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Original Article

Evaluation of Chinese Propolis Ethanolic Extracts on Potential Inhibitory Properties of COVID-19 Using Network Pharmacology and Molecular Docking

Songhao Tian[1,*], Xiaofeng Yang[2]

[1]Department of Medical Laboratory Science, Fenyang College of Shanxi Medical University, Fenyang, Shanxi, CHINA.
[2]Department of Biology, Xinzhou Teachers Normal College, Xinzhou, Shanxi, CHINA.

ABSTRACT

Background: Propolis is widely used in the pharmaceutical field for its biological properties such as antioxidant, immunomodulatory, anti-inflammatory, antiviral and antibacterial effects. The flavonoids contained in propolis can reduce virus replication and have anti-COVID-19 potential. This study aimed to explore the active compounds of propolis through network pharmacology and molecular docking and to reveal its mechanism of action against SARS-CoV-2. **Materials and Methods:** Prediction of target genes of Chinese Propolis Ethanolic Extracts (PEE) for COVID-19 treatment using the BATMAN database and the GeneCards database. Using Cytoscape to construct herbal-component-target networks. The hub targets of PEE were analyzed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The main active compounds of PEE were docked to SARS-CoV-2 3C-Like (3CL) protease hydrolase (SARS-CoV-2 3CL) and Angiotensin-Converting Enzyme II (ACE2). **Results:** The hub targets of PEE for COVID-19 treatment were AKT1, IL6, TNF, IL1B, CNR1 and PPARA. There were 166 GO items ($p<0.01$) in the GO enrichment analysis and 95 pathways ($p<0.01$) in the KEGG enrichment analysis and the key signaling pathways included: MAPK signaling pathway, FoxO signaling pathway, NF-κB signaling pathway and PI3K-AKT signaling pathway. Molecular docking results showed that both trans-isoferulic acid and apigenin had strong affinity for SARS-CoV-2 3CL and ACE2. **Conclusion:** The trans-isoferulic acid and apigenin in PEE may play a therapeutic role in COVID-19 by regulating AKT1 and MAPK signaling pathways.

Keywords: SARS-CoV-2, Chinese propolis ethanolic extracts, Network pharmacology, Molecular docking.

Correspondence:
Dr. Songhao Tian
Fenyang College of Shanxi Medical University, Fenyang-032200, CHINA.
Email: tshibm@163.com
ORCID: 0000-0002-9492-5826

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INTRODUCTION

COVID-19 is a global epidemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 is transmitted from person to person through saliva and droplets of respiratory secretions, spreading rapidly to many countries around the world and causing large numbers of human infections and significant economic losses.[1] To present, there are no effective treatments for COVID-19; the available treatments are largely supportive; there is still a lack of insight into the COVID-19 disease process; the understanding of the disease is still in its infancy; and there is great uncertainty about its etiology and management, both for the health care system and health professionals providing care as well as for patients and their families.[2] SARS-CoV-2 Spike protein enters the body by binding to ACE-2 in the human respiratory tract, thus causing infection.[3] Therefore, ACE2 inhibitors are critical for the treatment of infections caused by SARS-CoV-2. Based on the structure of SARS-CoV-2, SARS-CoV-2 3CL was identified as a potential target against COVID-19 because it can inhibit viral replication.[4]

Since ancient times, people have often used natural medicines to prevent and treat diseases.[5] Propolis is both animal and natural medicine with plant components and has been included in the Pharmacopoeia of the People's Republic of China (2020 edition). Propolis has a variety of properties, including anti-cancer, anti-oxidant, immunomodulatory, anti-inflammatory, anti-bacterial and anti-viral.[6] Because of its beneficial biological activity, propolis can be used to reduce the risk and impact of SARS-CoV-2 infection and as an adjunct to treatment.[7] Caffeic acid, myricetin and quercetin in propolis all inhibited human coronavirus activity in the body.[8] Studies have shown that flavonoids, the active ingredient in propolis, have a high inhibitory effect on Angiotensin-Converting Enzyme (ACE).[9] The model developed by Kumar *et al.* showed that Caffeic Acid

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Phenethyl Ester (CAPE) in propolis was able to interact with the protease of SARS-CoV-2 and thus inhibit the virus.[10] In addition, CAPE was able to inhibit coronavirus-induced pulmonary fibrosis.[11] Hesperidin, a polyphenol found in propolis and also in citrus juices, has an affinity for ACE2 receptors and is also able to interact with SARS-CoV-2 3CL, which may prevent viral invasion into host cells.[8]

So far, there are also no specific drugs for COVID-19 and it is crucial to explore alternative options that could help reduce infection and COVID-19 transmission. Network pharmacology is seen as a promising new way to predict potential new drugs or targets for specific diseases.[12] Molecular docking is an important avenue for computer-aided drug design and discovery.[13] This study was designed to explore the active compounds in PEE and reveal their anti-SARS-CoV-2 virus mechanism through network pharmacology and molecular docking, with the purpose of finding drug candidates that inhibit COVID-19 and provide an important role in minimizing the transmission of COVID-19.

