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ORIGINAL ARTICLE

Network pharmacology analysis of pharmacological mechanisms underlying the anti-type 2 diabetes mellitus effect of guava leaf

Li-Rong Jiang [a,b], **Yue Qin** [a,b,*], **Jia-Lin Nong** [a], **Hui An** [a,b,*]

[a] *College of Chemistry and Bioengineering, Hechi University, Hechi 546300, China*
[b] *Guangxi Key Laboratory of Microbiology and Plant Resources Development, Hechi 546300, China*

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Abstract The current study aimed to explore the anti-type 2 diabetes mellitus (T2DM) mechanism of guava leaf based on network pharmacology. The compounds contained in guava leaf was summarized from the literature, and a series of databases was used to identify the active components and corresponding potential targets. The intersection between diabetes-associated genes searched in the GeneCard database and the predicted targets of guava leaf active components was defined as target genes, which were then used to construct a "compound-active components-target genes" pharmacological network. The biological functions and pathway enrichment analyses of target genes were performed in KOBAS 3.0. The differential expression analysis of GSE76894 was performed to obtain the differential expressed genes (DEGs) in T2DM patients by comparing non-diabetic controls. Finally, the intersection between DEGs and target genes were named key genes, and the representative pathways in which these genes were involved were drawn through KEGG Mapper. We found that the active components of guava leaf may regulate the PI3K-AKT signaling pathway, T2DM regulation process, and insulin resistance pathway, which was evidenced by KEGG pathway analysis of key genes. These results implied that guava leaf has a potential anti-T2DM property and its mode of action may be exerted via regulating insulin secretion and reducing blood sugar level.

* Corresponding authors at: College of Chemistry and Bioengineering, Hechi University, No. 42 Longjiang Road, Yizhou district, Hechi 546300, Guangxi Zhuang Autonomous Region, China.
E-mail addresses: qinyuehechi@outlook.com (Y. Qin), anhuihcu@outlook.com (H. An).
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1. Introduction

Diabetes mellitus (DM) is a secretory and metabolic disease that seriously endangers human health. Such disease is characterized by hyperglycemia and disorder of carbohydrate, protein and fat metabolism, which are ascribed to abnormal insulin secretion, genetic factors and immune dysfunction (Petersmann et al,. 2019; Patel et al., 2012). At present, diabetes and its complications have become the third leading cause of human disability and death after tumors and cardiovascular diseases (Landgraf, 2000). The common clinical types of diabetes are type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus (GDM) (Petersmann et al., 2019). Compared with T1DM that is induced by autoimmune diseases, the incidence of T2DM has been continuously increasing. According to a previous report, approximately 170 million people were affected by T2DM worldwide, and the number of T2DM patients was expected to exceed 365 million by 2030 (Rathmann and Giani, 2004). Although the etiology and pathogenesis of T2DM remain unclear, insulin resistance was considered as a main pathological feature of T2DM patients (Guo et al., 2013). Therefore, to combat the T2DM, it is imperative to understand the mechanisms of insulin resistance and to discover new drugs that improve insulin sensitivity. Pharmacotherapy based on natural substances is considered a very promising therapeutic strategy for diabetes (Wińska et al., 2019), which might provide answers to the above questions.

Guava (*Psidium guajava*) is a famous tropical tree species, belonging to Myrtaceae family (Díaz-de-Cerio et al., 2017). Guava leaf extract contains a variety of effective components, including triterpenoids, flavonoids, tannins, sesquiterpenes, heteroterpenes, benzophenone glycosides, and meroterpenoid (Yang et al., 2007; de Souza et al., 2018). It was reported that guava leaf have many pharmacological effects such as hypoglycemic, anti-diarrheal, anti-oxidation, anti-tumor, anti-bacterial, and hypotensive activities (Gutiérrez et al., 2008; Naseer et al., 2018). A previous study in rat showed that the extract of guava leaf could improve insulin sensitivity and glucose metabolism by regulating insulin-related signal transduction (Guo et al., 2013). Similarly, through the T2DM rat model, researchers found that guava leaf extract could increase the activity of hepatic hexokinase (HKase), hepatic phosphofructokinase (PFKase) and hepatic glucose-6- phosphate dehydrogenase (G6PDHase) in the hepatic glycolysis pathway, thereby promoting hepatic glucose utilization (Shen et al., 2008). Moreover, due to the effectiveness and safety of guava leaf in treating T2DM, tea containing guava leaf extract was approved as a specific health food (beverage) in Japan in March 2000 (Deguchi and Miyazaki, 2010). However, the exact mechanism by which effective components of guava leaf work in concert to treat T2DM, has remained unclear. A meta-analysis conducted by Xu et al. evaluated the relationship between the intake of flavonoids and the risk of T2DM, and the results showed that participants with a high intake of flavonoids had a lower risk of T2DM (Xu et al., 2018). In addition, several previous studies have proved that triterpenoid might have anti-T2DM effects. For instance, Khanra et al. found that the triterpenoid from *Abroma augusta* leaf improved diabetic nephropathy in T2DM rats (Khanra et al., 2017). Besides, it was reported that triterpenoids in guava leaf could inhibit the enzymes involved in glucose metabolism, prevent the development of insulin resistance and normalize blood sugar/insulin levels aside from their hypolipidemic and anti-obesity activities (Nazaruk and Borzym-Kluczyk, 2015). Furthermore, through the experiments in mice, it was demonstrated that meroterpenoid (ganomycin I) could effectively reduce blood sugar, blood lipids, and promote insulin sensitivity (Wang et al., 2017).

The concept of network pharmacology has provided new ideas, theories and methods for the modern research on naturally-derived drugs (Hopkins, 2008). In recent years, network pharmacology has integrated bioinformatics, pharmacology, computer science and other multi-disciplinary tools to realize the network construction between naturally-derived drugs and diseases based on the interactions among effective components, functional pathways and target proteins, thereby providing a preliminarily perspective on the disease-specific pharmacological mechanism that support the development of new drugs (Hopkins, 2007; Luo et al., 2020).

As abovementioned, guava leaf contain many effective components, however, the underlying pharmacological mechanism has not been systematically analyzed. Therefore, through network pharmacology, this study aimed to explore the regulatory relationship between the main effective components in guava leaf and the corresponding target proteins in T2DM, in the hope of elucidating the underlying pharmacological mechanism.

2. Material and method

2.1. Database and software

2.1.1. Databases

The following databases were incorporated in the present study: database of traditional Chinese medicine systems pharmacology (TCMSP) (Ru et al., 2014), databases of substance and compound (PubChem) (Kim et al., 2016), pharmacophore mapping based web server for identifying potential drug target (PharmMapper) (Liu et al., 2010), the universal protein resource (UniProt) (UniProt, 2009), the human gene integrator (GeneCards) (Safran et al., 2010), the database for protein–protein interaction (PPI) networks (STRING) (Franceschini et al., 2013) and archive for high-throughput functional genomic data (GEO) (Barrett et al., 2009).

2.1.2. Softwares

Software for topological network analysis (Cytoscape 3.7.0) (Kohl et al., 2011), online interactive tool for drawing Venn diagram (Venny 2.1.0) (Oliveros, 2007), Gene ID conversion tool (g:Profiler) (Reimand et al., 2016), online software for functional enrichment and annotation (KOBAS 3.0) (Xie et al., 2011), pathway drawing tool (KEGG Mapper) (Kanehisa and Sato, 2020) were used in this current study.

2.2. Prediction of target genes of guava leaf active components

Active components of guava leaf and corresponding predicted targets were first retrieved from TCMSP according to a criterion of z′-score > 1.5. The 2- or 3-dimensional structures of candidate chemicals (defined in the previous step) were obtained from PubChem (Kim et al., 2016), and subsequently submitted to PharmMapper (Liu et al., 2010) for target prediction and classification of active compounds (they were roughly categorized into flavonoid, triterpenoid and meroterpenoid). The potential target genes of guava leaf components identified by both TCMSP and PubChem databases were used in our subsequent analyses.

