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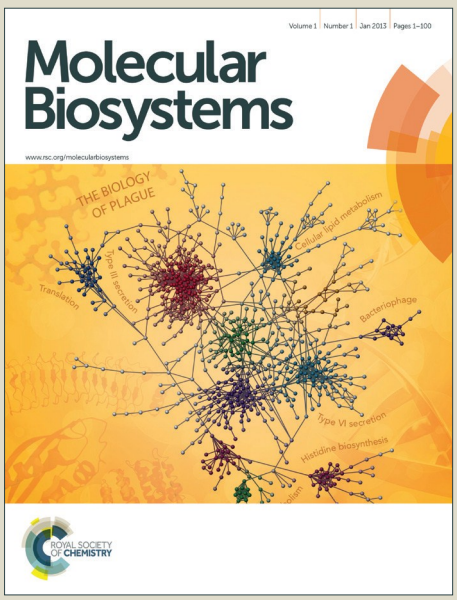

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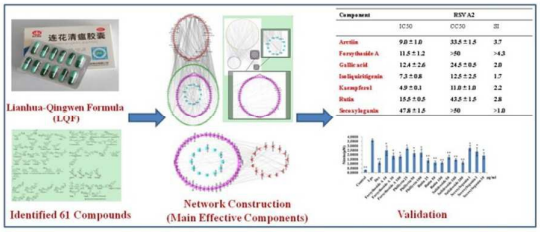
Graphical Abstract

We developed a novel approach to identify the main effective components in LQF and experimentally validated some of the predictions.



A Network Analysis of Chinese Medicine Lianhua-Qingwen Formula to Identify its Main Effective Components

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Abstract

Chinese Medicine is known to treat complex diseases with multiple components and multiple targets. However, the main effective components and their related key targets and functions remain to be identified. Herein, a network analysis method was developed to identify the main effective components and key targets of a Chinese medicine, Lianhua-Qingwen Formula (LQF). LQF is commonly used for the prevention and treatment of viral influenza in China. It is composed of 11 herbs, gypsum and menthol with 61 compounds being identified in our previous work. In this paper, these 61 candidate compounds were used to find their related targets and construct the predicted-targets (PT) network. Influenza-related protein-protein interaction (PPI) network was constructed and integrated with PT network. Then the compound-effective targets (CET) network and compound-ineffective targets network (CIT) were extracted respectively. A novel approach was developed to identify effective components by comparing CET and CIT networks. As a result, 15 main effective components were identified along with 61 corresponding targets. 7 of these main effective components were further experimentally validated to have antivirus efficacy *in vitro*. Main effective component-target (MECT) network was further constructed with main effective components and their key targets. Gene Ontology (GO) analysis of the MECT network predicted key functions such as NO production being modulated by LQF. Interestingly, five effective components were experimentally tested and exhibited inhibitory effects on NO production in LPS induced RAW 264.7 cell. In summary, we have developed a novel approach to identify the main effective components in a Chinese medicine LQF and experimentally validated some of the predictions.

1. Introduction

Chinese medicine has a long history of wide use in China for the prevention and treatment of various diseases by targeting and modulating multiple disease related pathways with multiple effective components.[1] It is thus a critical problem to identify the main effective components and the key targets they act upon to elucidate the mode-of-action of a Chinese medicine. With the advancements in medicine and pharmacology, it becomes more recognized that most diseases are caused by more than one single causal factor.[2] Based on this understanding, systematic and network-based approaches were developed and applied to study diseases as well as Chinese medicine formulae. One of the most important approaches is network pharmacology, which was first proposed by A. Hopkins in 2008[3] and developed as an efficient drug discovery method. Because of the complexity of Chinese medicine, many researchers have introduced it into Chinese medicine formula analysis in 2013.[4] Subsequently, the molecular mechanisms of many traditional Chinese medicines (TCMs) formulae were elucidated by this method.[5-8] It can facilitate the identification of the main active ingredients and synergistic ingredient pairs and lead to drug discoveries based on TCM in some cases.[4] Consequently, the development of network pharmacology provides us a possibility to identify the main effective components and their related key targets and biological functions.[9-12]