MATERIALS AND METHODS

Obtaining potential targets for PEE

InChI information for each compound was entered into the BATMAN database (http://bionet.ncpsb.org.cn/batman-tcm/) based on the previously reported composition of PEE,[14] with the score cutoff parameter set to 20 and the *p*-value after Benjamini-Hochberg multiple testing correction set to 0.05.

Prediction of COVID-19 targets

COVID-19 targets were collected using the GeneCards database (https://www.genecards.org/) by entering the keyword "COVID-19".

Building of Protein-Protein Interaction (PPI) networks

Venny 2.1 online website (https://bioinfogp.cnb.csic.es/tools/venny/) was used to obtain the intersection of PEE and COVID-19 as targets for PEE treatment of COVID-19. The obtained intersection targets were imported into the STRING database (https://cn.string-db.org/) and organisms were selected as "*Homo sapiens*" to construct the PPI network. Use Cytoscape 3.9.1 (http://www.cytoscape.org/) to build and visualize PPI networks and use CytoNCA, the network topology analysis plug-in in Cytoscape, to perform topology analysis of PPI networks. Betweenness Centrality (BC) represents the degree of interaction between a node and other nodes. In this study, the BC value is used as a reference to measure the importance of hub targets.

GO and KEGG enrichment analysis

GO and KEGG analysis of intersecting targets was performed using the DAVID database (https://david.ncifcrf.gov/home.jsp). Select Identifier select "OFFICIAL GENE SYMBOL" and species select "*Homo sapiens*". GO analysis includes Biological Process (BP), Molecular Function (MF) and Cellular Composition (CC). The statistical significance threshold for the enrichment analysis was set at $p<0.01$. The results of the GO and KEGG enrichment analyses were visualized using the bioinformatics online tool (http://www.bioinformatics.com.cn/).

Building of PEE components and target gene networks

Create visual component-target networks by Cytoscape 3.9.1, reflecting the complex relationships between active compounds and their potential targets. Screen the major active components in the PEE based on the magnitude of BC values. The screened compounds were used as ligands for molecular docking.

Molecular docking

SARS-CoV-2 3CL from 2019-nCoV and ACE2 in host cells are the most critical targets for inhibition of COVID-19.[15,16] Therefore, the screened compounds in PEE were used as ligands for molecular docking with the above two proteins. The 3D structures of SARS-CoV-2 3CL (PDB ID: 6lu7) and ACE2 (PDB ID: 1r42) were downloaded from the PDB database (https://www.rcsb.org/). The two-dimensional structures of the compounds trans-isoferulic acid (Compound CID: 736186) and apigenin (Compound CID: 5280443) were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The two-dimensional structure of the compound is processed by Open Babel and converted into PDB format. Using SARS-CoV-2 3CL and ACE2 as the receptors and the active compound as the ligand, the receptor and ligand molecules were molecularly docked using Auto Dock Tools 1.5.6 software after dehydration, hydrogenation and charge calculation of the receptor and the conformation with the best affinity was selected as the final docked conformation and visualized in Py mol 2.4.

RESULTS

Screening of active ingredients in PEE

Based on the pre-assayed Chinese PEE components, we collected a total of 20 active ingredients, including protocatechuic acid, chysin and apigenin. Fifteen active ingredients were screened by BATMAN database target prediction (Table 1).

Acquisition of intersection targets and building of PPI networks

A total of 155 non-duplicate active targets of PEE were screened from the BATMAN database. The latest COVID-19 target data from the DisGeNET database was used to obtain 5079 non-duplicate COVID-19 targets. Then, we generated Venn diagrams of the PEE and COVID-19 overlapping targets (Figure 1A). The Venn diagram showed that there were 64 intersecting targets for PEE and COVID-19. To clarify the interaction

Table 1: Screening information for 14 compounds in PEE.