2.3. Screening for potential target genes for guava leaf active components against T2DM

Keywords "type 2 diabetes mellitus" or "T2DM" were used as queries to search against GeneCards database, whereby known human genes associated with T2DM were retrieved. The search results were sorted by "relevance score" in descending order, the top 500 diabetes targets were filtered, and subsequently compared with the predicted targets of guava leaf

active components. In this way, the intersection between predicted targets of guava leaf active components and diabetes-associated genes, which might contribute to the anti-T2DM effect of guava leaf, were defined as target genes.

2.4. Construction of a "compound-active components-target genes" pharmacological network

Information concerning active compounds, their chemical classification and potential therapeutic targets in T2DM were visualized via a drug-target interaction network constructed using Cytoscape 3.7.0 (Kohl et al., 2011).

2.5. Topological analysis of the target genes

Next, the abovementioned target genes were submitted to STRING database, the protein–protein interaction (PPI) network was constructed according to the highest confidence level (interaction score > 0.9). The results of the STRING analysis were downloaded and visualized/analyzed locally using Cytoscape 3.7.0. In brief, two topological parameters "Node Degree Distribution" and "Betweenness Centrality" were calculated for each node, the former is proportional to the connections between indicated node and other nodes, whereas the latter indicates how frequent a node serves as a bridge between other two nodes. The resultant large network was subjected to analysis by another Cytoscape plug-in called "MCODE", whereby several sub-networks with stronger biological significance and central biological processes could be identified. In this way, pivotal players (we defined these genes as hub genes) in the PPI network that possess a high degree of connectivity and centrality were revealed.

2.6. GO and KEGG function enrichment analysis

Function enrichment analysis of target genes was performed based on GO terms and KEGG pathways using KOBAS 3.0 online tool (Xie et al., 2011). The results were downloaded and visualized using bubble plots.

2.7. Identification of differentially expressed gene (DEGs) in T2DM dataset from GEO

Keywords "type 2 diabetes" was used as a query to search against GEO database in compliance with the following criteria: 1, The experiments were associated with T2DM; 2, Number of experimental samples ≥ 10; 3, Availability of both T2DM and control samples. After defining control and case groups, online tool GEO2R was used for differential expression analysis. Then, after removal of replicated genes and genes with missing value, the remaining genes with p value < 0.05 were defined as DEGs

2.8. Drawing of pathway diagrams

The intersection of DEGs and target genes were named key genes, which deserves further investigation due to their strong biological significance. The key genes were mapped to KEGG database to retrieve the pathways in which the key genes were involved. Briefly, the HUGO IDs of key genes were first converted to Entrez IDs, and then imported into the pathway drawing tool (KEGG Mapper) (Kanehisa and Sato, 2020) to generate pathway diagrams, wherein up- and down-regulated key genes (obtained by DEG analysis) were colored yellow and red, respectively.

3. Results

3.1. Identification of targets for active components in guava leaf

According to our previous work (Jiang et al., 2020) and PubChem database, the chemical formula and structure of 68 active components were first downloaded and the corresponding *.sdf files were later input into the PharmMapper database to obtain the potential targets of active components of guava leaf. As a result, a total number of 530 predicted targets of guava leaf active components were identified at a threshold of z′-score ≥ 1.5, among which the numbers of targets of three predominant categories of compounds (triterpenoid, flavonoid, and meroterpenoid) were 411, 875, and 455, respectively. The information of the top 20 potential targets ordered by z′-score was shown in Table 1.

3.2. Identification of target genes for guava leaf active components against T2DM

Next, the gene name and UniProt ID of potential targets were obtained from UniProt database. A total of 500 diabetes-associated genes were obtained through searching the keywords "type 2 diabetes mellitus" or "T2DM" in the GeneCard database (**Supplementary** Table 1). As shown in Fig. 1, the intersection between the predicted targets of guava leaf active components ("Triterpenoid", "Meroterpenoid", and "Flavonoid") and diabetes-associated genes ("T2DM") was visualized by a Venn diagram. We obtained 179 potential targets for guava leaf active components against T2DM (the intersections between "Triterpenoid"/ "Meroterpenoid"/ "Flavonoid" and "T2DM"). These genes were defined as target genes. In addition, there were 73, 166 and 43 targets specific for guava leaf-derived triterpenoids, flavonoids and meroterpenoid in T2DM, respectively, accounting for 9.22%, 20.96% and 5.43% of the total number of T2DM-associated targets. The detailed information of the target genes was shown in Table 2.

3.3. Construction of a "compound-active components-target genes" pharmacological network

Cytoscape was used to visualize the interaction between the compounds, active components, and target genes involved in T2DM, with unconnected nodes being removed. The "compound-active components-target genes" pharmacological network (Fig. 2) included 249 nodes (3 compounds, 66 active components, 179 target genes) and 1880 edges. In the network, the red oval/blue diamond/green hexagonal nodes correspond to different guava leaf-derived compounds/active components/target genes. The average degree centrality of the blue nodes and the green nodes were 14 and 4, respectively, suggesting that one active component can potentially target multiple genes. The information of the active components and target genes with degree centrality > 10 was shown in Table 3.

Table 1 The information of the top 20 potential targets of guava leaf ordered by z′-score.

Compounds	Active components	Potential targets	Gene name	Normalized Fit Score	z′-score
Flavonoid	Apigenin	Cell division protein kinase 6	CDK6	0.9199	6.05915
Flavonoid	Kaempferol	Cell division protein kinase 6	CDK6	0.9249	5.94594
Flavonoid	Ononin	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	B3GAT1	0.7592	5.00886
Flavonoid	Morin-3-O-α-L-lyxopyranoside	Uridine-cytidine kinase 2	UCK2	0.7644	4.88183
Flavonoid	Quercetin	Cell division protein kinase 6	CDK6	0.8978	4.859
Flavonoid	Quercetin-3-O-(6″-feruloyl) -β-D-galactopyranoside	CD209 antigen	CD209	0.5827	4.84559
Flavonoid	Genistin	Aldehyde dehydrogenase, mitochondrial	ALDH2	0.9082	4.53315
Flavonoid	Isoquercitrin	Glucosamine-6-phosphate isomerase	GNPDA1	0.6748	4.49132
Flavonoid	Apigenin	cAMP-specific 3,5-cyclic phosphodiesterase 4D	PDE4B	0.6452	4.49097
Flavonoid	Daidzin	Aldehyde dehydrogenase, mitochondrial	ALDH2	0.8941	4.44701
Flavonoid	Kaempferol	cAMP-specific 3,5-cyclic phosphodiesterase 4D	PDE4B	0.6506	4.42388
Flavonoid	Daidzin	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	B3GAT1	0.7418	4.21266
Flavonoid	Ononin	Beta-hexosaminidase beta chain	HEXB	0.7022	4.14004
Flavonoid	Quercetin 3-O-beta-D-xylopyranoside	Histone acetyltransferase PCAF	KAT2B	0.4912	4.10724
Flavonoid	Reynoutrin	Carbonyl reductase [NADPH] 1	CBR1	0.8584	4.06041
Flavonoid	Apigenin	cGMP-specific 3,5-cyclic phosphodiesterase	PDE5A	0.531	3.97935
Flavonoid	Leucocyanidin	Glucocorticoid receptor	NR3C1	0.5577	3.97369
Flavonoid	Myricetin	Cell division protein kinase 6	CDK6	0.8552	3.9731
Flavonoid	Genistin	Galectin-3	LGALS3	0.7007	3.9062
Flavonoid	Kaempferol-3-glucoside	Cell division protein kinase 6	CDK6	0.9043	3.89581
Triterpenoid	Obtusinin	Eukaryotic translation initiation factor 4E	EIF4E	0.4978	4.10176
Triterpenoid	Goreishic acid I	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9576	3.70283
Triterpenoid	Uvoal	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9576	3.70283
Triterpenoid	Ilelatifol D	Epidermal growth factor receptor	EGFR	0.7682	3.43594
Triterpenoid	Obtusinin	Glutathione S-transferase A1	GSTA1	0.4895	3.42822
Triterpenoid	Psidiumoic acid	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PPP1CC	0.4055	3.42042
Triterpenoid	Corosolic acid	Trafficking protein particle complex subunit 3	TRAPPC3	0.8179	3.40365
Triterpenoid	Jacoumaric acid	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9251	3.39745
Triterpenoid	Asiatic acid	Interleukin-2	IL2	0.5246	3.37554
Triterpenoid	Corosolic acid	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9164	3.26152
Triterpenoid	Guavacoumaric acid	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9623	3.23795
Triterpenoid	Ilelatifol D	Sorbitol dehydrogenase	SORD	0.8007	3.16982
Triterpenoid	Isoneriucoumaric acid	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	0.9086	3.1421
Triterpenoid	2α-hydroxyoleanolic acid	Aldo-keto reductase family 1 member C3	AKR1C3	0.6586	3.10459
Triterpenoid	Guavanoic acid	cAMP-specific 3,5-cyclic phosphodiesterase 4D	PDE4B	0.7681	3.08659
Triterpenoid	Oleanolic acid	Trafficking protein particle complex subunit 3	TRAPPC3	0.7381	3.01578
Triterpenoid	Goreishic acid I	Sorbitol dehydrogenase	SORD	0.8779	2.99474
Triterpenoid	Uvoal	Sorbitol dehydrogenase	SORD	0.8779	2.99474
Triterpenoid	Guavanoic acid	Bile salt sulfotransferase	SULT2A1	0.9373	2.97436
Triterpenoid	Obtusinin	Eukaryotic translation initiation factor 4E	EIF4E	0.4978	4.10176
Meroterpenoid	Guajadial C	Adenosine kinase	ADK	0.746	4.81728
Meroterpenoid	Guajadial D	Adenosine kinase	ADK	0.746	4.81728
Meroterpenoid	Psidial C	Estrogen-related receptor gamma	ESRRG	0.7545	4.29261
Meroterpenoid	Psiguadial B	Bile acid receptor	NR1H4	0.6326	4.23767
Meroterpenoid	Guajadial	Renin	REN	0.5374	3.97044
Meroterpenoid	Guajadial B	Renin	REN	0.5374	3.97044
Meroterpenoid	Guavadial	Carbonic anhydrase 2	CA2	0.9737	3.8595
Meroterpenoid	Psiguadial D	Estradiol 17-beta-dehydrogenase 1	HSD17B1	0.8896	3.77446
Meroterpenoid	Psiguadial A	Histo-blood group ABO system transferase	ABO	0.6709	3.73128
Meroterpenoid	Psidial C	E3 ubiquitin-protein ligase Mdm2	MDM2	0.7637	3.72858
Meroterpenoid	Psidial B	Angiogenin	ANG	0.8709	3.47879
Meroterpenoid	Psiguadial C	Retinoic acid receptor gamma	RARG	0.6494	3.47538
Meroterpenoid	Psidial A	Nuclear receptor subfamily 1 group I member 3	NR1I3	0.6745	3.3536
Meroterpenoid	Guajadial E	Glutathione S-transferase A1	GSTA1	0.696	3.32108