Lianhua-Qingwen Formula (LQF) is commonly used for the prevention and treatment of viral influenza in China. The formula of LQF comes from two well-known TCM prescriptions *Maxing-Shigan-Tang* and *Yinqiao-San* containing 11 herbs including *Radix Isatidis* (Banlangen), *Fructus Forsythiae* (Lianqiao), *Flos Lonicerae Japonicae* (Jinyinhua), *Rhizoma Dryopteridis Crassirhizomatis* (Mianmaguanzhong), *Herba Ephedrae* (Mahuang), *Semen Armeniacae Amarum* (Kuxingren), *Herba Houttuyniae* (Yuxingcao), *Herba Pogostemonis* (Guanghuoxiang), *Radix et Rhizoma Rhodiolae Crenulatae* (Hongjingtian), *Radix et Rhizoma Rhei* (Dahuang) and *Radix et Rhizoma Glycyrrhizae* (Gancao) and a mineral medicine, *Gypsum Fibrosum* (Shigao) as well as menthol. Many components can be

found in methanol-water extracting solution of LQF, 61 of them have been isolated and identified by ultra performance liquid chromatography coupled with diode-array detector and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF-MS) in our previous report.[13]

In this study, these 61 compounds were used as the candidate effective components to find their related targets and construct the predicted-targets (PT) network. Influenza-related protein-protein interaction (PPI) network was constructed by collecting influenza-related targets from literature[14] and online Mendelian Inheritance in Man (OMIM) database. Then the PPI network was integrated with PT network followed by extracting subnetworks of compounds-effective targets (CET) network and compounds-ineffective targets (CIT) network. A novel approach was developed to identify effective components by comparing CET network and CIT network. For each candidate component, the number of its effective targets and the ratio of effective/ineffective targets were used to quantify the possibility of the candidate being an effective component. As a result, 15 main effective components (including arctiin, emodin, formononetin, forsythoside A, gallic acid, hesperidin, isoliquiritigenin, kaempferol, ononin, phillyrin, quercetin, rutin, salidroside, secoxyloganin and tricin) were predicted for LQF. These 15 effective components were connected to 61 key targets in the main effective component-target (MECT) network. 7 of these 15 main effective components (including arctiin, forsythoside A, gallic acid, isoliquiritigenin, kaempferol, rutin, secoxyloganin) were further validated to have antiviral effect by cytopathic effect (CPE) method *in vitro*. Further functional pathway enrichment analysis revealed several pathways modulated by LQF, including nitric-oxide synthase regulator activator. Interestingly, induction of nitric oxide has been found as an important factor contributing to the viral pathogenesis of the influenza virus infection. Five predicted effective components (including forsythoside A, phillyrin, rutin, salidroside, secoxyloganin) were experimentally validated to exhibit inhibitory effects on NO production *in vitro*.

2. Materials and Methods

2.1. Network construction

A network was constructed to analyze how the chemical components of LQF connect to their targets to achieve therapeutic effects against influenza. Using the 61 candidate components as the key words, related genes were identified as potential targets from 2 databases STITCH and Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP).[15] After manually removing duplicate items, 199 related targets were collected and input to the software Cytoscape[16] to construct predicted-targets (PT) network. Influenza-related genes were collected from literature[14] and OMIM database. 167 directly related genes were collected after manually removing duplicate items. Influenza-related protein-protein interaction (PPI) network was extracted from the Human Protein Reference Database (HPRD). PT and PPI network were merged to build a full network of LQF against influenza.

2.2. Gene category and networks refining

For the purpose of identifying disease related effective chemical components from the network analysis, all the collected genes were categorized to 5 categories. The first category referred to targets of the candidate compounds but unrelated to influenza; the second category referred to directly related genes of influenza but not targets of any candidate compounds; the third category referred to targets of the candidate compounds and directly related to influenza; the fourth category referred to indirectly related genes of influenza from influenza-related protein-protein interaction (PPI) network but not targets of any candidate compounds; the fifth category referred to targets of the candidate compounds and indirectly related to influenza from influenza-related PPI network. We define genes in the first category as ineffective targets (IT) while define the genes with the third and the fifth category as effective targets (ET). Refining PT network, influenza-related PPI network as well as the full vision network by using different color and shape marked nodes in different category.

2.3. Main effective components identification and validation

It is our hypothesis that components targeting disease related genes had a higher possibility of leading to treatment efficacy. A novel and simple method based on the number as well as the ratio of effective targets and ineffective targets was thus developed to identify effective components of a Chinese medicine.

Sub-network of compounds-effective targets (CET) network and compounds-ineffective targets (CIT) network were extracted from refined PT network. Main effective compounds were identified by setting the constraints as the ratio of candidate's effective targets and ineffective targets being no less than 1.5 or the component's effective targets being no less than 10. It should be noted that the filtering parameters should be empirically determined for different drugs.