Molecule Name	PubChem CID	International Chemical Identifier
Protocatechuic acid	91444309	1S/C14H12O6/c15-8-1-3-11(16)7(5-8)6-10-9(14(19)20)2-4-12(17)13(10)18/h1-5,15-18H,6H2,(H,19,20)
Apigenin	5280443	1S/C15H10O5/c16-9-3-1-8(2-4-9)13-7-12(19)15-11(18)5-10(17)6-14(15)20-13/h1-7,16-18H
Chrysin	5281607	1S/C15H10O4/c16-10-6-11(17)15-12(18)8-13(19-14(15)7-10)9-4-2-1-3-5-9/h1-8,16-17H
3,4-Dimethoxycinnamic acid	717531	1S/C11H12O4/c1-14-9-5-3-8(4-6-11(12)13)7-10(9)15-2/h3-7H,1-2H3,(H,12,13)/b6-4+
trans-Isoferulic acid	736186	1S/C10H10O4/c1-14-9-4-2-7(6-8(9)11)3-5-10(12)13/h2-6,11H,1H3,(H,12,13)/b5-3+
(Pinocembrin)	68071	1S/C15H12O4/c16-10-6-11(17)15-12(18)8-13(19-14(15)7-10)9-4-2-1-3-5-9/h1-7,13,16-17H,8H2/t13-/m0/s1
3-O-acetylpinobanksin	148556	1S/C17H14O6/c1-9z(18)22-17-15(21)14-12(20)7-11(19)8-13(14)23-16(17)10-5-3-2-4-6-10/h2-8,16-17,19-20H,1H3/t16-,17+/m1/s1
Pinobanksin	73202	1S/C15H12O5/c16-9-6-10(17)12-11(7-9)20-15(14(19)13(12)18)8-4-2-1-3-5-8/h1-7,14-17,19H/t14-,15+/m0/s1
Ferulic acid	445858	1S/C10H10O4/c1-14-9-6-7(2-4-8(9)11)3-5-10(12)13/h2-6,11H,1H3,(H,12,13)/b5-3+
Kaempferol	5280863	1S/C15H10O6/c16-8-3-1-7(2-4-8)15-14(20)13(19)12-10(18)5-9(17)6-11(12)21-15/h1-6,16-18,20H
Cinnamic acid	444539	1S/C9H8O2/c10-9(11)7-6-8-4-2-1-3-5-8/h1-7H,(H,10,11)/b7-6+
Quercetin	5280343	1S/C15H10O7/c16-7-4-10(19)12-11(5-7)22-15(14(21)13(12)20)6-1-2-8(17)9(18)3-6/h1-5,16-19,21H
Protocatechuic acid	72	1S/C7H6O4/c8-5-2-1-4(7(10)11)3-6(5)9/h1-3,8-9H,(H,10,11)
Hesperitin	72281	1S/C16H14O6/c1-21-13-3-2-8(4-10(13)18)14-7-12(20)16-11(19)5-9(17)6-15(16)22-14/h2-6,14,17-19H,7H2,1H3/t14-/m0/s1
Vanillic acid	8468	1S/C8H8O4/c1-12-7-4-5(8(10)11)2-3-6(7)9/h2-4,9H,1H3,(H,10,11)

relationships between these intersecting targets, we used the STRING database to filter out the 6 targets that were not interlinked. The interaction network of 58 intersecting targets was then constructed using Cytoscape 3.9.1 (Figure B). As shown in Figure B, the top 15 targets (Table 2), such as AKT1, IL6, TNF, IL1B, CNR1, PPARA, ALDOA, MPO and IL17A, were ranked as the hub targets of anti-COVID-19 based on the BC values of each target, which also play a vital role in the gene regulatory network.

GO and KEGG enrichment analysis

The GO enrichment analysis included three parts: BP, CC and MF. A total of 166 GO items were enriched and the top 10 items were selected in each part for display (Figure 2). BP mainly included positive regulation of cell proliferation, innate immune response and positive regulation of interleukin-6 production. The top three ranked CC were plasma membrane, extracellular region and extracellular space. The top three MF were cytokine activity, enzyme binding and G protein-coupled receptor activity, respectively.

A total of 95 KEGG pathways were significantly enriched ($p<0.01$) and the top 15 pathways were selected for display (Figure 3). According to the KEGG pathway enrichment analysis, the main pathways involved in PEE included MAPK signaling pathway, FoxO signaling, NF-κB signaling pathway and PI3K-AKT signaling pathway.

Construction of herb-component-target network

After removing the 6 compounds without targets, the herb-compound-target network was constructed, involving a total of 1 herb, 14 compounds and 64 targets (Figure 4). Analysis of the data showed that the median BC value of the maximum BC value compounds was 0.256 (Table 3). Two of the 14 compounds had BC values above the median, trans-isoferulic acid and apigenin, with BC values of 0.512 and 0.462, respectively. These results suggest that these two compounds play a crucial role in PEE against COVID-19.