Table 1 (*continued*)

Compounds	Active components	Potential targets	Gene name	Normalized Fit Score	z′-score
Meroterpenoid	Guajadial F	Glutathione S-transferase A1	GSTA1	0.696	3.32108
Meroterpenoid	Guajadial	Estrogen sulfotransferase	SULT1E1	0.6468	3.28588
Meroterpenoid	Guajadial B	Estrogen sulfotransferase	SULT1E1	0.6468	3.28588
Meroterpenoid	Psiguadial C	E3 ubiquitin-protein ligase Mdm2	MDM2	0.7796	3.26007
Meroterpenoid	Diguajadial	Interleukin-2	IL2	0.566	3.23955
Meroterpenoid	Guajadial C	Adenosine kinase	ADK	0.746	4.81728

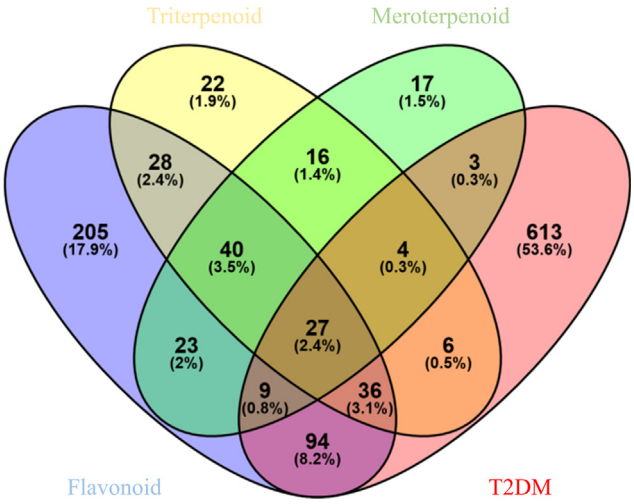

Fig. 1 The overlap of predicted target genes of guava leaf active components and diabetes genes.

3.4. Construction of the PPI network of target genes

The PPI network of 179 target genes was constructed using STRING database and the resultant *.TSV file was visualized by Cytoscape. As shown in Fig. 3, the network contained 166 nodes (13 nodes were removed due to the absence of biological interactions) and 961 edges, in which the thickness of the edge was proportional to the strength of protein interaction. A total of 37 hub genes, whose degrees and betweenness centrality exceeded the average, were identified in the topological network (colored in red). Sub-networks with stronger biological significance were identified by Cytoscape plug-in "MCODE", among which the largest sub-network was visualized by setting the shape of each sub-network member to triangle. The network topology parameters of the 37 hub genes were shown in Supplementary Table 2.

3.5. Functional enrichment analysis

To investigate the biological functions of target genes, we performed GO and KEGG functional enrichment analysis using KOBAS 3.0. The enriched biological functions of the target genes in 3 manually curated categories "Signal transduction pathways", "Metabolism process" and "Other processes" were shown in Fig. 4 **A-C**. The full list of enriched pathways of 179 target genes was provided in **Supplementary Table 4**. Moreover, the results of function enrichment analyses of all sub-networks (identified by "MCODE" in previous step) were provided in Supplementary Table 3. The most representative pathways were "regulation of the metabolic process", "regulation of leukocyte proliferation", "positive regulation of defense", and "regulation of signaling pathway".

3.6. Data processing and analysis of GSE76894

The differential expression analysis of the GSE76894 data was performed by GEO2R in NCBI. The DEGs were identified with a threshold of $P < 0.05$. As shown in Fig. 5**A**, there was a 49-gene overlap between DEGs and target genes, and these genes were defined as key genes. The expression profile of the 49 key genes were shown in Fig. 5**B**; and 13 genes (*SOD2, INSR, HSPB1, CCL2, CRP, RELA, EP300, GJA1, F3, TGFB2, TGFBR1, MET, VCAM1*) were significantly up-regulated and 4 genes (*INS, SOD1, RBP4, TIMP1*) were significantly down-regulated. The function enrichment (GO/KEGG) analyses of the 49 key genes were shown in Fig. 6. The most representative GO-BP terms (Fig. 6**A**) were "positive regulation of establishment of protein localization" and "positive regulation of protein transport" while the most representative GO-CC terms (Fig. 6**B**) were "membrane region", "membrane microdomain" and "membrane raft". The most representative GO-MF terms (Fig. 6**C**) were "signaling receptor activator activity" and "receptor ligand activity" while the most representative KEGG pathways (Fig. 6**D**) were "MAPK signaling pathway" and "AGE-RAGE signaling pathway".

3.7. Pathway analyses of key genes

To further investigate the way by which key genes contribute to the anti-T2DM effect of guava leaf, we uploaded the Entrez IDs of 49 key genes to the KEGG Mapper for pathway analyses. The pathway diagram of one of the most representative pathway, namely PI3K-AKT pathway, was shown in Fig. 7, which demonstrated the involvement of 13 key genes through Toll-like receptor, B cell receptor, JAK/STAT, Focal adhesion and Chemokine signaling pathways; up- or down-regulated key genes were colored by yellow or red background, respectively. In the T2DM regulation process (**Supplementary figure 1**), 4 genes were found in the regulation of adipocytokine signaling pathway, insulin pathway, and mitochondria pathway. We also found 8 genes that participated in the regulation of the insulin resistance pathway (**Supplementary figure 2**), including the biological processes of muscle cell, liver cell, and O-GlcNAc regulation of insulin resistance. The corresponding detailed information was summarized in **Supplementary Table 5.**

Table 2 The information of 179 target genes.