26 of 61 candidate components are commercially available from Sigma Aldrich (St. Louis. MO. USA). The antiviral potentials of these 26 components were tested by the cytopathic effect (CPE) method in *vitro* against RSV A2 virus to evaluate the identification accuracy of main effective components.[17,18] Briefly, HEp-2 cells were seeded in 96-well plate. After 24 h, cell monolayers were inoculated with the mixture of compounds and virus suspension (100 $TCID_{50}$).The viral control were not treated with compounds. Afterwards, the plate was incubated for 3 days. The RSV-induced CPE was observed under light microscopy. The CC_{50} represents the concentration of inhibiting 50% CPE with respect to virus control. Similarly, The cytotoxicity of the compounds on HEp-2 cells were evaluated by CPE method.

2.4. Main effective component-target (MECT) network

Main effective component-target (MECT) network was extracted by selecting the nodes of main effective components in LQF as well as their directly connected nodes in CET network. MECT network could help to visually explain the interactions between components and targets. In order to understand the biological functions exerted by LQF on the treatment of influenza, key targets were analyzed for their functional enrichment using WEB-based GEne SeT AnaLysis Toolkit

(WebGestalt).[19,20] The significance level was set as 0.05 and the functions enriched on the third level of the tree structure were taken into account.

2.5. NO Production Inhibitory Rate Testing

Mouse monocyte-macrophage RAW 264.7 cells (ATCC) were suspended in RPMI 1640 (DMEM/High Gluose) medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat inactive fetal bovine serum. The cells were harvested with cell scraper and diluted to a suspension in fresh medium. The cells were seeded in 24-well plates with 1×10^6 cells/well and allowed to adhere for 2 h at 37□ in 5% CO_2 in air. Then, the cells were treated with 100 ng/mLof LPS for 16 h with or without various concentrations of testing compounds. NO Production were determined by measuring the accumulation of nitrite in culture supernatants using Griess reagent.[21] Briefly, 150 μL of the culture supernatants from the incubates were mixed with 130 μL deionized water and 20 μL Griess reagent (0.1% N-[1-naphthyl] ethylenediamine and 1% sulfanilamide in 5% H_3PO_4). Cytotoxicity was determined by the MTT colorimetric assay after 16 h incubation with test compounds.

The concentration of NO_2^- was calculated by using 0, 1.04, 3.13, 6.25, 12.5, 25, 50, 100 μM sodium nitrite solutions, and the inhibitory rate on NO production induced by LPS was calculated by the NO_2^- levels as follows:

$$\text{Inhibitory rate (\%)} = 100\times[NO_2^-]_{LPS}-[NO_2^-]_{LPS+sample}/[NO_2^-]_{LPS}-[NO_2^-]_{untreated}$$

Experiments were performed in triplicate and the data were expressed as the mean ± SD of the three independent experiments.

3. Results and Discussion

3.1. Network construction

In total, 199 candidate targets and 167 influenza related genes were collected. The PT network includes 260 nodes along with 477 edges (Figure 1. and Supplementary Information File Table S1.). This network contained the information of compounds, targets as well as their relationships between each other. It should be

noted that these candidate targets are collected from databases and these targets have been proved effected by LQF's components. However, it needs further study on whether the compounds in LQF act on these targets directly or not.

The influenza-related PPI network was extracted from HPRD related with these 167 genes. It contained 1210 nodes and 1758 edges (Figure 2. and Supplementary Information File Table S2.). The PPI network provided the information of these predicted targets of LQF and a database to find indirect effective targets of influenza.

The full network was constructed by merging PT network and PPI network (Supplementary Information File Figure S1.). It contained 1400 nodes and 2190 edges.

3.2. Main effective components identification and validation

CET and CIT network were extracted from refined PT network. By calculating the number of effective targets as well as the ratio of effective targets and ineffective targets (Table 1.), 15 components (arctiin, emodin, formononetin, forsythoside A, gallic acid, hesperidin, isoliquiritigenin, kaempferol, ononin, phillyrin, quercetin, rutin, salidroside, secoxyloganin and tricin) were identified as main effective components.