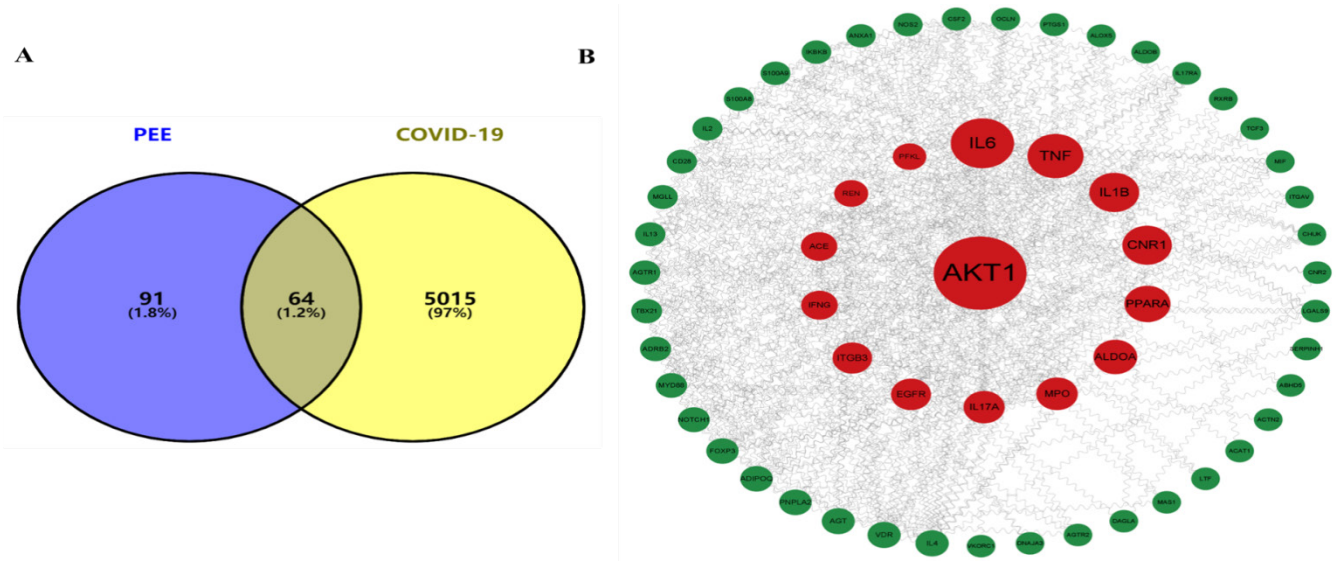

Figure 1: Acquirement of hub targets for PEE treatment of COVID-19.

A: Venn diagram of PEE and COVID-19 intersection targets; B: PPI network predicts hub targets of PEE effect on COVID-19.