NO.	Potential targets	Gene name	UniProt ID
1	Acetyl-CoA carboxylase 1	ACACA	Q13085
2	Acetylcholinesterase	ACHE	P22303
3	Beta-2 adrenergic receptor	ADRB2	P07550
4	Aldose reductase	AKR1B1	P15121
5	RAC-alpha serine/threonine-protein kinase	AKT1	P31749
6	RAC-beta serine/threonine-protein kinase	AKT2	P31751
7	Aldehyde dehydrogenase, mitochondrial	ALDH2	P05091
8	Arachidonate 5-lipoxygenase	ALOX5	P09917
9	Intestinal-type alkaline phosphatase	ALPI	P09923
10	Annexin A5	ANXA5	P08758
11	Adenomatous polyposis coli protein	APC	P25054
12	Apolipoprotein A-I	APOA1	P02647
13	Apolipoprotein B-100	APOB	P04114
14	Androgen receptor	AR	P10275
15	Serine-protein kinase ATM	ATM	Q13315
16	Apoptosis regulator BAX	BAX	Q07812
17	Cholinesterase	BCHE	P06276
18	Apoptosis regulator Bcl-2	BCL2	P10415
19	Bcl-2-like protein 1	BCL2L1	Q07817
20	Baculoviral IAP repeat-containing protein 5	BIRC5	O15392
21	Caspase-3	CASP3	P42574
22	Caspase-8	CASP8	Q14790
23	Catalase	CAT	P04040
24	Caveolin-1	CAV1	Q03135
25	C-C motif chemokine 2	CCL2	P13500
26	G1/S-specific cyclin-D1	CCND1	P24385
27	CD209 antigen	CD209	Q9NNX6
28	Platelet glycoprotein 4	CD36	P16671
29	CD40 ligand	CD40LG	P29965
30	Cell division protein kinase 4	CDK4	P11802
31	Cyclin-dependent kinase inhibitor 1	CDKN1A	P38936
32	Cystic fibrosis transmembrane conductance regulator	CFTR	P13569
33	Serine/threonine-protein kinase Chk2	CHEK2	O96017
34	Collagen alpha-1(I) chain	COL1A1	P02452
35	Carnitine O-palmitoyltransferase 1, liver isoform	CPT1A	P50416
36	C-reactive protein	CRP	P02741
37	Cathepsin D	CTSD	P07339
38	C-X-C motif chemokine 10	CXCL10	P02778
39	Interleukin-8	CXCL8	P10145
40	Cytochrome *c*	CYCS	P99999
41	Cytochrome P450 19A1	CYP19A1	P11511
42	Cytochrome P450 1A1	CYP1A1	P04798
43	Cytochrome P450 1A2	CYP1A2	P05177
44	Steroid 21-hydroxylase	CYP21A2	P08686
45	Cytochrome P450 2C8	CYP2C8	P10632
46	Cytochrome P450 2C9	CYP2C9	P11712
47	Cytochrome P450 3A4	CYP3A4	P08684
48	Dipeptidyl peptidase 4	DPP4	P27487
49	Pro-epidermal growth factor	EGF	P01133
50	Epidermal growth factor receptor	EGFR	P00533
51	Histone acetyltransferase p300	EP300	Q09472
52	Receptor tyrosine-protein kinase erbB-2	ERBB2	P04626
53	Estrogen receptor	ESR1	P03372
54	Prothrombin	F2	P00734
55	Tissue factor	F3	P13726
56	Coagulation factor VII	F7	P08709
57	Fatty acid-binding protein, adipocyte	FABP4	P15090
58	Tumor necrosis factor ligand superfamily member 6	FASLG	P48023
59	Fatty acid synthase	FASN	P49327
60	Heparin-binding growth factor 2	FGF2	P09038
61	Basic fibroblast growth factor receptor 1	FGFR1	P11362
62	Fibronectin	FN1	P02751
63	Glucose-6-phosphatase	G6PC	P35575

Table 2 (*continued*)

NO.	Potential targets	Gene name	UniProt ID
64	Glucosylceramidase	GBA	A0A068F658
65	Vitamin D-binding protein	GC	P02774
66	Glucokinase	GCK	P35557
67	Glial fibrillary acidic protein	GFAP	P14136
68	Somatotropin	GH1	P01241
69	Growth hormone receptor	GHR	P10912
70	Gap junction alpha-1 protein	GJA1	P17302
71	Beta-galactosidase	GLB1	P16278
72	Glycogen synthase kinase-3 beta	GSK3B	P49841
73	Glutathione S-transferase Mu 1	GSTM1	P09488
74	Glutathione S-transferase P	GSTP1	P09211
75	Beta-hexosaminidase subunit beta	HEXB	P07686
76	Hexokinase-1	HK1	P19367
77	Hexokinase-2	HK2	P52789
78	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	P04035
79	Heme oxygenase 1	HMOX1	P09601
80	GTPase HRas	HRAS	P01112
81	Corticosteroid 11-beta-dehydrogenase isozyme 1	HSD11B1	P28845
82	Heat shock protein beta-1	HSPB1	P04792
83	Intercellular adhesion molecule 1	ICAM1	P05362
84	Interferon gamma	IFNG	P01579
85	Insulin-like growth factor 1 receptor	IGF1R	P08069
86	Insulin-like growth factor II	IGF2	P01344
87	Insulin-like growth factor-binding protein 3	IGFBP3	P17936
88	Inhibitor of nuclear factor kappa-B kinase subunit beta	IKBKB	O14920
89	NF-kappa-B essential modulator	IKBKG	Q9Y6K9
90	Interleukin-10	IL10	P22301
91	Interleukin-1 alpha	IL1A	P01583
92	Interleukin-1 beta	IL1B	P01584
93	Interleukin-2	IL2	P60568
94	Interleukin-4	IL4	P05112
95	Interleukin-6	IL6	P05231
96	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2	INPPL1	O15357
97	Insulin	INS	P01308
98	Insulin receptor	INSR	P06213
99	Tyrosine-protein kinase JAK2	JAK2	O60674
100	Transcription factor AP-1	JUN	P05412
101	Potassium voltage-gated channel subfamily H member 2	KCNH2	Q12809
102	ATP-sensitive inward rectifier potassium channel 11	KCNJ11	Q14654
103	Vascular endothelial growth factor receptor 2	KDR	P35968
104	Neutrophil gelatinase-associated lipocalin	LCN2	P80188
105	Low-density lipoprotein receptor	LDLR	P01130
106	Dual specificity mitogen-activated protein kinase kinase 1	MAP2K1	Q02750
107	Mitogen-activated protein kinase 1	MAPK1	P28482
108	Mitogen-activated protein kinase 14	MAPK14	Q16539
109	Mitogen-activated protein kinase 3	MAPK3	P27361
110	Mitogen-activated protein kinase 8	MAPK8	P45983
111	Hepatocyte growth factor receptor	MET	P08581
112	Interstitial collagenase	MMP1	P03956
113	72 kDa type IV collagenase	MMP2	P08253
114	Stromelysin-1	MMP3	P08254
115	Neutrophil collagenase	MMP8	P22894
116	Matrix metalloproteinase-9	MMP9	P14780
117	Myeloperoxidase	MPO	P05164
118	Microsomal triglyceride transfer protein large subunit	MTTP	P55157
119	Myc proto-oncogene protein	MYC	P01106
120	Nuclear factor erythroid 2-related factor 2	NFE2L2	Q16236
121	NF-kappa-B inhibitor alpha	NFKBIA	P25963
122	Nitric oxide synthase, inducible	NOS2	P35228
123	Nitric-oxide synthase, endothelial	NOS3	P29474
124	NAD(P)H dehydrogenase [quinone] 1	NQO1	P15559
125	Oxysterols receptor LXR-beta	NR1H2	P55055
130	Progesterone receptor	PGR	P06401

(*continued on next page*)

Table 2 (*continued*)