Arctiin is a lignan found in many plants of Asteraceae family. It has anti-inflammation, anti-microbial, anti-carcinogenic effects and it was proven having the therapeutic effect of anti-influenza function by blocking hydrogen peroxide-induced senescence and cell death.[22] This function could be important to help keep normal metabolic state and maintain homeostasis when infected by influenza virus.[23] Emodin is a natural anthraquinone derivative found in numerous plants' roots and rhizomes. It has been found to have effects on modulating mammalian cell cycle in oncogene-overexpressing cell cycle through inhibiting tyrosine kinase.[24] Formononetin is a plant-derived phytoestrogen. It has been reported to activate T-cell cytoplasmic 1 signaling pathway and increase the expression and secretion of T cell,[25] with a potential role to activate immune response rapidly after infection by influenza virus. Forsythoside A is a polyphenolic constituent existing in

Forsythia suspense. It is widely used for anti-inflammatory[26] and anti-virus[27] use in Chinese medicine. Gallic acid is one type of phenolic acid and it can be found in numbers of land plants as well as some aquatic plants. It has also been reported having the anti-inflammatory function.[28] Hesperidin is a flavanone glycoside existing in the fruits of citrus. Its anti-inflammatory and antioxidant function[29] can lead to the therapeutic effects of influenza. Isoliquiritigenin is a licorice chalconoid, one type of natural phenols. It has been proved as a potent inhibitor of inflammasome activation.[30] Kaempferol is a natural flavonol isolated from many plant sources. It has exhibited high activity against two types of influenza viruses, H1N1 and H9N2.[31] Ononin is a kind of isoflavones. It can be found in many herbs of *Glycyrrhiza* genus. It has been studied as a component of anti-asthmatic drug,[32] however, there have been no reports on its effect of treating influenza. Phillyrin is the main chemical constituent of *Forsythia suspense* and it is an endophytic fungal. It has been reported that phillyrin can be used to attenuate pulmonary inflammation.[33] Quercetin is a flavonol exist in many fruits, leaves of plants and its anti-influenza virus function has been reported [23]. Rutin is the glycoside between the flavonol quercetin and the disaccharide rutinose. It exhibits anti-H5N1 virus[34] and anti-oxidant activity[35] in different studies. Salidroside is a glucoside of tyrosol existing in *Radix et Rhizoma Rhodiolae Crenulatae*. It has been used as a component of antidepressant and anxiolytic,[36,37] while there is no report about its therapeutic effects on influenza. Secoxyloganin is a kind of secoiridoid with the anti-bacterial and anti-oxidant activities.[38,39] Tricin is a type of flavonoid. It also has been reported the anti-influenza virus activity.[40]

26 commercially available components were tested for their antiviral potential to evaluate the accuracy of effective components prediction by network analysis. The CPE results (Table 2.) showed that 7 of the 15 predicted main effective components (arctiin, forsythoside A, gallic acid, isoliquiritigenin, kaempferol, rutin, secoxyloganin) exhibited antivirus effects *in vitro*. It indicates the high accuracy of the approach to identify effective components with network analysis by comparing CET and CIT.

The MECT network with 15 main effective components and 61 directly connected genes were extracted from CET network (Figure 3). In order to understand the LQF's mechanism on treating influenza, these 61 key targets of LQF were analyzed for their related key functions with GO analysis (Table 3). Enriched GO terms include biological processes (Regulation of molecular, single organism signaling and response to stimulus), molecular function (Protein binding and nitric-oxide synthase regulator activity), cellular components (Protein complex, extracellular region part and membrane-enclosed lumen). The GO analysis shows that LQF acts on molecular regulation, single organism signaling as well as response to stimulus. LQF acts on protein complex, extracellular region part and membrane-enclosed lumen so that it can inhibit the protein binding between IVs and host cells, keep IVs outside of cells. Interestingly, LQF were predicted to have the function of modulating nitric-oxide synthase regulator activator, and induction of nitric oxide has been found as an important factor contributing to the viral pathogenesis of the influenza virus infection.[21] We further experimentally tested the inhibitory effects of the main effective components on NO production. 5 out of the 15 effective components exhibited significant inhibitory effects as shown in Figure 4.

Besides the anti-virus and anti-inflammation activities, anti-microbial and immune-modulating effects were also reported in the literatures.[41,42] In this study, arctiin was reported having anti-microbial activity as well as formononetin with the immune modulating effect out of the 15 identified main effective components in LQF. Moreover, LQF is mainly used in clinical for viral colds, cough and pneumonia.[42] Therefore, hesperidin, rutin and secoxyloganin with their antioxidant activity as well as ononin's anti-asthmatic activity are related to the clinical use of LQF. In addition, anti-tumor, anti-antidepressant and anxiolytic activities were also uncovered by our network investigation and these bioactivities await further study in the future.