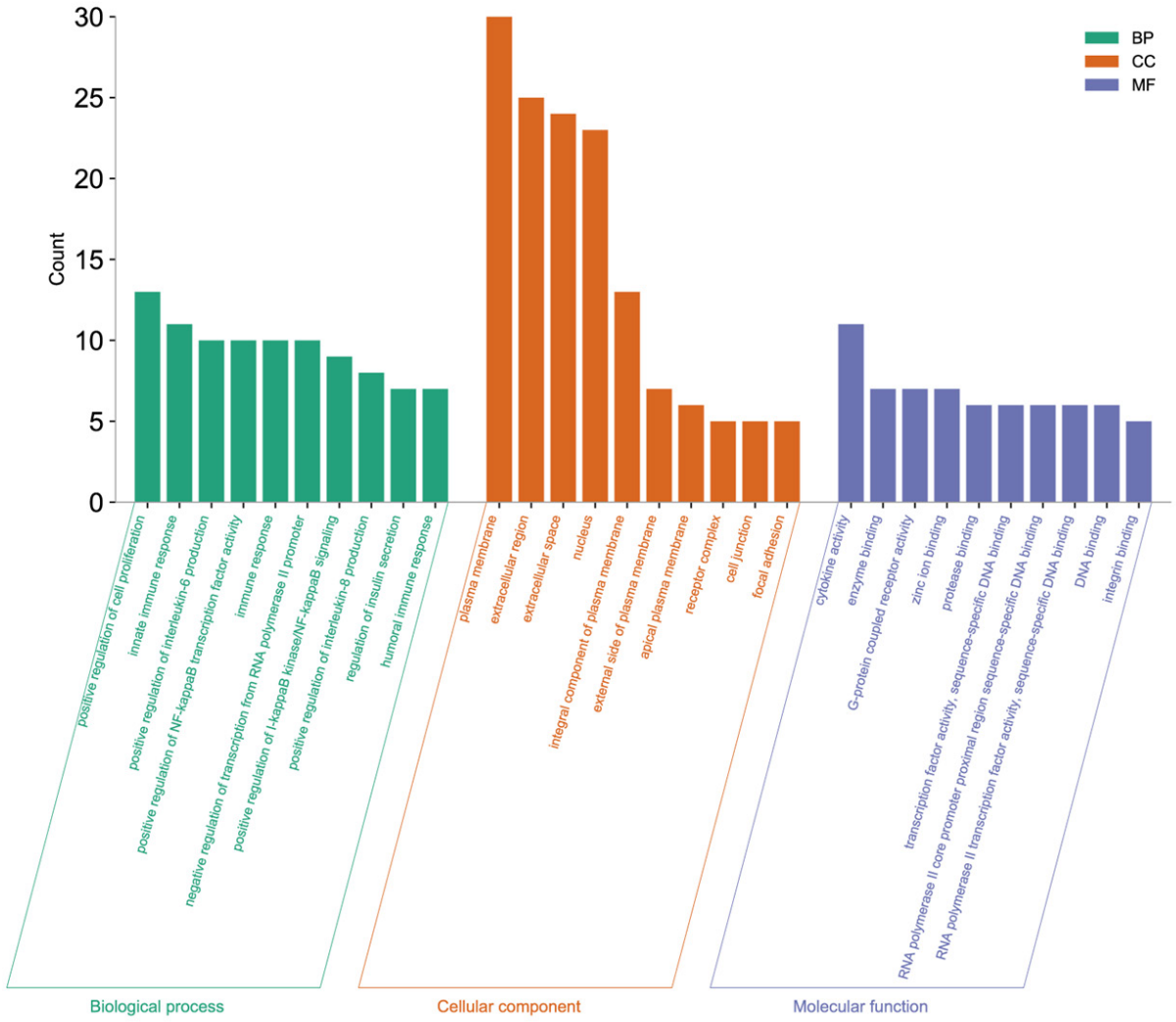

Figure 2: GO enrichment analysis.

Molecular Docking

Molecular docking was used to verify whether the two compounds have a significant role in the regulation of SARS-CoV-2 3CL and ACE2. The results showed that both compounds, trans-ferulic acid and apigenin, had strong affinity for SARS-CoV-2 3CL and ACE2 (Figure 5). The binding energy and binding site information are shown in Table 4 (SARS-CoV-2 3CL) and Table 5 (ACE2).

DISCUSSION

Natural products and their derivatives are the source of new drug discovery and many of the drugs currently on the market are derived from natural products or their derivatives.[17] Propolis is widely used in the pharmaceutical field because of its unique biological properties.[18,19] Flavonoid components of Egyptian propolis showed high affinity for COVID-19 3CL protease and S1 spike protein, showing antiviral potential.[20] Five compounds in Sulawesi propolis serve as effective inhibitors of ACE2.[3] The flavonoids in the ethanolic extract of propolis have a greater potential to bind to ACE2 receptors, suggesting that natural propolis has the potential to treat COVID-19.[9] A clinical study found that administration of Brazilian green propolis was effective in reducing COVID-19-related kidney injury.[21] COVID-19 patients taking propolis had earlier viral clearance, faster recovery of symptoms, faster discharge from the hospital and lower mortality rates relative to other patients[8]. Therefore, the use of propolis for COVID-19 alleviation is theoretically feasible, but further clinical trials are needed to confirm the efficacy on COVID-19.

In this study, a network pharmacology approach was used to predict the targets of PEE for the treatment of COVID-19, which are related to inflammation, immunity, cancer and viruses, such as AKT1, IL6, TNF, IL1B, CNR1 and PPARA. Recent studies have

Table 2: BC values for hub targets.

Target	Betweenness Centrality, BC
AKT1	692.48
IL6	373.87
TNF	296.79
IL1B	228.80
CNR1	225.11
PPARA	188.94
ALDOA	163.00
MPO	139.23
IL17A	134.72
EGFR	123.02
ITGB3	117.95
IFNG	87.07
ACE	84.19
REN	57.25
PFKL	54.00

Table 3: BC values for PEE components.

Component	Betweenness Centrality, BC
trans-Isoferulic acid	0.512149985
Apigenin	0.461587729
3-O-acetylpinobanksin	0.081401589
Protocatechuic acid	0.065017627
Hesperitin	0.060864484
Chrysin	0.048189014
Caffeic acid	0.040922827
Quercetin	0.040922827
Ferulic acid	0.028639299
Kaempferol	0.024565894
3,4-Dimethoxycinnamic acid	0.007832812
Pinocembrin	0.007832812
Cinnamic acid	0.007832812
Vanillic acid	0.005495333

Table 4: Docking results of PEE active ingredient with SARS-CoV-2 3CL.

Ligand	Number of hydrogen bonds	Bound amino acid residues	binding energies (kcal/mol)
Trans-Isoferulicacid	3	ASN-63、PHE-66、VAL-77	-3.89
Apigenin	5	LEU-141、SER-144、HIS-163、MET-165、THR-190	-5.63

Table 5: Docking results of PEE active ingredient with ACE2.