NO.	Potential targets	Gene name	UniProt ID
131	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit, gamma isoform	PIK3CG	P48736
132	Phosphatidylinositol 3-kinase regulatory subunit alpha	PIK3R1	P27986
133	Tissue-type plasminogen activator	PLAT	P00750
134	Urokinase-type plasminogen activator	PLAU	P00749
135	Serum paraoxonase/arylesterase 1	PON1	P27169
136	Peroxisome proliferator-activated receptor alpha	PPARA	Q07869
137	Peroxisome proliferator-activated receptor delta	PPARD	Q03181
138	Peroxisome proliferator activated receptor gamma	PPARG	P37231
139	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	PPARGC1B	Q86YN6
140	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	PTEN	P60484
141	Cystathionine beta-synthase	PTGS1	P23219
142	Prostaglandin G/H synthase 2	PTGS2	P35354
143	Tyrosine-protein phosphatase non-receptor type 1	PTPN1	P18031
144	Tyrosine-protein phosphatase non-receptor type 11	PTPN11	Q06124
145	Retinol-binding protein 4	RBP4	P02753
146	Lithostathine-1-alpha	REG1A	P05451
147	Transcription factor p65	RELA	Q04206
148	Renin	REN	P00797
149	Transforming protein RhoA	RHOA	P61586
150	Runt-related transcription factor 2	RUNX2	Q13950
151	Retinoic acid receptor alpha/Retinoic acid receptor beta	RXRA	P19793
152	Protein S100-A9	S100A9	P06702
153	E-selectin	SELE	P16581
154	P-selectin	SELP	P16109
155	Plasminogen activator inhibitor 1	SERPINE1	P05121
156	Sex hormone-binding globulin	SHBG	P04278
157	NAD-dependent deacetylase sirtuin-1	SIRT1	Q96EB6
158	Solute carrier family 2,facilitated glucose transporter member 4	SLC2A4	P14672
159	Sodium-dependent serotonin transporter	SLC6A4	P31645
160	Superoxide dismutase [Cu-Zn]	SOD1	P00441
161	Superoxide dismutase [Mn], mitochondrial	SOD2	P04179
162	Osteopontin	SPP1	P10451
163	Proto-oncogene tyrosine-protein kinase Src	SRC	P12931
164	Signal transducer and activator of transcription 1-alpha/beta	STAT1	P42224
165	Signal transducer and activator of transcription 3	STAT3	P40763
166	Angiopoietin-1 receptor	TEK	Q02763
167	Transforming growth factor beta-1	TGFB1	P01137
168	Transforming growth factor beta-2	TGFB2	P61812
169	TGF-beta receptor type-1	TGFBR1	P36897
170	Tyrosine 3-monooxygenase	TH	P07101
171	Thrombomodulin	THBD	P07204
172	Metalloproteinase inhibitor 1	TIMP1	P01033
173	Tumor necrosis factor	TNF	P01375
174	Cellular tumor antigen p53	TP53	P04637
175	Triosephosphate isomerase	TPI1	P60174
176	UDP-glucuronosyltransferase 1–1	UGT1A1	P22309
177	Vascular cell adhesion protein 1	VCAM1	P19320
178	Vitamin D3 receptor	VDR	P11473
179	Vascular endothelial growth factor A	VEGFA	P15692

4. Discussion

Mounting evidence suggested that guava leaf have potential against T2DM (Okpashi et al., 2014; Shen et al., 2008; Shabbir et al., 2020), whereas the underlying mechanism remains obscure. This study explored the mechanism underlying the efficacy of guava leaf component in the treatment of T2DM with the help of network pharmacology. Initially, a total of 530 target genes of 68 guava leaf active compounds were obtained from the TCMSP and PharmMapper database. Subsequently, 179 target genes were obtained by intersecting the acquired guava leaf targets with T2DM-asscociated genes obtained from the GeneCard database. Then, a pharmacological network of guava leaf active components and target genes was constructed using Cytoscape and the results showed that target genes could be co-regulated by multiple active components. This finding indicated that guava leaf have the characteristics common to Chinese medicine in the treatment of T2DM, such as multi-component and multi-target effects, which was consistent with the research strategy based on network pharmacology (Zhou et al., 2020).

To summarize the collective effect of target genes at the protein level, and explain the treatment effect of guava leaf against T2DM, we constructed a ‘‘compound-active

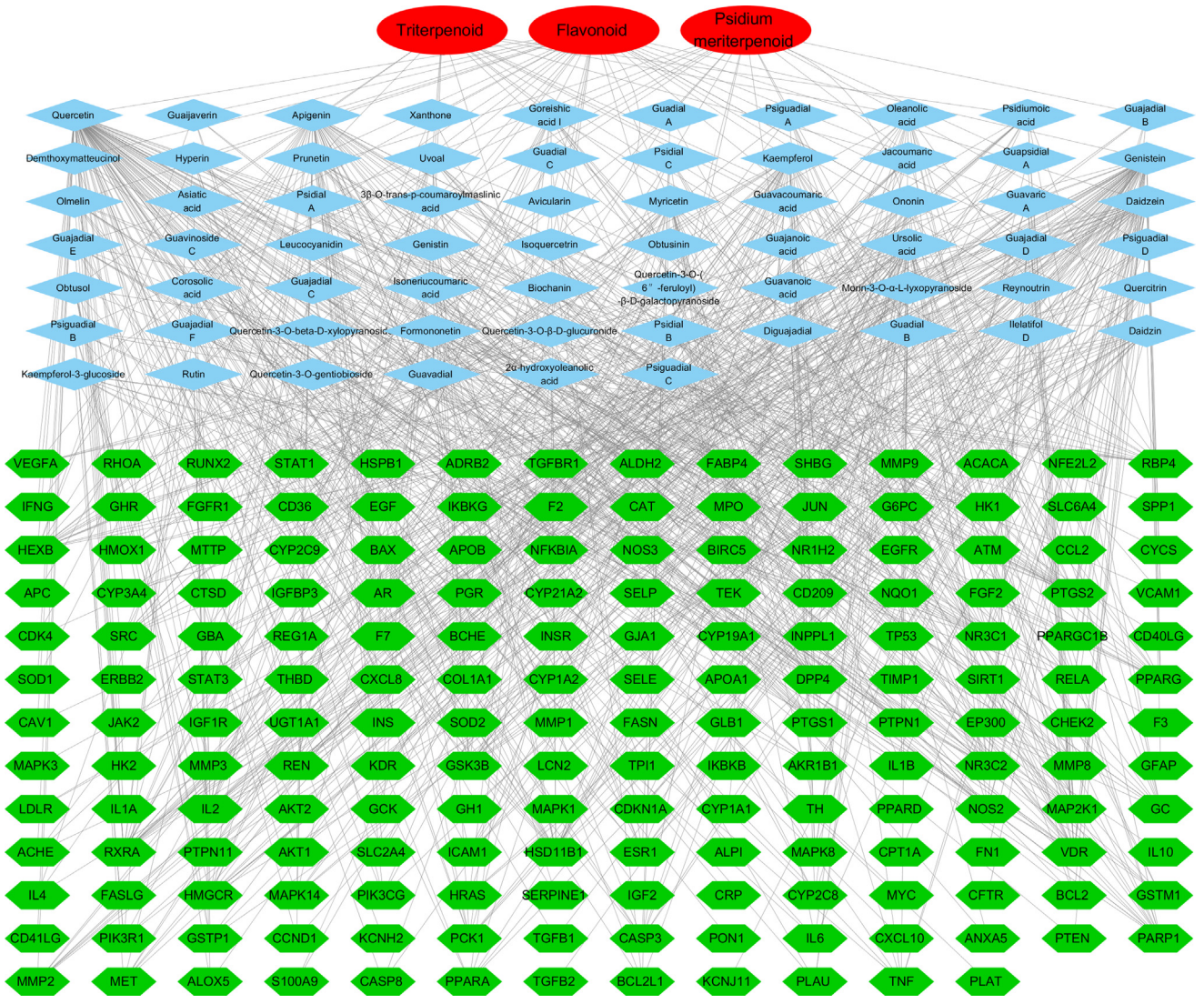

Fig. 2 The "compound-active components-target genes" pharmacological network.

