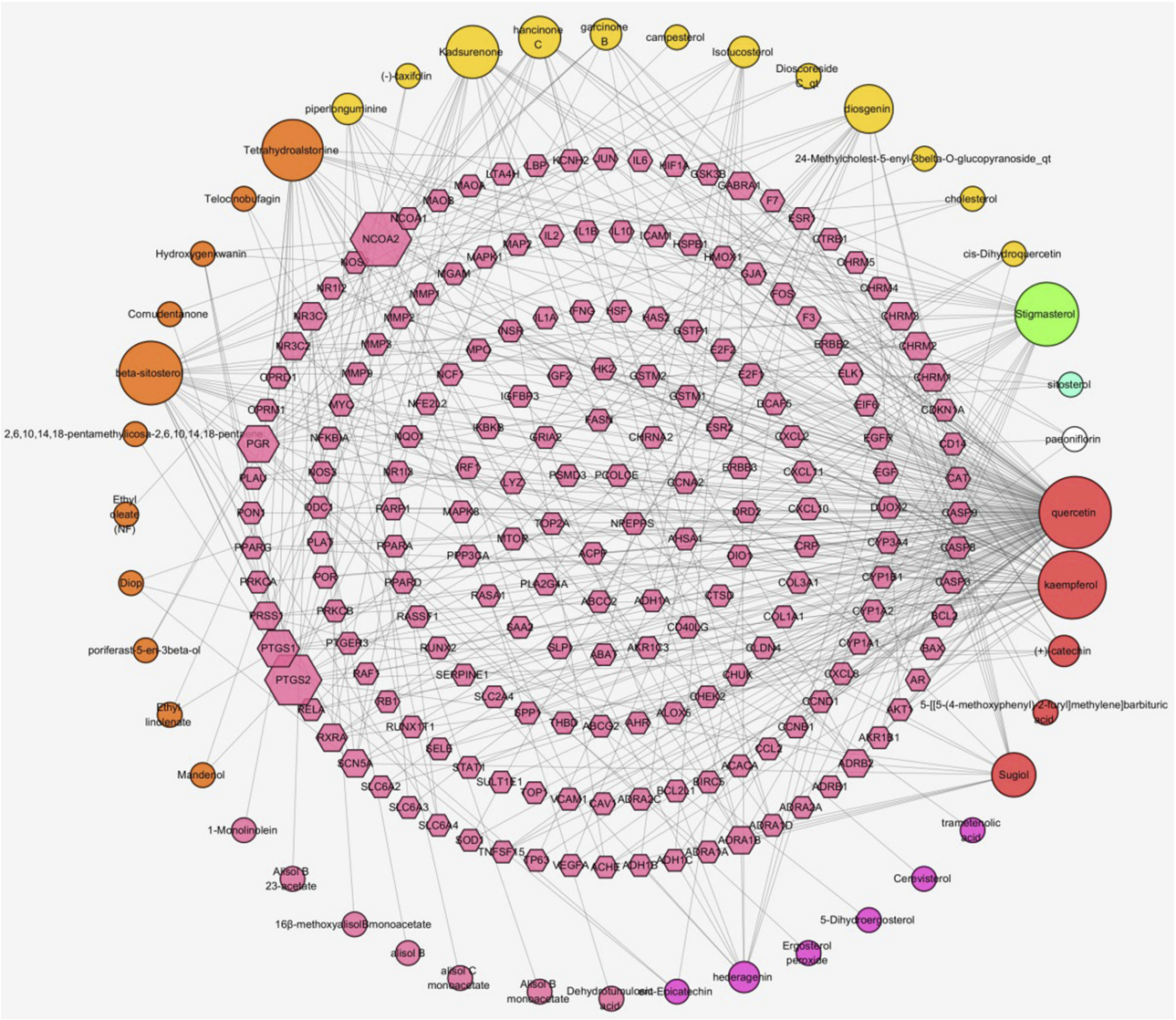


Figure 1 Compound–compound target network of LWDH Pill. Compound–compound target network of LWDH Pill consists of 185 compound target nodes and 45 compounds. The gene targets are described as a pink hexagon. Red, orange, yellow, pink, and purple circles represent MDP, SZY, SY, ZX, and FL, respectively. Green circle stands for common compound of SDH, SZY, and SY. Blue circle is employed to stand for common compound of SDH, SZY, MDP, and ZX. White circle represents common compound of MDP and FL. Node size of gene targets and compounds is proportional to the number of constituent. Lines stand for relation of compounds and target nodes.

we get compound–T2DM target PPI network with higher degree (degree ≥ 0.700) of connectivity (Figure 1), which contains 163 nodes and 2735 edges (Figure 2).

In PPI network, some nodes (AKT1, MAPK1, MAPK8, IL6, JUN, VEGFA, EGF, MMP9, and CXCL8) have higher degrees. The number of edges of each node is quite large (66 in AKT1, 55 in MAPK1, 54 in MAPK8, 53 at IL6, 52 in JUN, 45 in VEGFA, 42 in EGF, and 41 in MMP9 and CXCL8).

Cluster Of Compound–T2DM PPI Network

Targets information in PPI network queried from STRING protein database was analyzed by MCODE tool returned 8 central gene clusters (Figure 3 and Table 2). The top 3 modules with score > 6 were selected to detect significant modules in this PPI network. There are some T2DM-related targets in those clusters. Cluster 1 includes AR, HMOX1, AKT1, NOS3, ESR1, STAT1, IL1B, IL10, ICAM1, IL6, EGF, and MMP1. Cluster 2 gets CXCL8, EGFR, MAPK8, VEGFA, MMP9, MAPK1, PTGS2, MYC, MMP2, and FOS with high degree value. CYP1A2, CYP1A1, GSTM2, GSTP1, CYP1B1, CYP3A4, and GSTM1 were involved in Cluster 3.