4. Conclusion

We developed a simple and effective algorithm in this paper. The method combined data mining, network analysis and experimental validation to study

multi-components and multi-targets drugs such as Chinese medicine. The constraint setting for identifying main components was based on the intuition that components with more influenza-related targets should exert more positive effects. Thus the number of effective targets of a candidate components and the ratio of effective and ineffective targets were taken into consideration. Every target was treated equally in this study without consideration of the biological function they involve. To improve this algorithm, the relative amount of each components and the importance of each potential target should also be considered.

Acknowledgement

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Figure Legends

Figure 1. Refined LQF predicted-targets network. Different colors represent nodes with different attributions. Red nodes represent candidate compounds; Triangles represent predicted targets, cyan ones refer to direct effective targets, purple ones refer to indirect effective targets and green ones represent ineffective targets.

Figure 2. Refined Influenza-related PPI network. Different colors represent nodes with different attributions and each attribution cluster as a cycle. Cycle with cyan nodes refer to direct effective targets while cycle with purple nodes refer to indirect effective targets; Cycle's nodes filled with blue color represent genes direct related to influenza while could not be effected by LQF; Cycle's nodes filled with yellow color represent genes indirect related to influenza either could not be effected by LQF.

Figure 3. Main effective component-target network. Different colors represent nodes with different attributions. Red nodes represent main effective components; Triangles represent key targets, cyan ones refer to direct effective targets and purple ones refer to indirect effective targets.

Figure 4. Inhibitory effects of the five compounds on LPS-induced nitric oxide (NO) production (* $P < 0.05$, ** $P < 0.01$ compared to LPS-induced group; No cytotoxicity to RAW 264.7 cells at their respective experimental concentrations).