components-target genes" pharmacological network based on 68 guava leaf active components and target genes using the STRING database. The network showed that guava leaf are closely related to various target proteins in T2DM, suggesting that the therapeutic efficacy of guava leaf in the treatment of T2DM depends on collective effect of multiple target proteins, rather than the regulation of a single target. Here, we selected the top 5 active components according to their degree within the PPI network, and subsequently identified their target proteins, which included RXRA, HSD11B1, VDR, NR3C1, DPP4, PTGS2, and IL2. RXRA is a retinoid-X receptor that involves in the regulation of metabolic processes including lipid metabolism (Stossi et al., 2019). Several studies revealed that the methylation of RXRA was closely related to diabetes (Franzago et al., 2019; Castellano-Castillo et al., 2019). HSD11B1 is generally distributed in the liver and adipose, where it functions as the gate-keeper of activity levels of local glucocorticoid (Chapman et al., 2013). A previous study conducted by Masuzaki and co-workers demonstrated that HSD11B1 overexpression in mice can lead to dyslipidemia, visceral obesity, and even insulin-resistant diabetes (Masuzaki et al., 2001), highlighting that Hsd11b1 is closely related to the development of adverse metabolic features. As known, VDR is involved in maintaining insulin secretion and vitamin D metabolism process (Zeitz et al., 2003; Ogunkolade et al., 2002). Recently, an increasing body of literature reported that polymorphisms in the VDR gene might significantly reduce both mRNA and protein levels of VDR, which increases the risk of diabetes development (Angel et al., 2018; Xia et al., 2017). As a key regulatory enzyme of the incretin system, DPP4 has gained interest in the treatment of T2DM in the last decade (Röhrborn et al., 2015). It has been confirmed that DPP4 inhibitor not only improve glycemic control, but also preserve β-cell function in T2DM, highlighting that DDP4 inhibitor is a novel and promising anti-diabetic agent (Wang et al., 2018). Therefore, we speculated that guava leaf might play a therapeutic role in T2DM by regulating levels of insulin, vitamin D and DPP4, as well as retinoid-X receptor expression. Besides, there is few studies about the association between T2DM and several other guava leaf target proteins including PTGS2, NR3C1 and IL2. We suggested that these proteins might be used as novel targets in T2DM therapy.

Table 3 The information of the active components and target genes with degree > 10 in the pharmacological network.

NO	Name	Composition type	Degree	Category
1	Quercetin	Flavonoid	94	Compound
2	Genistein	Flavonoid	60	Compound
3	Apigenin	Flavonoid	46	Compound
4	Ursolic acid	Triterpenoid	46	Compound
5	Daidzein	Flavonoid	41	Compound
6	Kaempferol	Flavonoid	38	Compound
7	Myricetin	Flavonoid	28	Compound
8	Formononetin	Flavonoid	23	Compound
9	Daidzin	Flavonoid	20	Compound
10	Ononin	Flavonoid	19	Compound
11	Oleanolic acid	Triterpenoid	18	Compound
12	Prunetin	Flavonoid	16	Compound
13	Rutin	Flavonoid	16	Compound
14	Isoquercetrin	Flavonoid	15	Compound
15	Goreishic acid I	Triterpenoid	15	Compound
16	Uvoal	Triterpenoid	15	Compound
17	Psidiumoic acid	Triterpenoid	14	Compound
18	Genistin	Flavonoid	13	Compound
19	Quercitrin	Flavonoid	13	Compound
20	2α-hydroxyoleanolic acid	Triterpenoid	13	Compound
21	Guavacoumaric acid	Triterpenoid	13	Compound
22	Olmelin	Flavonoid	12	Compound
23	Guavaric A	Flavonoid	12	Compound
24	Hyperin	Flavonoid	12	Compound
25	Guavanoic acid	Triterpenoid	12	Compound
26	Quercetin-3-O-β-D-glucuronide	Triterpenoid	11	Compound
27	Corosolic acid	Triterpenoid	11	Compound
28	Isoneriucoumaric acid	Triterpenoid	11	Compound
29	Psidial B	Meroterpenoid	11	Compound
30	Guajadial C	Meroterpenoid	11	Compound
31	Guajadial D	Meroterpenoid	11	Compound
32	RXRA	–	23	Target
33	HSD11B1	–	21	Target
34	VDR	–	21	Target
35	NR3C1	–	20	Target
36	DPP4	–	19	Target
37	PTGS2	–	19	Target
38	IL2	–	18	Target
39	NR3C2	–	17	Target
40	HEXB	–	17	Target
41	SHBG	–	16	Target
42	GSK3B	–	15	Target
43	PPARG	–	14	Target
44	PARP1	–	14	Target
45	CD209	–	14	Target
46	RBP4	–	13	Target
47	HMGCR	–	13	Target
48	PTGS1	–	13	Target
49	MAP2K1	–	13	Target
50	REN	–	12	Target
51	GC	–	12	Target
52	NOS2	–	12	Target
53	AR	–	12	Target
54	ESR1	–	11	Target

Then, we applied the Cytoscape software and KOBAS 3.0 platform to carry out functional enrichment analysis of target genes. Results of KEGG pathway analysis indicated that these genes were mainly enriched in several biological processes including the metabolic pathway, apoptosis, insulin resistance, and PI3K-Akt signaling pathways, implying that the anti-T2MD role of guava leaf might be achieved by regulation of these processes.

To further improve the biological significance of the currently identified 179 target genes, the intersection between DEGs (resulting from differential expression analysis of T2DM patients versus non-diabetic controls) and target genes

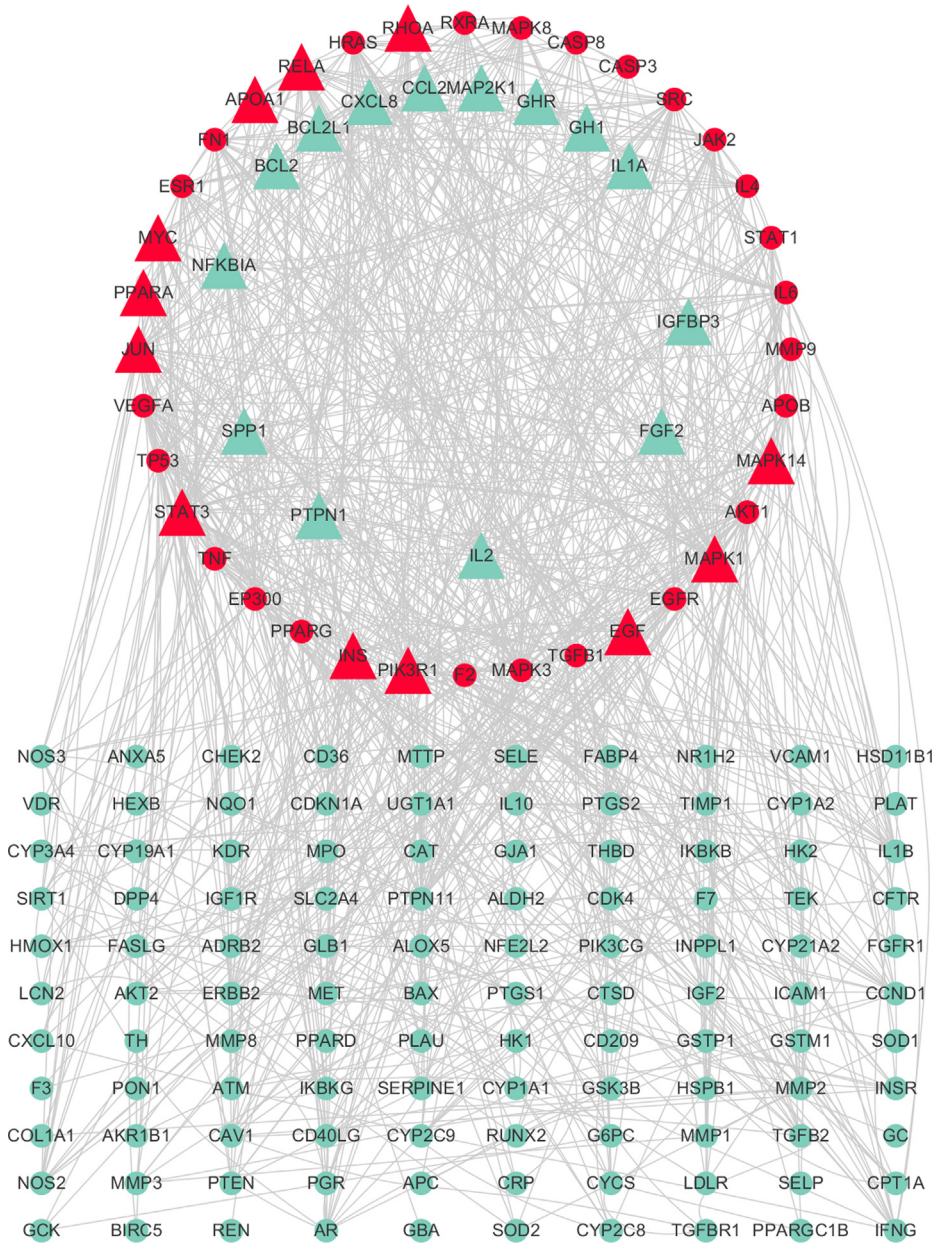

Fig. 3 The PPI network of 179 target genes.