In retrospective analysis, T(−413)A single nucleotide polymorphism (SNP) of Heme oxygenase 1 (HMOX1) promoter was significantly more susceptible

Table 1 Compound–Compound Target Network Information

Mol ID	Compound	MW	OB (%)	DL	Medicine	Database Source
MOL000359	Sitosterol	414.79	36.91	0.75	SDH, SZY, MDP, ZX	TCMSP
MOL000449	Stigmasterol	412.77	43.83	0.76	SDH, SZY, SY, ZX	TCMSP
MOL001494	Mandenol	308.56	42	0.19	SZY	TCMSP
MOL001495	Ethyl linolenate	306.54	46.1	0.2	SZY	TCMSP
MOL001771	Poriferast-5-en-3beta-ol	414.79	36.91	0.75	SZY	TCMSP
MOL002879	Diop	390.62	43.59	0.39	SZY	TCMSP
MOL002883	Ethyl oleate (NF)	310.58	32.4	0.19	SZY	TCMSP
MOL000358	Beta-sitosterol	414.79	36.91	0.75	SZY	TCMSP
MOL005481	2,6,10,14,18-pentamethylicosa-2,6,10,14. 18-pentaene	342.67	33.4	0.24	SZY	TCMSP
MOL005503	Cornudentanone	378.56	39.66	0.33	SZY	TCMSP, Taiwan, BATMAN-TCM
MOL005530	Hydroxygenkwanin	300.28	36.47	0.27	SZY	TCMSP
MOL005531	Telocinobufagin	402.58	69.99	0.79	SZY	TCMSP, Taiwan, BATMAN-TCM
MOL008457	Tetrahydroalstonine	352.47	32.42	0.81	SZY	TCMSP, Taiwan, BATMAN-TCM
MOL000098	Quercetin	302.25	46.43	0.28	MDP	TCMSP
MOL000211	Betulinic acid	456.78	55.38	0.78	MDP	TCMSP
MOL000422	Kaempferol	286.25	41.88	0.24	MDP	TCMSP
MOL000492	(+)-Catechin	290.29	54.83	0.24	MDP	TCMSP
MOL007374	5-[[5-(4-methoxyphenyl)-2-furyl]methylene] barbituric acid	312.3	43.44	0.3	MDP	TCMSP
MOL001924	Paeoniflorin	480.51	53.87	0.79	MDP, FL	BATMAN-TCM
MOL002222	Sugiol	300.48	36.11	0.28	MDP	BATMAN-TCM, Taiwan
MOL001559	Piperlonguminine	273.36	30.71	0.18	SY	TCMSP
MOL001736	(-)-Taxifolin	304.27	60.51	0.27	SY	TCMSP
MOL000322	Kadsurenone	356.45	54.72	0.38	SY	TCMSP
MOL005430	Hancinone C	400.51	59.05	0.39	SY	TCMSP
MOL005435	24-Methylcholest-5-enyl-3belta-O-glucopyranoside_qt	400.76	37.58	0.72	SY	TCMSP
MOL005438	Campesterol	400.76	37.58	0.71	SY	TCMSP, BATMAN-TCM
MOL005440	Isofucosterol	412.77	43.78	0.76	SY	TCMSP
MOL005458	Dioscoreside C_qt	444.72	36.38	0.87	SY	TCMSP
MOL000546	Diosgenin	414.69	80.88	0.81	SY	TCMSP, BATMAN-TCM
MOL005465	Aids180907	394.45	45.33	0.77	SY	TCMSP
MOL000953	Clr	386.73	37.87	0.68	SY	TCMSP
MOL004580	Cis-Dihydroquercetin	304.27	66.44	0.27	SY	BATMAN-TCM
MOL000275	Trametenolic acid	456.78	38.71	0.8	FL	TCMSP
MOL000279	Cerevisterol	430.74	37.96	0.77	FL	TCMSP
MOL000282	5-dihydroergosterol	398.74	43.51	0.72	FL	TCMSP
MOL000283	Ergosterol peroxide	430.74	40.36	0.81	FL	TCMSP
MOL000285	Dehydrotumulosic acid	482.77	38.26	0.82	FL	TCMSP
MOL000296	Hederagenin	414.79	36.91	0.75	FL	TCMSP
MOL000073	Ent-Epicatechin	290.29	48.96	0.24	FL	Taiwan
MOL000830	Alisol B	472.78	34.47	0.82	FL, ZX	Taiwan
MOL000831	Alisol B monoacetate	514.82	35.58	0.81	ZX	TCMSP, Taiwan, BATMAN-TCM
MOL000849	16β-methoxyalisol B monoacetate	544.85	32.43	0.77	ZX	TCMSP
MOL000856	Alisol C monoacetate	514.77	33.06	0.83	ZX	TCMSP
MOL002464	1-monolinolein	354.59	37.18	0.3	ZX	TCMSP
MOL000862	Alisol B 23-acetate	514.82	35.58	0.81	FL, ZX	TCMSP, Taiwan

