

Identification of the *Tagetes erecta* Linn. Extract's potentials against influenza A based on bioinformatics analysis and experimental study

Minjee Kim

Konkuk University

Aleksandra Nowakowska

Konkuk University

Jaebum Kim

Konkuk University

Young Bong Kim

kimera@konkuk.ac.kr

Konkuk University

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Abstract

Background: *Tagetes erecta* Linn. (TE) is used to treat cardiovascular, renal, and gastrointestinal diseases. In this study, we aimed to investigate the active compounds and targets of the TE extract in influenza A infections.

Method: Active compounds and targets of the TE extract were identified using network pharmacology. Pathway and target studies were conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Expression Omnibus (GEO) gene difference, and molecular docking analyses. *In vitro* validation of the TE extract was performed using antiviral and plaque reduction assays.

Results: From 6 compounds, 95 active targets, 887 influenza-associated target genes, and 14 intersecting core targets were identified. A compound–target network was constructed and two compounds (lutein and beta-carotene) were identified. Protein–protein interaction and GEO analysis revealed interleukin (IL)-6 as a hub gene. *In vitro* experimental results revealed that the TE extract exerted therapeutic rather than protective effects.

Conclusions: Overall, we identified the two main active compounds in the TE extract, lutein and beta-carotene, and IL-6 as an important target associated with influenza. Moreover, our findings confirmed the activity of the TE extract against the influenza A virus.

Introduction

Influenza is an acute respiratory disease characterized by continuous antigenic drift and sporadic shift (1, 2). Its negative-sense RNA genome lacks a proofreading mechanism, which leads to continuous mutations. Vaccines are the first line of defense against influenza; therefore, antigenic, sequence, and population susceptibility analyses are commonly used to develop effective global vaccines (2). Therapeutic interventions are also important for influenza treatment, particularly when the vaccines are unavailable or fail to provide protection. However, to date, only one class of drugs, neuraminidase (NA) inhibitors (NAIs), has been approved for antiviral treatment. NAIs are virus-specific drugs that bind to the active site of the influenza virus. However, NAIs need to be administered within 48 h of symptom onset, which is challenging in many countries and one of its main drawbacks (3). In addition, emergence of NAI-resistant influenza virus strains impacts the efficacy of currently available antiviral agents (4). Therefore, new and effective drugs need to be developed for better treatment of influenza infection.

Natural products are extensively used in medicine because of their bioactive metabolites that modulate cellular targets in various diseases (5, 6). Low adverse effects, affordability, and accessibility are the major benefits of medicinal plants (6). High-throughput screening is used to assess the pharmacological effects of natural products that may be used as herbal medicines and facilitate new drug development (7). The one drug–one target–one disease strategy in drug discovery is inefficient and time-consuming (8). The concept of developing multi-target drugs for complex diseases has led to the emergence of

“network pharmacology,” which facilitates the prediction of gene networks regulated by bioactive compounds (9).

Tagetes erecta Linn. (TE), a member of the *Asteraceae* family, exerts various biological effects, such as antioxidant, antiproliferative, and antidiabetic effects (10). TE flower extracts are traditionally used to treat cardiovascular, renal, and gastrointestinal diseases in South America (11). TE flower extracts reduce inflammation and accelerate the gastric healing process in rodents (12). Owing to their high lutein content, TE extracts are also used as nutritional supplements to prevent the loss of visual activity due to aging or eye diseases (13).

In this study, we aimed to determine the effects of the TE extract against influenza virus and identify its major compounds and targets using network pharmacology and molecular docking. We constructed a compound–target network to identify the active compounds and analyze the targets and pathways associated with the influenza virus. Additionally, we performed molecular docking analysis to identify the potential therapeutic candidates. Furthermore, we evaluated the *in vitro* cytotoxicity and antiviral and plaque reduction effects of the TE extract.

Materials and methods

In silico analyses

Identification of potential active compounds in the TE extract

TE compounds were identified using Dr. Duke’s Phytochemical and Ethnobotanical databases (version 1.10.07; <https://phytochem.nal.usda.gov/phytochem>) and the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (version 2.3; <http://tcmssp.com/tcmssp.php>). Active compounds were identified using the in silico absorption, distribution, metabolism, and excretion screening model according to the oral bioavailability (OB) and drug-likeness (DL) results obtained using the TCMSP database.