Ligand	Number of hydrogen bonds	Bound amino acid residues	binding energies (kcal/mol)
Trans-Isoferulicacid	4	ARG-482、GLU-489、GLU-495、TYR-613	-3.55
Apigenin	4	ASP-615、UNK-912、UNK-917	-4.81

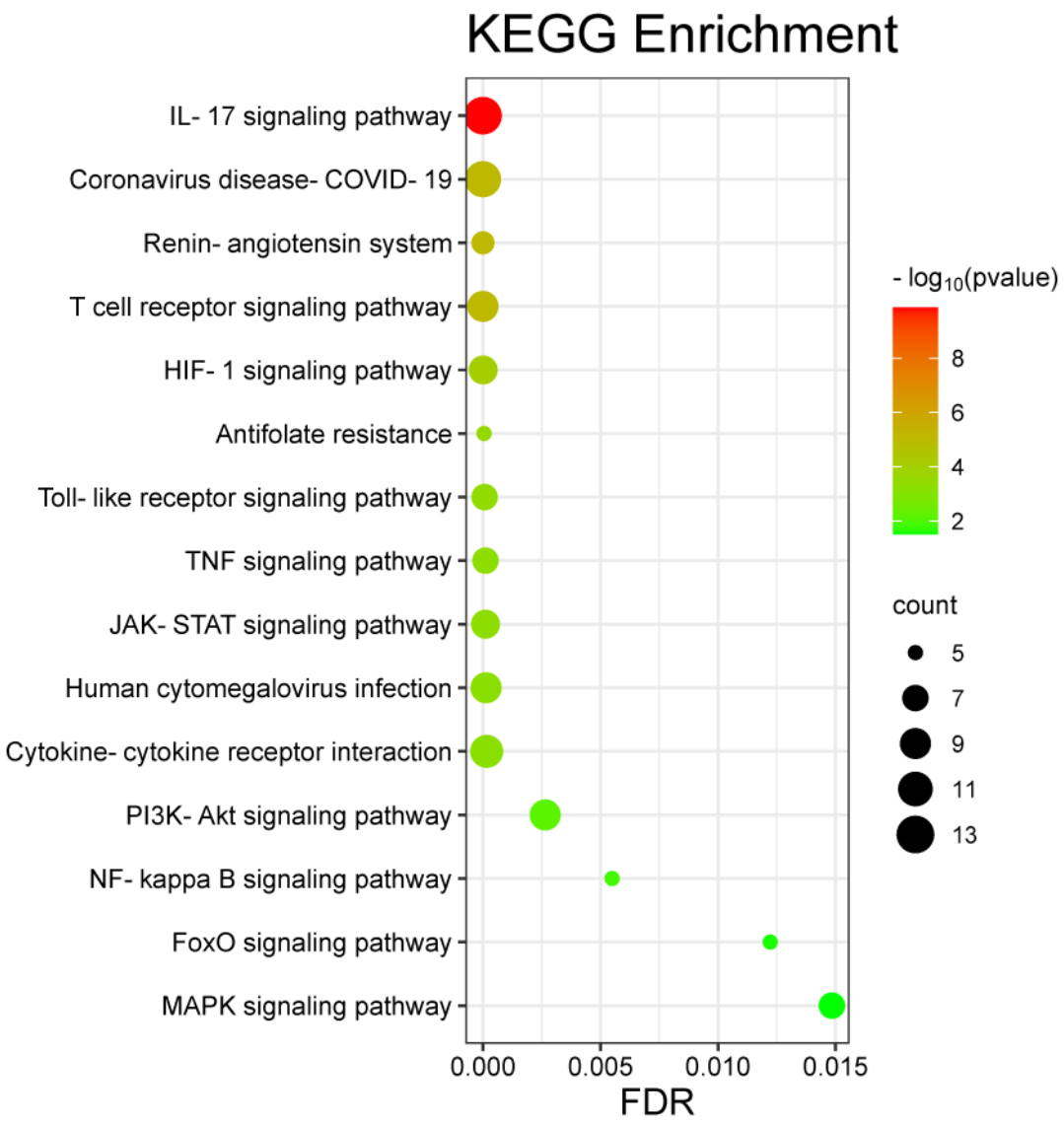

Figure 3: KEGG enrichment analysis.

shown that Akt is activated in a dose-dependent manner during SARS-CoV-2 infection and that Akt may be a therapeutic target for COVID-19.[22] Lianhua Qingwen Capsule (LQC) has shown good therapeutic effects in patients with COVID-19.[23] Akt1 is the main hub gene regulated by LQC and LQC may play a role in the treatment of COVID-19 by reducing tissue damage and helping to eliminate viral infection through the Akt1 target.[23] Xuebijing Injection was found to improve COVID-19 clinical symptoms and reduce the severity of the disease.[24] Xuebijing Injection inhibits COVID-19 by affecting AKT1.[24] AKT1 is a member of the AKT kinase family that regulates metabolism, proliferation, cell survival, growth and angiogenesis through a range of downstream substrates. Previous studies have shown that AKT1 promotes lung fibroblast proliferation and that AKT1 dominant-negative mutants significantly inhibit viral RNA expression, further reducing viral capsid protein expression and viral release.[25,26] The hub target predicted in our study for PEE treatment of COVID-19 is AKT1, which may be an ideal target with broad-spectrum antiviral effects. During SARS-CoV-2 infection, the levels of IL6, TNF and IL1β were elevated in patients.[27] Elevated cytokine levels can lead to infectious shock and multi-organ failure.[28] As an anti-inflammatory substance, propolis is effective in inhibiting and down-regulating the levels of pro-inflammatory cytokines IL-1β, IL-6, IFN-γ and TNF-α in the body.[29] Therefore, the predicted targets of PEE for COVID-19 in this study are reliable and this study validates the effectiveness of PEE in the treatment of COVID-19 from a bioinformatics perspective.