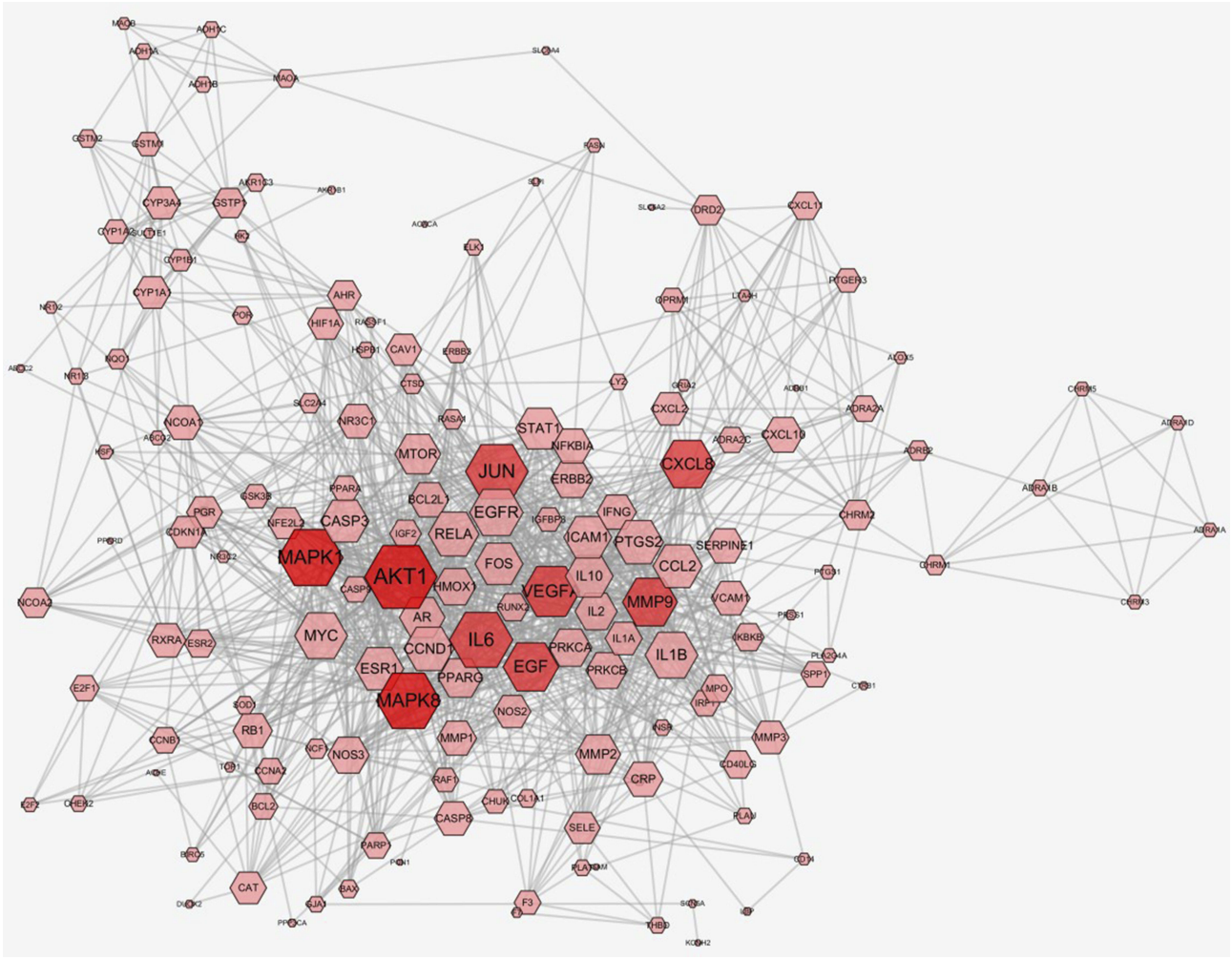


Figure 2 Compound–Type 2 diabetes mellitus PPI network. Compound–T2DM target PPI network contains 163 gene nodes and 2735 edges. Pink hexagon represents gene targets. The red nodes (AKT1, MAPK1, MAPK8, IL6, JUN, VEGFA, EGF, MMP9, and CXCL8) have higher degrees. Node size of gene targets is proportional to the number of degrees.

to albuminuria development than those carrying the A allele, especially in T2DM with longer duration and poor glycemic control.[41–43] Yin et al, investigate that blocking of George et al, suggested that the role of Akt deficiency in human type 2 diabetes was underscored by the discovery of a family with type 2 diabetes associated with an inherited loss of function Akt2 mutation that seems to act in a dominant-negative manner to inhibit other Akt isoforms.[44] Chen et al discovered that the haplodeficiency of Akt1 is sufficient to convert the mild insulin resistance in Akt2-/- mice to an overt type 2 diabetes, indicating a complementary role of Akt1 in the genesis of type 2 diabetes initiated by the deficiency of Akt2.[45] Yin et al also investigated that blocking of AKT1 expression in pancreatic β-cells and peripheral tissues may lead to hyperglycemia and diabetes in Chinese Han population.[46] Thus, these results showed that Akt1 and AKT2 are relatived to diabetes. The study reported inflammatory cytokines disorder was used as critical early biomarkers for estimating the T2DM risks and complications.[47,48] T2DM patients had significantly higher levels of inflammatory markers interleukin-1B (IL-1B) and IL-6, while IL-10 showed lower levels, compared with non-diabetic patients.[47,49] STAT1 is highly expressed in patients with T2DM. It can induce beta-cell injury by regulating IL-1B-activated beta-cell gene network in coordination with NF-kappaB.[50,51] Yamada et al. showed that the 13989A→G(Ile118Val) polymorphism on CYP3A4 was significantly associated with T2DM sensitivity or drug resistance.[52]

The production of reactive oxygen species (ROS) abnormally increases in pancreatic β-cells when exposed to high concentrations of glucose and/or free fatty acids for a