Potential targets of the TE extract and influenza virus

The main regulatory target genes associated with the identified TE compounds were investigated using TCMSP and DrugBank (version 5.1.10; <https://www.drugbank.ca>) databases. Influenza virus-associated target genes (*Homo sapiens*) were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and GeneCards (version 5.14.0; <https://www.genecards.org/>) databases.

Network construction and pathway analysis

Compounds and intersecting core target networks were constructed using Cytoscape (version 3.9.0). The network was analyzed to identify the hub compounds and targets. Information on the pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG), and biological process (BP) in gene ontology (GO)

associated with the predicted targets were retrieved from the Database for Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov>) and KEGG database.

Gene Expression Omnibus (GEO) gene difference and gene enrichment analysis

GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) from NCBI was used to further screen the influenza targets from clinical case information. We obtained influenza clinical case samples from the GSE48466 data set of geo database (The data set contains 12 clinical samples, of which 9 samples of human influenza H1N1 infected persons and 3 healthy controls were compared). Through the gene difference analysis of GSE clinical samples, we constructed an intuitive gene difference heatmap for target screening again.

Molecular docking analysis

The major compounds identified in the TE extract were docked into two main targets retrieved from the network analysis and three enzymes involved in skin aging. Three-dimensional structures of the five proteins were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (for the targets, IL-6 (PDB ID: 1P9M), Transforming growth factor beta 1 (TGFB1; PDB ID: 5VQP), and IL-8 (CXCL8, PDB ID: 3IL8). Chemical structures of ligands were obtained from the PubChem database. Molecular docking was performed using the AutoDock Vina (version 1.1.2) option in Pyrx (version 0.9.6) based on scoring functions. Ligand-Target 2D interaction was performed using Biovia Discovery Studio (version 20.1.0.19295).

In vitro experiment

Plant material and extraction

TE flowers were grown in Woori Bio Smart farm (Gyeongsan, Korea). The plant materials were ground and extracted using 100% ethanol (maceration extraction). The extracts were filtered and evaporated at a low temperature under reduced pressure. The research on plants complies with institutional and national guidelines. The voucher specimen is deposited at Konkuk University (Seoul, Korea).

Cells and viral infection

Mardin–Darby canine kidney (MDCK) cells were obtained from The American Type Culture Collection (ATCC, Manassas, VA) and cultured in the Eagle's Minimum Essential Medium (EMEM; ATCC) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin at 37°C and 5% CO₂. Human influenza type A/California/07/09 (H1N1) virus was provided by the Centers for Disease Control and Prevention (Korea). Infected MDCK cells were maintained in the virus growth medium (EMEM) supplemented with 0.3% bovine serum albumin, 1% penicillin/streptomycin, and 0.5 µg/mL trypsin-TPCK (Sigma). Virus titer was defined as the 50% tissue culture infectious dose (TCID₅₀), according to Reed and Muench endpoint method.

Antiviral assay: Pre-treatment, blockage of virus attachment, co-treatment, and post-treatment

Antiviral activity of the TE extract against influenza H1N1 virus infection was determined using four methods: pretreatment, attachment inhibitory, co-treatment, and post-treatment assays. Briefly, MDCK cells were seeded in a 96-well plate and incubated for 24 h. In the pre-treatment assay, cells were first treated with different concentrations of TE extract and incubated for 1 h at 37°C. Then, samples were removed and infected with the virus (100 TCID₅₀/well) for 1 h. Attachment inhibitory assay was performed via pre-absorption of the virus (100 TCID₅₀/well) into cells with different concentrations of TE extract for 1 h at 4°C. Co-treatment assay was performed by incubating the cells with the virus (100 TCID₅₀/well) and extract for 1 h at 37°C. In the post-treatment assay, cells were pre-incubated with the virus (100 TCID₅₀/well) for 1 h at 37°C and subsequently incubated for 48 h with various concentrations of TE extract prepared in the virus growth media. After 48 h, cell viability was measured using the WST method with the EZ-Cytox kit.

Plaque reduction assay

MDCK cells were seeded in a 6-well plate and cultured for 24 h. Cells were infected with the virus at a dose of 50 plaque-forming units (PFUs) per well and incubated at 37°C and 5% CO₂ for 1 h. The inoculum was removed, and cells were treated for 1 h with different concentrations of the TE extract. The mixture was removed and 3 mL of an overlay medium containing 0.6% low melting agar was added. Overlaid plates were incubated for 48 h at 37°C and 5% CO₂. Finally, the overlay medium was removed, and cells were stained with the crystal violet staining solution for plaque counting.