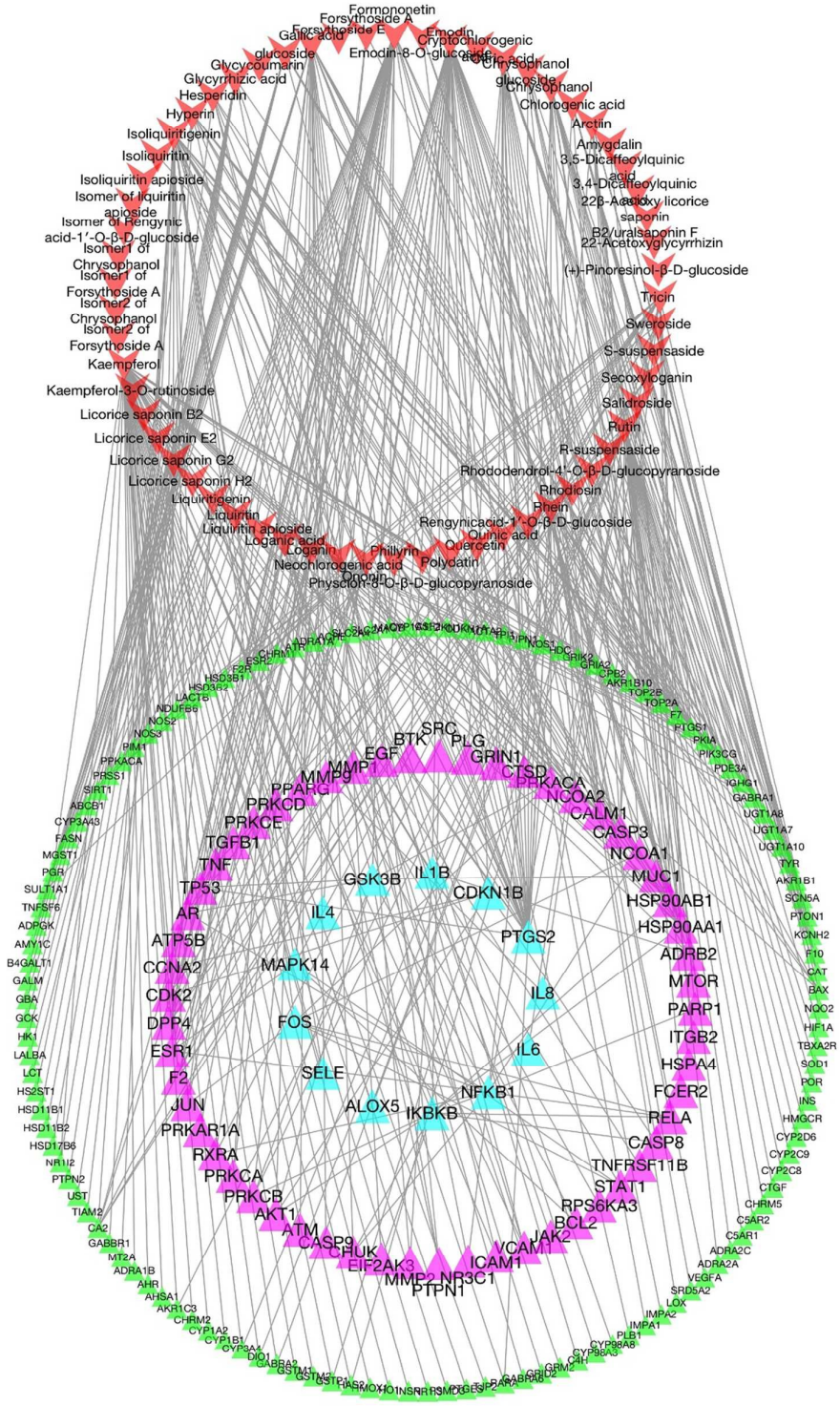

Figure 1. LQF predicted-targets network

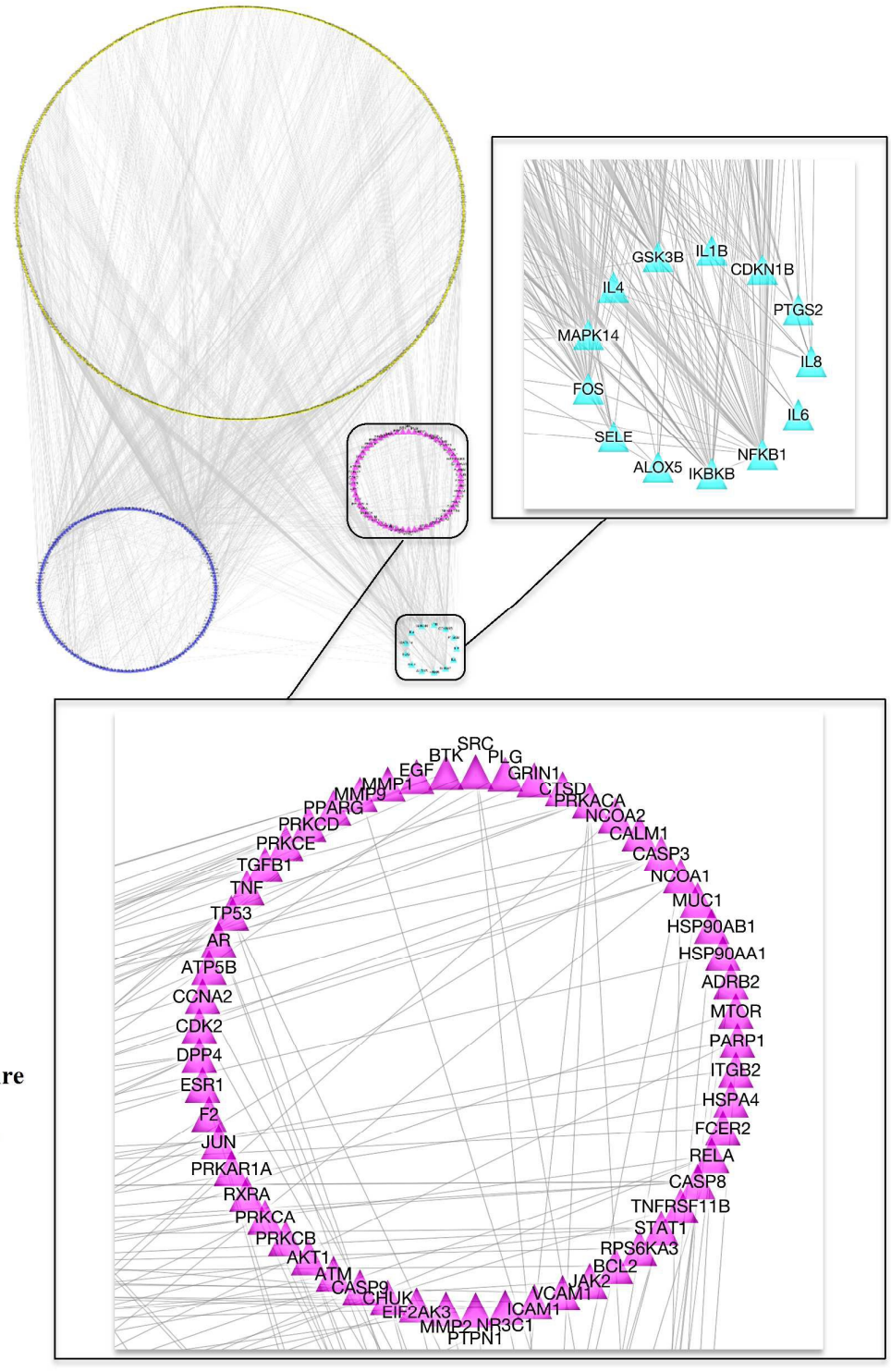

Figure 2.