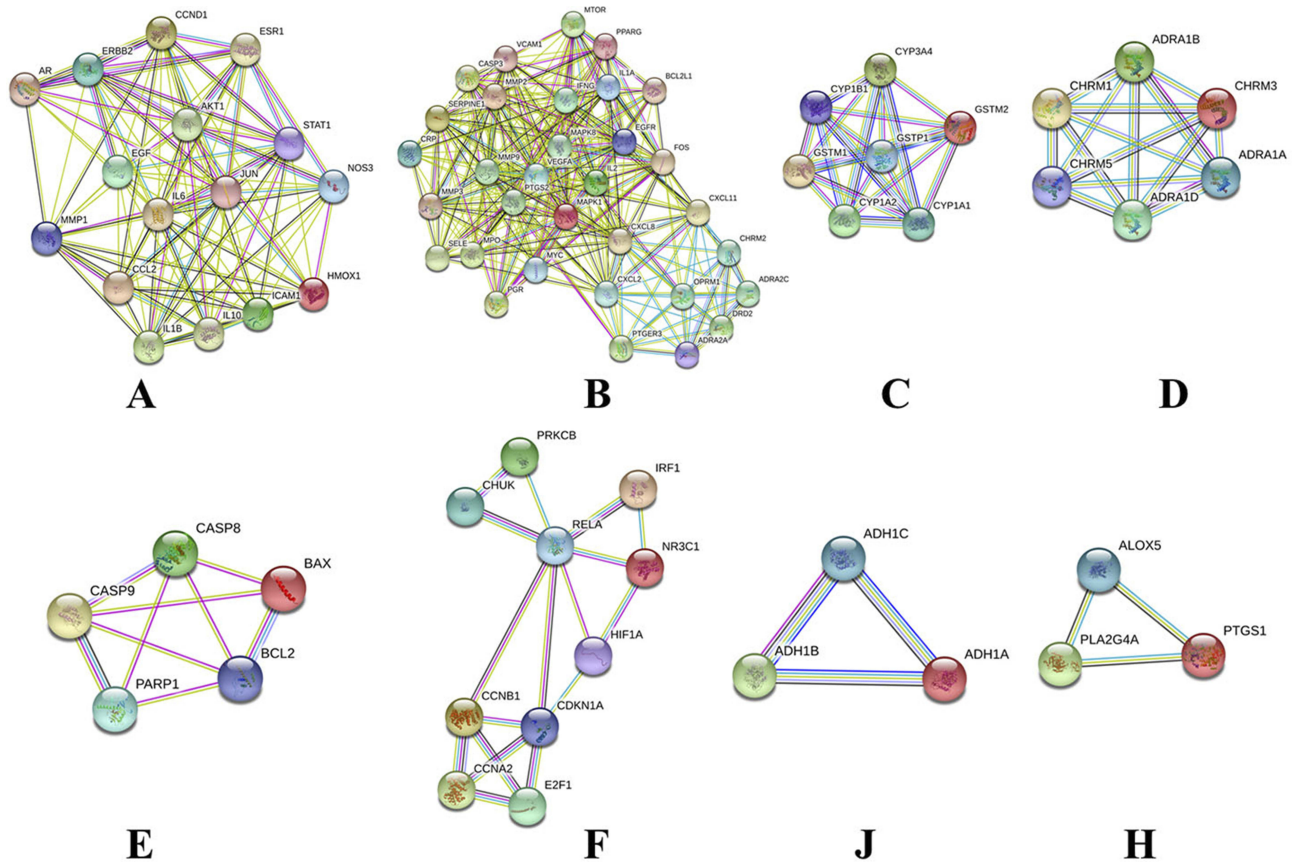


Figure 3 Cluster of compound–Type 2 diabetes mellitus PPI network. Eight clusters were identified in compound–Type 2 diabetes mellitus PPI network (**A**, **B**, **C**, **D**, **E**, **F**, **G**, and **H**) stand for clusters 1, 2, 3, 4, 5, 6, 7, and 8).

prolonged period of time, resulting in a burden of oxidative stress, further contributing to dysfunction of pancreatic β-cells.[53] Oxidative stress increases intracellular matrix metalloproteinases including MMP1, MMP9, and MMP2 expression and intracellular activity, which response to induce impaired insulin secretion, declined glucose utilization, insulin resistance, and abnormal hepatic glucose production.[54–56] Glutathione S-transferases (GSTs) are a family of multi-gene and multifunctional detoxification enzymes, which enable to remove ROS and S-thiolated protein regeneration caused by oxidative stress, metabolites, and chemicals.[57–59] In Cluster 3, two families of related genes were found, mu and pi encoded by GSTM and GSTP genes. Tang et al confirmed by meta-analysis that 1354 T2DM from literature and database were significantly associated with Glutathione S-transferases mu 1 (GSTM1) polymorphism, with a pooled OR=1.66 (95% CI=1.17–2.37).[60] Andrea et al illustrated that GSTM2 is one of the male-specific genes and plays an important role in liver metabolism, detoxification, and secretion.[61] Adina's data suggest that Val allele of the GSTP1 Ile105Val polymorphism (OR = 2.0, 95% CI=1.20–3.35, p=0.007) and GSTP1 Val/Val genotype (diabetic patients and controls: 13.1% vs 5.1%, resp.) may be associated with higher risk of T2DM in Romanian population.[62]

This suggests that LWDH Pill may be involved in the regulation of these genes, which are key or central genes in the development of T2DM.

Pathway Of Compounds–T2DM PPI Network

The KEGG pathway enrichment analysis was performed on the genes in the above mentioned three modules (Cluster 1, Cluster 2, and Cluster 3), displayed in Figure S2.

Clusters 1, 2, and 3 were enriched to 10 KEGG pathways, respectively, which were enriched respectively and mainly in AGE-RAGE signaling pathway in diabetic complications (p=5.08E^{-15}), Pathways in cancer (p= 1.37E^{-12}), Fluid shear stress and atherosclerosis (p= 4.42E^{-10}), Chagas disease (p= 3.77E^{-9}), TNF signaling pathway (p= 4.85E^{-9}), Kaposi's sarcoma-associated herpesvirus infection (p= 1.22E^{-11}), IL-17 signaling pathway (p= 1.03E^{-13}). The details are described in Table S4.

Table 2 Cluster Of Compound–Type 2 Diabetes Mellitus PPI Network

Cluster	Score (Density*#Nodes)	Nodes	Edges	Genes
1	10.267	16	77	*JUN, AR, ERBB2, HMOX1, CCL2, AKT1, NOS3, CCND1, ESR1, STAT1, IL1B, IL10, ICAM1, IL6, EGF, MMP1*
2	10.194	32	158	*MYC, MAPK1, VEGFA, SELE, MPO, MMP9, IFNG, CXCL11, OPRM1, DRD2, EGFR, CXCL2, PTGER3, FOS, ADRA2A, ADRA2C, MAPK8, MMP2, CHRM2, BCL2L1, IL2, MTOR, SERPINE1, PTGS2, CASP3, PPARG, MMP3, CRP, VCAM1, PGR, IL1A, CXCL8*
3	6.667	7	20	*CYP1B1, GSTM2, CYP1A1, CYP1A2, GSTP1, CYP3A4, GSTM1*
4	6	6	15	*CHRM1, CHRM5, ADRA1A, ADRA1B, ADRA1D, CHRM3*
5	3.5	5	7	*PARP1, BCL2, CASP9, BAX, CASP8*
6	3.111	10	14	*RELA, NR3C1, CHUK, E2F1, IRF1, CCNB1, HIF1A, CDKN1A, CCNA2, PRKCB*
7	3	3	3	*ADH1A, ADH1C, ADH1B*
8	3	3	3	*PLA2G4A, ALOX5, PTGS1*

LWDH Pill–T2DM Network Analysis

LWDH Pill–T2DM Network

To shed light on the potential mechanisms of LWDH Pill acting on T2DM, the putative LWDH Pill target–T2DM-related target network consisting of LWDH herbs, compound–disease interactional proteins, and KEGG pathways was constructed based on PPI databases. Our pathway-act-network demonstrates interactions in multiple pathways including cross-talk of the pathway terms and the transitive relation. There are 125 nodes (6 herb nodes, 94 T2DM-related target nodes, and 25 pathway nodes) and 681 edges in LWDH Pill–T2DM network. The details of 25 signaling pathway nodes are shown in Figure 4 and Table 3.