GO and KEGG enrichment analyses of target genes suggested that innate immune response and MAPK signaling pathway may be involved in PEE treatment of COVID-19. There is evidence that propolis exerts regulatory activity in the immune response,

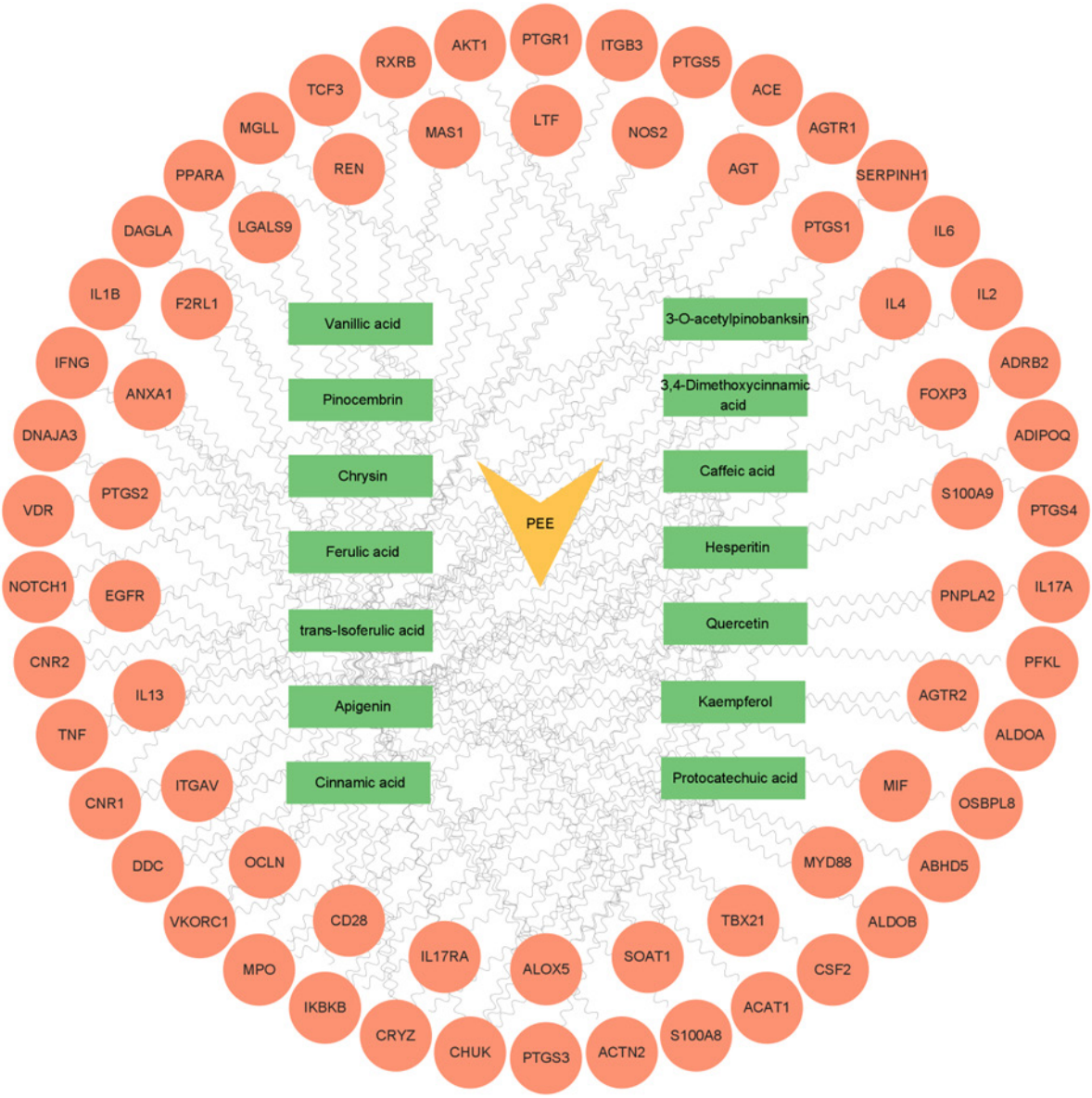

Figure 4: Herb-compound-target gene network for PEE.