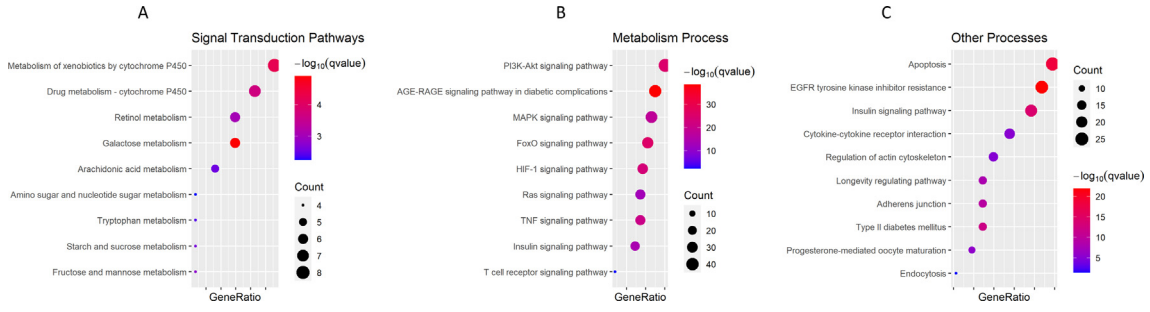

Fig. 4 **GO analysis of target genes (A)** Metabolic process. **(B)** Signal transduction pathways. **(C)** Other processes.

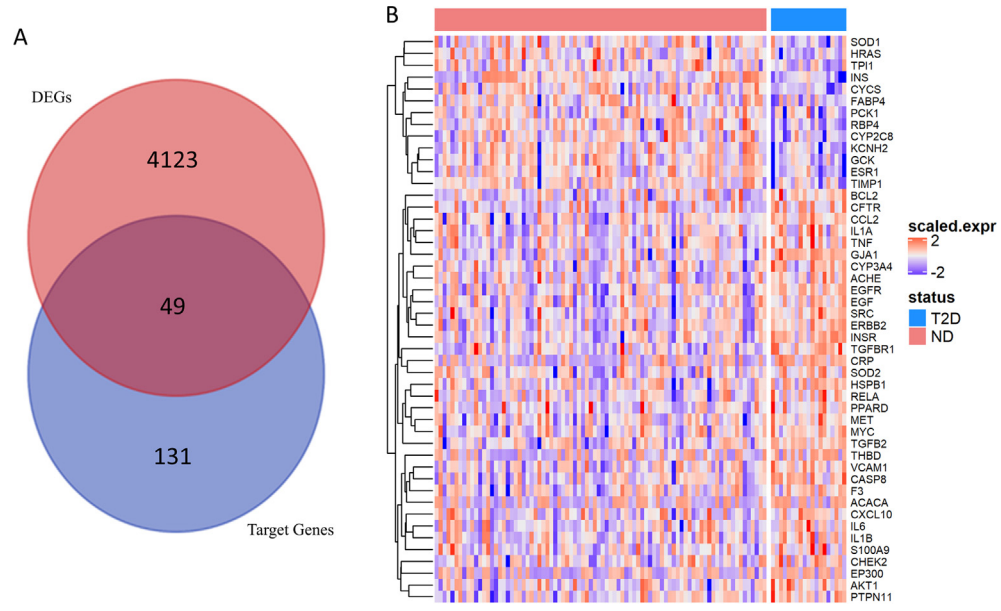

Fig. 5 Data processing and analysis of GSE76894. (A) Venn diagram showing the overlaps between DEGs and target genes. **(B)** Heatmap showing Z-score scaled expression profile of 49 key genes in GSE76894. A red-blue gradient signifies whether a gene is up- or down-regulated.

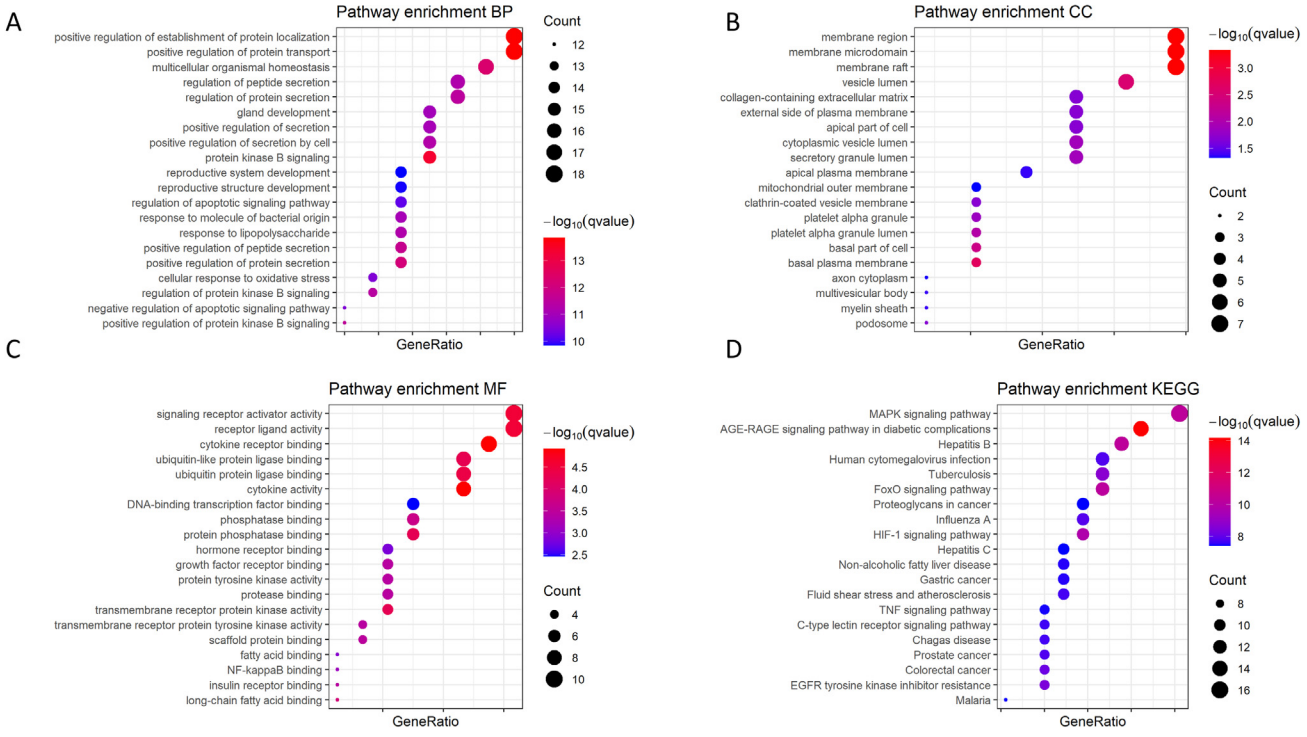

Fig. 6 Functional enrichment analysis of key genes (A) Biological process. **(B)** Cellular Component. **(C)** Molecular function. **(D)** KEGG enrichment.

was defined as key genes and used in subsequent analyses. Briefly, bioinformatics tool KEGG Mapper was used to depict how these key genes contribute to the anti-T2DM effect of guava leaf on the signaling pathway level. As the primary signal transduction pathway of insulin, the PI3K/AKT signaling pathway has been proved to reinforce the sensitivity of insulin and exert a role in regulating sugar lipid metabolism (Gao et al., 2019). Many studies revealed that amounts of active compounds of traditional Chinese medicine show therapeutic effect against diabetes via PI3K/Akt signaling pathway (Chan and Ye, 2013; Li et al., 2014; Dai et al., 2016). Given that insulin resistance would cause β-cell dys-