LWDH–T2DM network analysis showed that six herbs in LWDH act synergistically to treat T2DM and its complications. The study by Yokozawa et al shows that the glucose content of the renal tissue in high blood glucose group was 3.89 mg/g, but this declined to 3.27 mg/g when treated with dried *Rehmanniae Radix* extract.[63] SDH and SZY have been well evidenced to play a beneficial synergistic interaction in the ameliorating of renal injury in C57BL/KsJ-db/db diabetic mice by inhibiting AGEs/RAGE/SphK1 signaling pathway.[64] Qian et al demonstrated that alcohol extract of SZY can increase GLUT4 mRNA and its protein expression in T2DM rats through promoting proliferation of islet and increasing postprandial secretion of insulin and therefore accelerate glucose transport.[65] Qian et al demonstrated that alcohol extract of SZY can accelerate glucose transport by promoting islet proliferation and increasing postprandial secretion of insulin, thereby increasing GLUT4 mRNA and protein expression in T2DM rats.[65] Zhang's findings indicated that MDP decreased significantly the overexpression of IL-6, MCP-1, TGF-β1, ICAM-1, and RAGE in high-glucose-fat diet and streptozotocin (STZ)-induced diabetic nephropathy rats.[66] In addition, previous studies showed that polysaccharides of SY, FL, and ZX perform the potential antidiabetic effect.[67,68]

Pathway Of LWDH Pill–T2DM Network

In LWDH Pill–T2DM network, some signaling pathways (such as AGE-RAGE signaling pathway in diabetic, IL-17 signaling pathway, TNF signaling pathway, HIF-1 signaling pathway, endocrine resistance, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, and EGFR tyrosine kinase inhibitor resistance) with higher degrees are closely related to the development, metastasis, and drug resistance of T2DM.

Pro-inflammatory and/or oxidative stress mediators are directly interlinked with the pathogenesis of T2DM and development of insulin resistance.[69,70] The AGE-RAGE signaling pathway plays a key role in the production of vascular complications (eg. atherosclerosis and kidney disease) that induce T2DM.[71,72] The formation and accumulation of advanced glycation end products (AGEs) was accelerated upregulated with increased circulating glucose as well as other reducing sugars, such as galactose and fructose, in T2DM, and subsequently AGEs-their receptor RAGE interaction to a signaling cascade, stimulating NAD(P)H oxidase and increasing

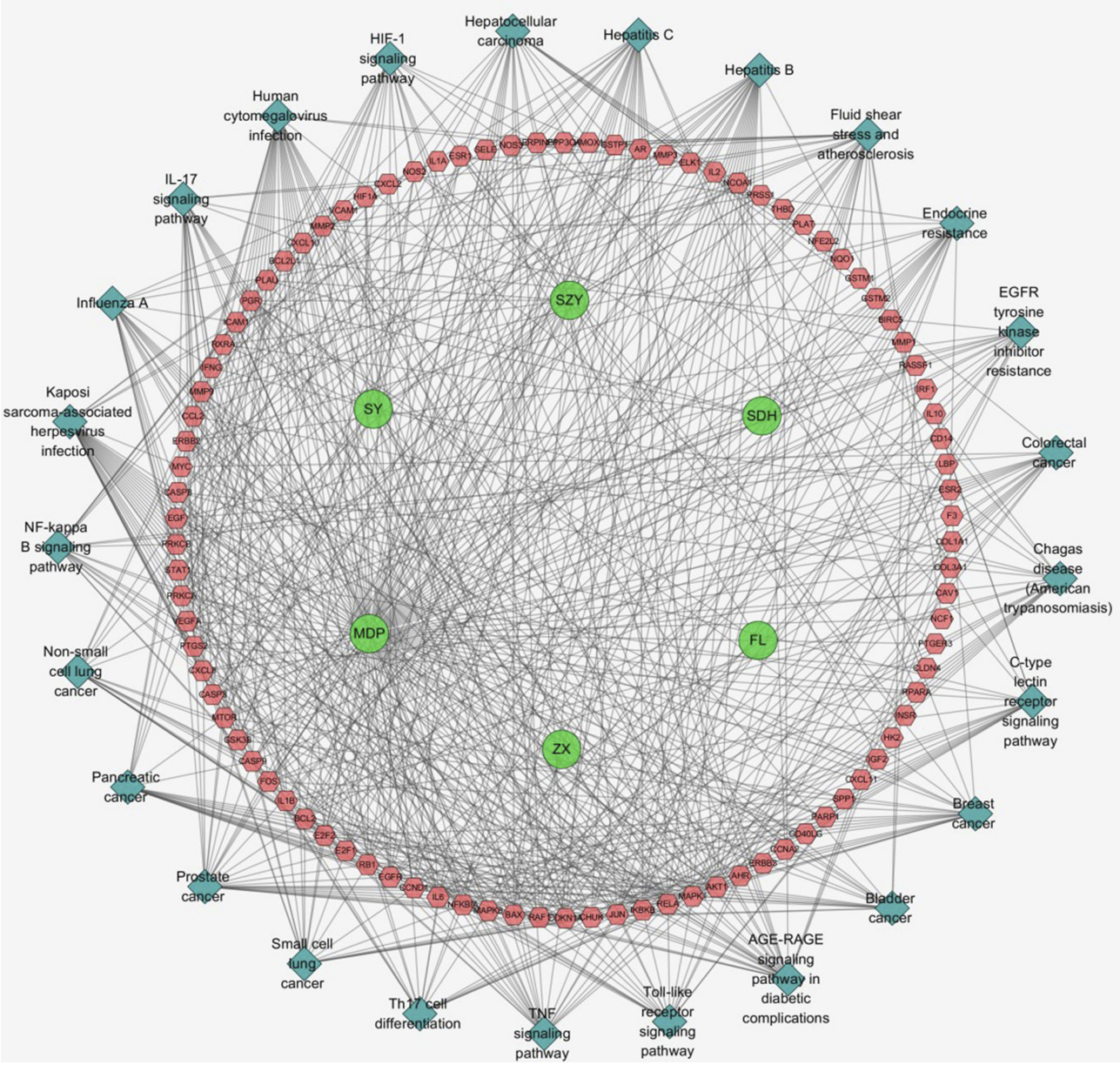


Figure 4 LWDH Pill–Type 2 diabetes mellitus network. Pathway of LWDH Pill–T2DM network includes 125 nodes (6 herb nodes represented by green circles, 94 T2DM-related target nodes represented by pink hexagon, and 25 pathway nodes described by blue diamond) and 681 edges.