Statistical analysis

All experiments in this study were performed more than three times and are presented as the average values. Statistical significance was analyzed using one-way analysis of variance and Dunnett's test. $P < 0.05$ was considered to be significant. Data processing and statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA).

Results

In silico findings

Active compounds and target prediction

A total of six compounds were selected from the TCMSP database based on the criteria on OB (≥ 20), and DL (≥ 0.1) according to the database suggestion (Table 1). Targets regulated by the active compounds were queried using the TCMSP and National Center for Biotechnology Information databases and 95 targets of the main active ingredients of TE were obtained.

Table 1
Active compounds in the *Tagetes erecta* (TE) extract.

Compound	MW	OB	DL
Alpha-carotene	536.96	34.51	0.58
Beta-carotene	536.96	37.18	0.58
Beta-sitosterol	414.79	36.91	0.75
Campesterol	400.76	37.58	0.71
Lutein	568.96	22.59	0.55
Stigmasterol	412.77	43.83	0.76

MW: molecular weight; OB: oral bioavailability; DL: drug-likeness.

Influenza target prediction

Using KEGG and GeneCards databases, 160 and 720 targets were obtained with the search term “influenza” and “*Homo sapiens*” species, respectively. After removing the duplicates, 14 intersecting target genes associated with both the TE extract and influenza were retrieved. The retrieved targets were further analyzed using a compound–disease target network and four compounds (lutein, beta-carotene, beta-sitosterol, and alpha-carotene). In the network, lutein showed the highest degree, followed by beta-carotene (Fig. 1).

Construction of the protein–protein interaction (PPI) network of potential targets

Fourteen intersection targets of TE–active compounds associated with influenza were analyzed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<https://string-db.org/>) and imported into Cytoscape for compound–target network analysis (Fig. 2).

Network analysis revealed that the network contained 14 nodes linked to 49 edges. Specific information on the targets is listed based on the number of degrees in Table 2. From the PPI network analysis, IL-6 showed the highest degree.

Table 2
Intersection target prediction.

Target	Description	Degree
IL-6	Interleukin-6	11
HIF1A	Hypoxia inducible factor 1 subunit alpha	10
IL-1 β	Interleukin-1 beta	10
CXCL8	Interleukin-8	9
RELA	RELA proto-oncogene, NF-kB subunit	9
HMOX1	Heme oxygenase 1	8
TGFB1	Transforming growth factor beta 1	8
APP	Amyloid beta precursor protein	7
BAX	Apoptosis regulator BAX	6
CTGF	Cellular communication network factor 2	6
TNFRSF10B	Tumor necrosis factor receptor superfamily member 10B	6
ATM	ATM serine/threonine kinase	5
CAPN2	Calpain 2	2
BAG3	BAG cochaperone 3	1

GEO analysis

Through the NCBI GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), we obtained a total of 20,396 H1N1 influenza-related disease genes from the GSE48466 dataset. We discovered the expression data of the 7 overlapping targets from the case samples. A heatmap of gene differences was constructed using SRplot (<http://www.bioinformatics.com.cn/en>) (Fig. 3). The average expression levels in H1N1 patients of these 7 targets were ranked high to low by CXCL8, BAX, TGFB1, TNFRSF10B, RELA, IL6 and ATM. However, compared to healthy control, the expression levels were highly increased in IL6, TGFB1, CXCL8, RELA, TNFRSF10B, BAX, and ATM.

KEGG pathway and GO analysis

From the KEGG pathway analysis, 81 signaling pathways were screened, and the top 10 pathways were plotted in bubble diagrams according to the enriched gene ranking of the pathways (Fig. 4). Influenza showed the third highest correlation in the KEGG pathway analysis after diabetic complications and non-alcoholic fatty liver disease.

For gene ontology (GO) biological process (BP) analysis, we observed 7 targets and drew a chord diagram with 4 biological regulatory processes (Fig. 5). Among 7 targets, 6 targets were enriched in GO chord. We identified IL6, TGFB1, CXCL8, RELA were associated with inflammatory response (GO:0006954) and IL6, TGFB1, BAX were involved in cellular response to virus (GO:0098586).

Molecular docking of active compounds with hub targets

Here, we conducted molecular docking to evaluate the antiviral potential of the major compounds in the TE extract, lutein and beta-carotene (Table 3). Three main targets retrieved from the GEO analysis, (IL-6, TGFB1, and CXCL8) were docked with the main compounds. IL-6 showed the strongest binding affinities among other targets. Lutein exhibited stronger binding to IL-6, and Beta-carotene showed stronger bindings to TFGB1 and CXCL8. The 2D ligand-target binding of lutein-IL-6 was performed using Biovia Discovery Studio (Fig. 6). The key bonds were alkyl bonds with LYS66, PHE74, CYS73, ALA180, PHE78 and pi-alkyl bonding with PHE74 and ARG179.