acting in different events of innate and adaptive immunity and that propolis may influence innate immunity by down-regulating the pro-inflammatory activity of monocytes.[30] MAPK signaling pathway plays a key role in regulating cell growth, differentiation, apoptosis, stress and inflammation responses. Propolis and its component caffeic acid inhibit LPS-stimulated pro-inflammatory responses by blocking NF-κB and MAPK activation to inhibit LPS-stimulated pro-inflammatory responses.[31] Chinese propolis can protect Vascular Endothelial Cells (VECs) from LPS-induced oxidative stress and inflammation, which may be related to the inhibition of autophagy and MAPK/NF-κB signaling pathway.[32] In SARS-CoV-2 infection, the virus may directly activate p38 MAPK, leading to possible abnormal upregulation of the p38 MAPK pathway.[33] p38 MAPK inhibitors are expected to be used in the treatment of COVID-19. These results all suggest that propolis is able to play a functional role in the treatment of COVID-19.

We hypothesized that trans-isoferulic acid and apigenin are the most important active components affecting the efficacy of PEE for COVID-19 treatment by constructing an herb-component-target network. After molecular docking, it was verified that these two compounds could bind tightly to SARS-CoV-2 3CL and ACE2. Apigenin analogs act as potent inhibitors of Main protease of SARS-CoV-2 (M^{pro})[34] and this is in general agreement with our findings. Our study is based on data already available in a specific database and the use of a different database may yield different

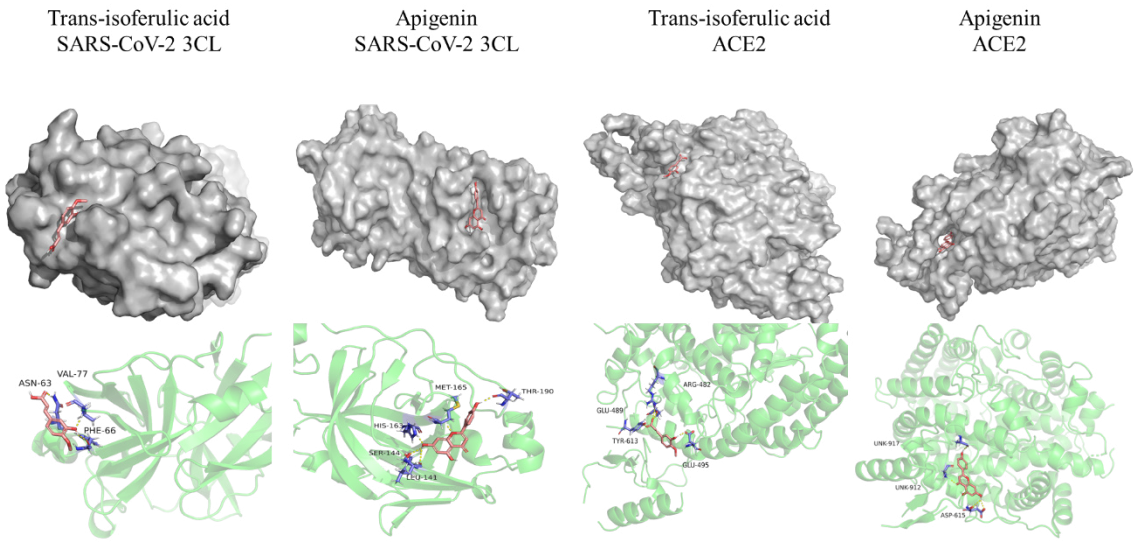

Figure 5: Molecular docking results of compounds in PEE with receptor proteins.

results. The effect of PEE on COVID-19 needs to be confirmed by more experiments.

CONCLUSION

In summary, this study predicted the active ingredients trans-Isoferulicacid and Apigenin for PEE treatment of COVID-19 by network pharmacology and molecular docking analysis. PEE may affect the body's immune response by modulating AKT1 and MAPK signaling pathways, thus ultimately inhibiting the excessive inflammatory response associated with COVID-19. The results of this study need to be validated in subsequent experiments to provide a basis for PEE treatment of COVID-19.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PEE: Propolis ethanolic extracts; **GO:** Gene Ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **SARS-CoV-2 3CL:** SARS-CoV-2 3C-like (3CL) protease hydrolase; **ACE2:** Angiotensin-converting enzyme II; **PPI:** Protein-protein interaction; **BP:** Biological process; **MF:** Molecular function; **CC:** Cellular composition; **LQC:** Lianhua Qingwen capsule; **VECs:** Vascular endothelial cells.

SUMMARY

The mechanism of PEE for COVID-19 and the core components of PEE for COVID-19 were predicted by network pharmacology. The binding ability of the core components in PEE to SARS-CoV-2 3CL and ACE2 was verified by molecular docking. Provide new insights into PEE treatment of COVID-19.

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