the production of ROS eventually lead to the vascular endothelial dysfunction.[73–77] There are also accumulating evidence that the interaction of AGE/RAGE and tumor necrosis factor-alpha (TNF-α) signaling, perhaps even by amplifying one another, induces production of superoxide in T2DM.[73,78–80] Nuclear factor-κB (NF-κB) was characterized as a transcription factor activated through inflammation and oxidative stress, and involved in cytokines (e.g., TNF-α, IL-6, and IL-1B) and adhesion molecules [e.g., intercellular adhesion molecule-1 (ICAM1)] expression.[81] Akash et al proposed that the level of TNF-α disordered raised in adipocytes and/or peripheral tissues induces insulin resistance by impairing the insulin signaling through serine phosphorylation leading the development of T2DM.[70] Zhang et al confirmed that PDTC pretreatment, a specific NF-kappa B inhibitor, was able to attenuate the translocation of NF-κBp65 protein into the nucleus of human endothelial cells after high glucose treatment.[81] Apoptosis of β cell damage, caused by antigen-presenting cell activation mediated by the Toll-like receptor 2 (TLR2) pathway, was an initial event for the development of autoimmune diabetes.[82,83]

Table 3 KEGG Pathway Analysis Based On LWDH Pill–Type 2 Diabetes Mellitus Network

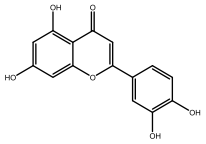
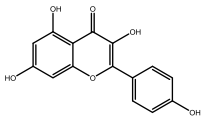
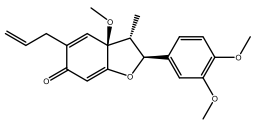
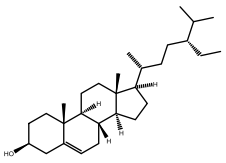
No.	Pathway ID	Pathway Name	*P* value	Count	Gene Name
1	05167	Kaposi sarcoma-associated herpesvirus infection	4.39E-20	31	*PTGS2, IL6, RELA, AKT1, VEGFA, CCND1, FOS, CDKN1A, BAX, CASP9, MAPK1, RB1, JUN, CASP3, NFKBIA, CASP8, RAF1, HIF1A, STAT1, MYC, ICAM1, CXCL8, CXCL2, CHUK, E2F1, E2F2, IKBKB, MAPK8, PPP3CA, GSK3B, MTOR*
2	05163	Human cytomegalovirus infection	1.35E-17	31	*PTGS2, IL6, RELA, EGFR, AKT1, VEGFA, CCND1, CDKN1A, BAX, CASP9, MAPK1, RB1, CASP3, ELK1, NFKBIA, CASP8, RAF1, PRKCA, MYC, IL1B, CCL2, PTGER3, CXCL8, PRKCB, CHUK, E2F1, E2F2, IKBKB, PPP3CA, GSK3B, MTOR*
3	05418	Fluid shear stress and atherosclerosis	1.11E-21	29	*RELA, AKT1, VEGFA, BCL2, FOS, MMP2, MMP9, JUN, HMOX1, CAV1, ICAM1, IL1B, CCL2, SELE, VCAM1, NOS3, PLAT, THBD, IFNG, IL1A, NCF1, GSTP1, NFE2L2, NQO1, CHUK, GSTM1, GSTM2, IKBKB, MAPK8*
4	05161	Hepatitis B	1.23E-19	29	*IL6, RELA, AKT1, BCL2, FOS, CDKN1A, BAX, CASP9, MMP9, MAPK1, RB1, JUN, CASP3, ELK1, NFKBIA, CASP8, RAF1, PRKCA, STAT1, MYC, CXCL8, PRKCB, BIRC5, CHUK, E2F1, E2F2, IKBKB, MAPK8, CCNA2*
5	04933	AGE-RAGE signaling pathway in diabetic complications	8.84E-25	28	*IL6, RELA, AKT1, VEGFA, CCND1, BCL2, BAX, MMP2, MAPK1, JUN, CASP3, PRKCA, STAT1, F3, ICAM1, IL1B, CCL2, SELE, VCAM1, CXCL8, PRKCB, NOS3, THBD, SERPINE1, COL1A1, IL1A, COL3A1, MAPK8*
6	05160	Hepatitis C	5.23E-17	26	*RXRA, RELA, EGFR, AKT1, CCND1, CDKN1A, BAX, CASP9, MAPK1, EGF, RB1, CASP3, NFKBIA, CASP8, RAF1, STAT1, MYC, IFNG, CLDN4, PPARA, CXCL10, CHUK, E2F1, E2F2, IKBKB, GSK3B*
7	05215	Prostate cancer	3.33E-21	25	*AR, MMP3, RELA, EGFR, AKT1, CCND1, BCL2, CDKN1A, CASP9, PLAU, MMP9, MAPK1, EGF, RB1, NFKBIA, RAF1, ERBB2, PLAT, GSTP1, CHUK, E2F1, E2F2, IKBKB, GSK3B, MTOR*
8	05225	Hepatocellular carcinoma	3.91E-14	24	*EGFR, AKT1, CCND1, BCL2L1, CDKN1A, BAX, MAPK1, RB1, ELK1, RAF1, PRKCA, HMOX1, MYC, PRKCB, GSTP1, NFE2L2, NQO1, E2F1, E2F2, IGF2, GSTM1, GSTM2, GSK3B, MTOR*
9	04668	TNF signaling pathway	2.32E-17	23	*PTGS2, IL6, MMP3, RELA, AKT1, FOS, MMP9, MAPK1, JUN, CASP3, NFKBIA, CASP8, ICAM1, IL1B, CCL2, SELE, VCAM1, CXCL2, CXCL10, CHUK, IRF1, IKBKB, MAPK8*
10	04657	IL-17 signaling pathway	7.05E-18	22	*PTGS2, IL6, MMP3, RELA, FOS, MMP9, MAPK1, JUN, CASP3, NFKBIA, CASP8, MMP1, IL1B, CCL2, CXCL8, IFNG, CXCL2, CXCL10, CHUK, IKBKB, MAPK8, GSK3B*
11	05164	Influenza A	4.33E-12	22	*IL6, PRSS1, RELA, AKT1, CASP9, MAPK1, JUN, NFKBIA, RAF1, PRKCA, STAT1, ICAM1, IL1B, CCL2, CXCL8, PRKCB, IFNG, IL1A, CXCL10, IKBKB, MAPK8, GSK3B*
12	05212	Pancreatic cancer	9.09E-19	21	*RELA, EGFR, AKT1, VEGFA, CCND1, BCL2L1, CDKN1A, BAX, CASP9, MAPK1, EGF, RB1, RAF1, STAT1, ERBB2, CHUK, E2F1, E2F2, IKBKB, MAPK8, MTOR*
13	04066	HIF-1 signaling pathway	5.70E-16	21	*IL6, RELA, EGFR, AKT1, VEGFA, BCL2, CDKN1A, MAPK1, EGF, PRKCA, HIF1A, ERBB2, HMOX1, PRKCB, NOS3, SERPINE1, IFNG, INSR, HK2, NOS2, MTOR*
14	05224	Breast cancer	1.75E-12	21	*PGR, ESR1, EGFR, AKT1, CCND1, FOS, CDKN1A, BAX, MAPK1, EGF, RB1, JUN, RAF1, ERBB2, MYC, E2F1, E2F2, GSK3B, NCOA1, ESR2, MTOR*

(*Continued*)

Table 3 (Continued).