Influenza-related PPI network.

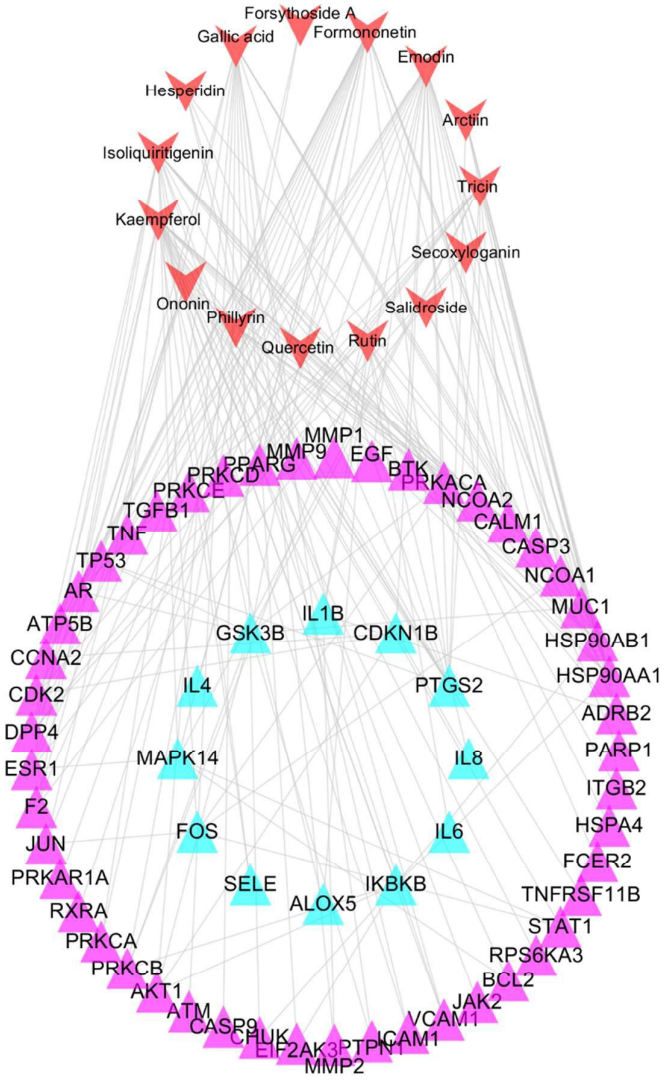

Figure 3. Main effective component-target network

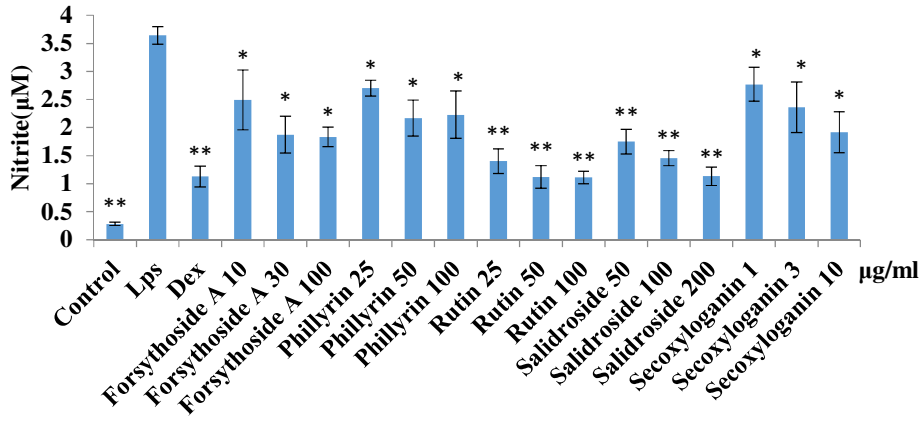

Figure 4. Inhibitory effects of the five compounds on LPS-induced nitric oxide (NO) production.