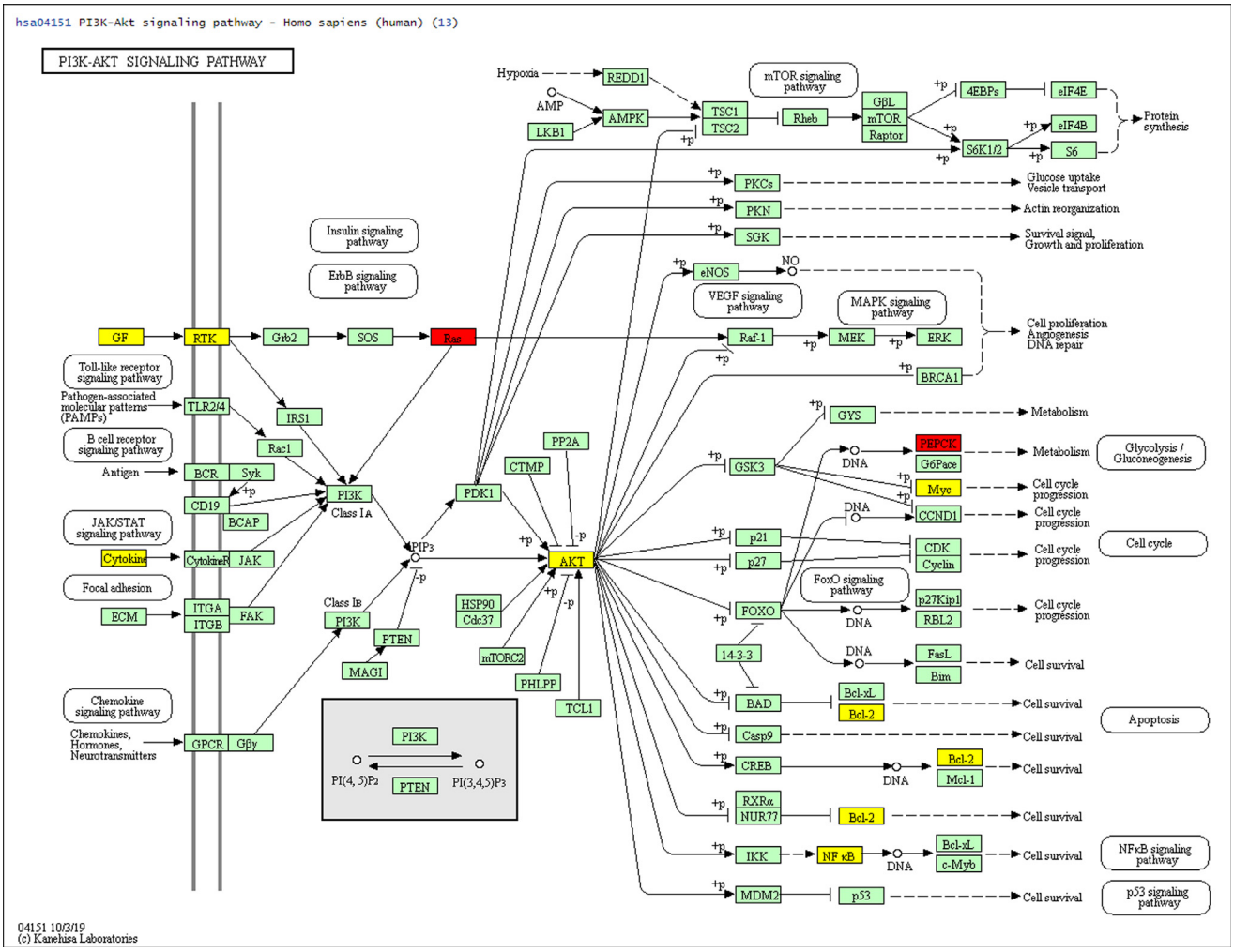

Fig. 7 Pathway diagram of PI3K-AKT pathway and the involvement of 13 key genes.

function even in the development of T2DM, targeting insulin resistance was proved to be a valuable therapeutic strategy for ameliorating the progression of T2DM (Arnold et al., 2018). Combined with our current findings and previous literature, we selected three signaling pathways including PI3K/Akt signaling, T2DM, and insulin resistance pathway for further interpretation. Functional enrichment analysis showed that the 49 key genes mainly participated in "mitogen-activated protein kinase (MAPK) signaling pathway" and "AGE-RAGE signaling pathway in diabetic complications. MAPK has been proposed to maintain glucose homeostasis (Schultze et al., 2012), and is therefore associated with obesity and diabetes. The "AGE-RAGE signaling pathway" is a well-studied regulatory axis in diabetes (Kay et al., 2016). Specifically, RAGE is accountable for perturbed myocardial functions in diabetes; it also promotes other cardiovascular diseases such as atherosclerosis in the context of diabetes (Ramasamy et al., 2011). The present results indicated that the key genes are highly involved in diabetes and are promising candidate targets for guava leaf, and, thus, deserve further investigation.

Finally, representative diagrams of pathways where key genes were involved were drawn, among 49 key genes, 13 were found in PI3K/Akt signaling pathway, whereas 4 and 8 were found in the T2DM pathway and insulin resistance pathway, respectively. These results suggested that guava leaf is effective against T2DM mainly through regulating the PI3K/Akt signaling pathway. Notably, one of the diabetes specific targets of guava leaf active components, PI3K, was found in all three pathways. Thus, we speculated that PI3K plays a crucial role in the anti-T2DM effect of guava leaf. Besides, INS, INSR and TNF were simultaneously found in insulin resistance and T2DM pathways; while PEPCK, NFKB and AKT were simultaneously found in PI3K-Akt signaling and insulin resistance pathways. Therefore, we hypothesized that guava leaf components regulate these genes (including INS, INSR, TNF, PEPCK, NFKB, and AKT) to alleviate insulin resistance and thereby exert anti-T2DM activity.

By taking the results of the above three pathways into consideration, it can be concluded that insulin binds with the insulin receptors to trigger signal transduction for activation of PI3K, thereby facilitating GLUT4 synthesis and transport. In muscle cells and liver cells, GLUT4 transports glucose from the blood to the cells for consumption, which lowers the blood sugar level, and regulates the insulin resistance (Gao et al., 2019; Beg et al., 2017). Meanwhile, PIP3 produced by PI3K can activate the Akt family, and activated Akt regulates cell growth cycle and metabolism and gluco-

neogenesis/glycolysis by phosphorylation. TNF plays a central role in insulin resistance in T2DM (Borst, 2004). In the insulin resistance pathway, TNF is capable of increasing free fatty acids to enhance the production of glycogen in liver cells (El-Moselhy et al., 2011). TNF acts as an activator to trigger NFKB action, then the activated NFKB can in turn further activate TNF, forming a positive feedback mechanism to interfere with the signal transduction of insulin receptors, thereby leading to insulin resistance (Yang et al., 2009). Collectively, our study revealed that the active components of guava leaf mainly target the PI3K-Akt signaling pathway to modulate various T2DM related genes and regulate levels of insulin, vitamin D and DPP4, as well as retinoid-X receptor expression in the treatment of T2DM. However, there are some limitations in our study. First, bioactive components and the corresponding targets of guava leaf reported in our study were not fully comprehensive as we merely focused on flavonoid, triterpenoid, and meroterpenoid. Second, this study lacks experimental evidence, therefore, in the future, further validations are required to consolidate the findings of this study.

In conclusion, for the first time, we uncovered the hypoglycemic mechanism of guava leaf through network pharmacology. This study preliminarily revealed that the active compounds of guava leaf are mainly involved in the PI3K/AKT signaling pathway, acting on TNFα, PEPCK and other factors to regulate insulin resistant and other biological pathways to achieve therapeutic effects against T2DM, providing a scientific base for further research on the molecular mechanism underlying guava leaf anti-T2DM effects.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103143.

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