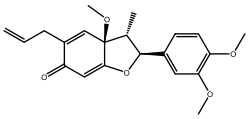
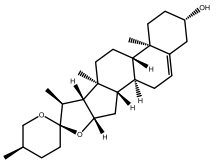
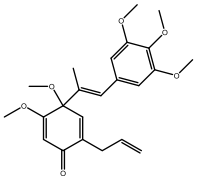
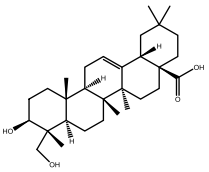
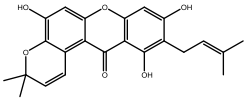
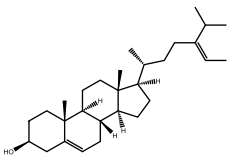
No.	Pathway ID	Pathway Name	*P* value	Count	Gene Name
15	01522	Endocrine resistance	5.32E-15	20	*ESR1, EGFR, AKT1, CCND1, BCL2, FOS, CDKN1A, BAX, MMP2, MMP9, MAPK1, RB1, JUN, RAF1, ERBB2, E2F1, E2F2, MAPK8, ESR2, MTOR*
16	05222	Small cell lung cancer	2.61E-14	19	*PTGS2, RXRA, RELA, AKT1, CCND1, BCL2, BCL2L1, CDKN1A, BAX, CASP9, RB1, CASP3, NFKBIA, MYC, CHUK, E2F1, E2F2, NOS2, IKBKB*
17	05142	Chagas disease (American trypanosomiasis)	1.87E-13	19	*IL6, RELA, AKT1, FOS, MAPK1, IL10, JUN, NFKBIA, CASP8, IL1B, CCL2, CXCL8, IL2, SERPINE1, IFNG, CHUK, NOS2, IKBKB, MAPK8*
18	04620	Toll-like receptor signaling pathway	2.25E-13	19	*IL6, CD14, LBP, RELA, AKT1, FOS, MAPK1, JUN, NFKBIA, CASP8, STAT1, IL1B, CXCL8, CXCL11, CXCL10, CHUK, SPP1, IKBKB, MAPK8*
19	05210	Colorectal cancer	8.32E-14	18	*EGFR, AKT1, CCND1, BCL2, FOS, CDKN1A, BAX, CASP9, MAPK1, EGF, JUN, CASP3, RAF1, MYC, BIRC5, MAPK8, GSK3B, MTOR*
20	04064	NF-kappa B signaling pathway	5.17E-13	18	*PTGS2, CD14, LBP, RELA, BCL2, BCL2L1, PLAU, NFKBIA, ICAM1, IL1B, VCAM1, CXCL8, PRKCB, PARP1, CXCL2, CHUK, CD40LG, IKBKB*

Table 4 Virtual Docking Of Ten Bioactive Ingredients From LWDH Pill For Type 2 Diabetes Mellitus Targets

No.	Compound	Structure	Binding Energy/(kcal mol^{-1})			
			AKT1	MAPK1	MAPK8	MMP9
1	Quercetin		−6.8181	−6.3394	−7.1100	−6.2667
2	Kaempferol		−6.5715	−6.3819	−6.3977	−5.9005
3	Stigmasterol		−6.8343	−7.9176	−5.5400	−6.2369
4	Beta-sitosterol		−6.8563	−7.6574	−5.5483	−6.0089

(*Continued*)

Table 4 (Continued).

No.	Compound	Structure	Binding Energy/(kcal mol^{-1})			
			AKT1	MAPK1	MAPK8	MMP9
5	Kadsurenone		−7.0874	−7.3170	−6.1939	−5.7586
6	Diosgenin		−5.5341	−7.1899	−5.3117	−6.1969
7	Hancinone C		−7.7971	−7.0791	−5.6103	−5.9619
8	Hederagenin		−3.7120	−6.3780	−5.3367	−5.3195
9	Garcinone B		−7.1429	−7.2783	−6.7518	−6.5401
10	Isofucosterol		−7.4410	−8.4075	−5.8677	−5.6977

Note: The combination with lower binding energy scores is more stable.

Additionally, previous reports have shown that vascular dysfunction of T2DM will also occur when EGFR tyrosine kinase activity is enhanced.[84,85] Patricia et al have demonstrated that use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in T2DM can significantly improve glucose tolerance, reduce insulin resistance, reduce circulating levels of TNF-α, IL-6, and decrease macrophage infiltration in free fatty acids.[86,87]