Tables

Table 1. Main effective components identification

Candidate	Effective targets number	Ineffective targets number	Ratio
Arctiin	6	4	1.5
Forsythoside A	2	1	2
Gallic acid	15	12	1.25
Isoliquiritigenin	15	14	1.07
Kaempferol	23	37	0.62
Rutin	10	22	0.45
Secoxyloganin	2	1	2
Emodin	20	14	1.43
Hesperidin	4	2	2
Phillyrin	6	2	3
Quercetin	2	0	/
Salidroside	2	1	2
Formononetin	20	19	1.06
Ononin	7	4	1.75
Tricin	12	7	1.71
Chlorogenic acid	2	5	0.4
Chrysophanol glucoside	2	4	0.5
Cryptochlorogenic acid	0	0	0
Hyperin	5	6	0.83
Neochlorogenic acid	1	3	0.33
(+)-Pinoresinol-β-D-glucoside	0	0	0
Amygdalin	1	2	0.5

Emodin-8-O-glucoside	1	2	0.5
Glycyrrhizic acid	2	8	0.25
Loganic acid	3	6	0.5
Quinic acid	1	2	0.5
Rhein	9	9	1
Sweroside	1	5	0.2
22-Acetoxyglycyrrhizin	0	0	0
22β-Acetoxy licorice saponin B2/uralsaponin F	0	0	0
3,4-Dicaffeoylquinic acid	0	0	0
3,5-Dicaffeoylquinic acid	0	0	0
Chrysophanol	6	7	0.86
Citric acid	5	12	0.42
Forsythoside E	0	0	0
glucoside	1	13	0.08
Glycycoumarin	0	0	0
Isoliquiritin	0	1	0
Isoliquiritin apioside	0	0	0
Isomer of liquiritin apioside	0	0	0
Isomer of Rengynic acid-1'-O-β-D-glucoside	0	0	0
Isomer1 of Chrysophanol	0	0	0
Isomer1 of Forsythoside A	0	0	0
Isomer2 of Chrysophanol	0	0	0
Isomer2 of Forsythoside A	0	0	0
Kaempferol-3-O-rutinoside	0	0	0
Licorice saponin B2	0	0	0
Licorice saponin E2	0	0	0

Licorice saponin G2	0	0	0
Licorice saponin H2	0	0	0
Liquiritigenin	5	8	0.63
Liquiritin	0	0	0
Liquiritin apioside	0	0	0
Loganin	2	2	1
Physcion-8-O-β-D-glucopyranoside	0	0	0
Polydatin	2	2	1
Rengynicacid-1'-O-β-D-glucoside	0	0	0
Rhodiosin	0	0	0
Rhododendrol-4'-O-β-D-glucopyranoside	0	0	0
R-suspensaside	0	0	0
S-suspensaside	0	0	0

The first 15 candidate components were identified as main effective components.

Table2 Antiviral activities of the 12 compounds against RSV A2 (n = 3)

Compounds	RSV A2		
	IC_{50}[a] (μg/ml)	CC_{50}[b] (μg /ml)	SI[c]
Arctiin	9.0 ±1.0	33.5 ±1.5	3.7
Chlorogenic acid	19.3 ±0.8	42.5 ±2.5	2.2
Chrysophanol glucoside	21.0± 1.0	>50	>2.3
Cryptochlorogenic acid	23.8 ±1.3	25.0 ±2.3	1.1
Forsythoside A	11.5 ±1.2	>50	>4.3
Gallic acid	12.4 ±2.6	24.5 ±0.5	2.0
Hyperoside	22.0 ±2.0	38.0 ±2.0	1.7
Isoliquiritigenin	7.3 ±0.8	12.5 ±2.5	1.7
Kaempferol	4.9 ±0.1	11.0 ±1.0	2.2
Neochlorogenic acid	37.5 ±2.5	>50	>1.3
Rutin	15.5 ±0.5	43.5 ±1.5	2.8
Secoxyloganin	47.8±1.5	>50	>1.0

a CC_{50} represents the concentration of inducing 50% cell death (CPE) compare to no treated group; data were expressed as mean ± SD.

b IC_{50} was detected by CPE reduction assay with RSV A2 strain at 100 $TCID_{50}$; data are expressed as mean ± SD.

c SI value equals CC_{50}/IC_{50}.

Table 3. Key functions of LQC enriched by GO analysis

Function	Number of enriched genes /Number of key targets	Adjusted p-Value
Regulation of molecular	40/61	1.49e-20
Single organism signaling	53/61	6.52e-18
Response to stimulus	59/61	1.01e-17
Protein binding	58/61	1.28e-14
Nitric-oxide synthase regulator activity	4/61	2.69e-08
Protein complex	24/61	8.00e-04
Extracellular region part	15/61	3.71e-05
Membrane-enclosed lumen	32/61	2.55e-07