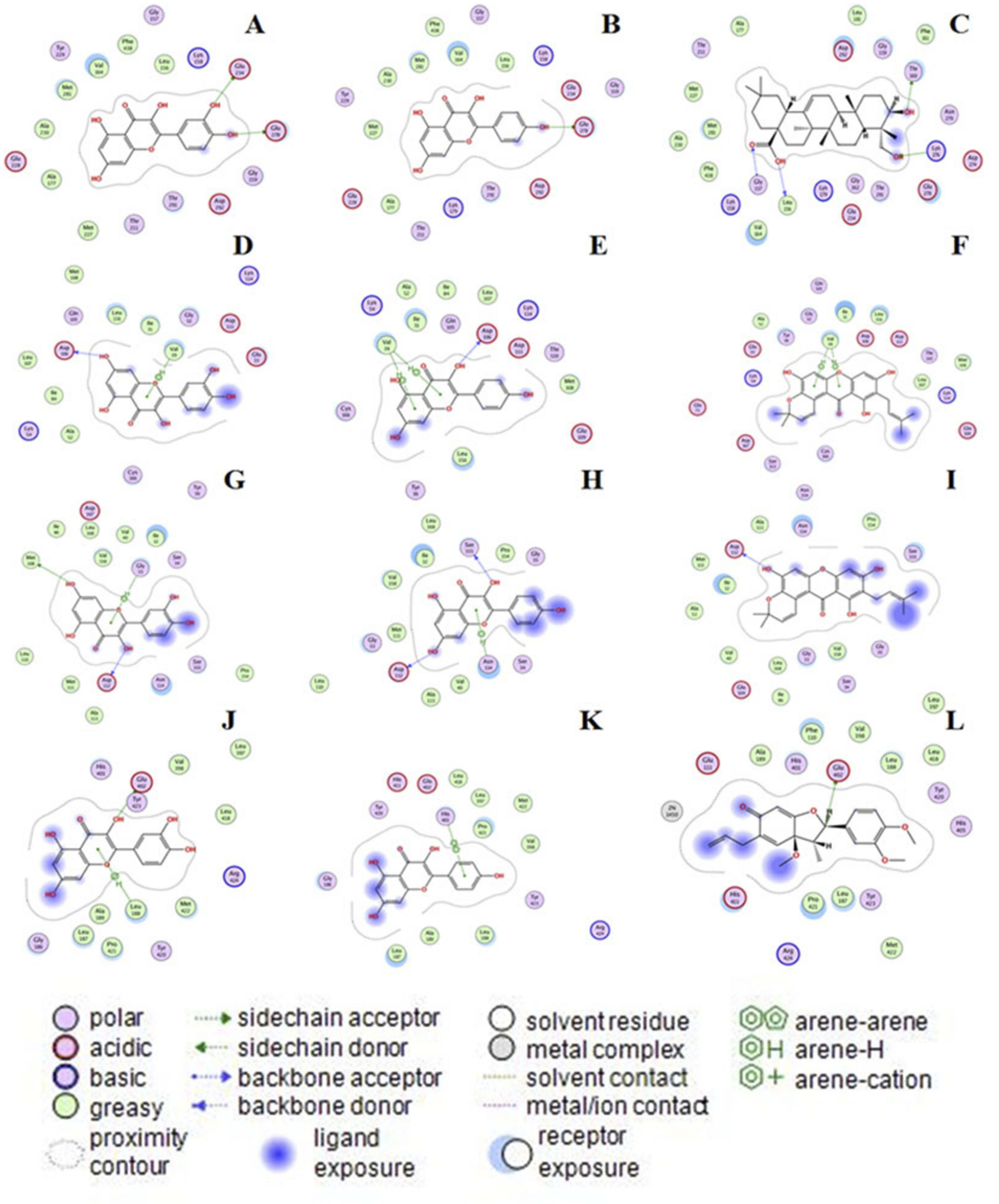


Figure 5 Virtual docking of bioactive ingredients from LWDH Pill for Type 2 diabetes mellitus targets. The virtual docking of quercetin with AKT1, MAPK1, MAPK8, and MMP9 was represented by **A**, **D**, **G**, and **J**, respectively. **C** stands for the docking of AKTI and Hederagenin. The virtual docking of Kaempferol with AKT1, MAPK1, MAPK8, and MMP9 was represented by **B**, **E**, **H**, and **K**, respectively. The virtual docking of Garcinone B with MAKP1 and MAKP8 was represented by F and I, respectively. L stands for the virtual docking of MMP9 and Kadsurenone.

Our research confirmed that LWDH Pill can achieve the potential characteristics of multi-target- multi-disease through the LWDH Pill-T2DM network.

Validation Of Compound–Target Interaction

Molecular docking was further applied in our study to assess the interaction between components and targets, reducing complexity and improving the accuracy of the constituent-target network. Virtual screening using MOE was performed to determine the binding affinity between protein models and 10 potentially active compounds (including Quercetin, Kaempferol, Stigmasterol, Beta-Sitosterol, Kadsurenone, Diosgenin, Hancinone C, Hederagenin, Garcinone B, Isofucosterol) obtained from compound–compound target network. Molecular docking analyzed four targets (AKT1, MAPK1, MAPK8 and MMP9) and 10 compounds (Table 4 and Figure 5). The results of these signal transductions have been reported to be the possible mechanism for LWDH Pill in treating diabetic complications.

The three-dimensional structures of the proteins 4kl, 3w55, 2g01, and 1gkc were obtained by searching AKT1, MAPK1, MAPK8, and MMP9 from the PDB protein database, respectively. Quercetin has been demonstrated to downregulate the expression of RNA and protein levels of MAPK, TNF-α, IL-6, and IL-1B in rat pancreatic histopathological damage.[88,89] The virtual docking of kaempferol with AKT1, MAPK1, MAPK8, and MMP9 was represented by B, E, H, and K, respectively. Kaempferol fixed the binding cavity of T2DM target AKT1 through H-bonds with residues Glu 278 (2.89 Å). Xu et al have validated that the anti-Diabetic retinopathy (DR) effect of Kaempferol (5–25 mM) was mediated via downregulating the expression of Erk1/2 and AKT1.[90] The complex Kaempferol–MAPK1 was stabilized by one H-bond and two π-H bonds with residues including Asp 106 (2.94 Å), Val 39 (4.41 Å), and Val 39 (3.86 Å), respectively, which further verifies Kaempferol in LWDH Pill anti-T2DM effects. Same as the complex of Kaempferol–MAPK8 was stabilized by one π-H and two H-bonds with residues including Ser 155 (2.90 Å), Asp 112 (2.84 Å), and Asn 114 (4.23 Å), respectively, and Kaempferol–MMP9 was stabilized by π-π bonds with residues HIS 401 (3.96 Å).

Conclusion

In the past three decades, the prevalence of T2DM, a constellation of metabolic aberrations, has risen dramatically worldwide.-[91] The global consensus is that the health goal by 2025 is to stop the rise of diabetes and obesity.[92] LWDH Pill is described as a commonly used drug for treatment and prevention of diabetes for thousands of years. Our research is the first report to explain the mechanisms of LWDH Pill and validate that LWDH Pill plays an active therapeutic role in T2DM, especially in diabetic complications (atherosclerosis and nephropathy). A total of 7822 genes were found related to T2DM by using bioinformatics analysis. Among them, 163 hub genes were selected; furthermore, AKT1, MAKP1, MAPK8, and MMP9 might be the core genes for LWDH Pill in treating T2DM and its complications. Although we have examined the possible role of LWDH Pill in treating T2DM, there are some limitations in the present study. Further in vitro and in vivo experimental validation is needed to support our research.

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Disclosure

The authors report no conflicts of interest in this work.

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