Table 3
Binding affinities of the active compounds of the TE extract with influenza-associated genes.

Compound	Binding affinity (kcal/mol)		
	IL-6	TGFB1	CXCL8
Lutein	-8.6	-7.3	-7.3
Beta-carotene	-8.2	-7.6	-7.6

In vitro findings

Antiviral activity of the TE extract

Four different experiments were performed to determine whether the TE extract exhibits anti-influenza activity at different stages of the viral life cycle (Fig. 7). Our *in vitro* results revealed that the TE extract exerted effects on attachment inhibition and co-treatment assays (B and C). In both cases, extract at doses 1000 and 500 µL/mL significantly improved the cell viability compared to that in the untreated group. However, no antiviral activity was detected in the pre-treatment assay (A), indicating that the TE extract is unlikely to elicit protective effects against influenza infection. Antiviral properties of the TE extract are prominent during the late stages of influenza infection. Post-treatment yielded the best results among the four assays. Cell viability increased to 31 µg/mL. Interestingly, incubation of the extract with the infected cells not only inhibited the viral activity but also increased the cell growth, which was indicated by cell viability > 100% compared to that in the control group.

Plaque reduction assay

To confirm the antiviral activity of the TE extract against influenza, a plaque reduction assay was performed (Fig. 8). The number of plaques in infected (50 PFU/well) and treated cells was compared to that in infected (50 PFU/well) and untreated cells. The differences were not as critical as in the case of the post-treatment assay but remained significant. Compared with the infected-only control group, the plaque number decreased to approximately 60% after treatment with 1000 μ L/mL of the TE extract and stayed at approximately 70% for other groups.

Discussion

Influenza virus is an evolving pathogen that is a serious threat to medical and public health systems (14). In this study, we used network pharmacology to identify the active compounds and targets associated with influenza. We also determined the binding affinities between the active compounds and targets using molecular docking. The antiviral effects were experimentally validated using MDCK cells. After filtering using the TCMSP database criteria, six compounds from the TE flower extract were selected for target prediction. To determine the active compounds associated with influenza, we constructed a compound–target network between the main compounds and intersecting genes. From this network, we identified two core active compounds: lutein and beta-carotene. The network was further analyzed using a PPI network to identify the hub genes associated with influenza (IL-6, HIF1A, and IL-1 β) and GEO data showed (IL-6, TGFB1, and CXCL8). In our antiviral assays, post-treatment with the TE extract had the most potent effect, indicating its potential as an anti-influenza agent.

In influenza H1N1, disease severity is strongly associated with high levels of circulating IL-6, IL-10, IFN- γ -induced proteins, and monocyte chemotactic protein (15, 16). Particularly, IL-6 is the major cytokine in the early stages of influenza. IL-6 levels are significantly increased in the sera of patients with uncomplicated influenza (15). Elevated levels of the pro-inflammatory cytokine, IL-6, are correlated with a high number of hospital admissions (17). Here, we identified the two main compounds in the TE extract via network analysis and further conducted molecular docking to assess target binding. We found that lutein exhibited the stronger binding with IL-6 compared to beta-carotene.

Finally, we validated the anti-influenza effects of the TE extract in MDCK cells and assessed their effects on the viral cycle. In the antiviral test, the most potent effects were observed in the post-treatment assay, indicating the potential of the TE extract for influenza treatment. It was effective upto a concentration of 31 μ g/mL, which was very low compared to that obtained in the other three assays. Moreover, incubation of the extract with the infected cells increased the cell growth, as indicated by the cell viability, which was > 100% compared to that of the control cells. The improved cell conditions may be due to the antioxidant activity of the natural compounds in the TE extract.

In conclusion, we identified the two main compounds, lutein and beta-carotene, in the TE extract that prevent influenza infection by targeting IL-6. Our results demonstrated the antiviral effects of the TE flower extract against the influenza virus, suggesting its potential for influenza treatment.

Declarations

Conflict of interest statement

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.

Contributors

M K: analysis and interpretation of in silico data, drafting the article, A.N: acquisition of in vitro data, J.K: acquisition of in silico data, Y.B: The conception and design of the study

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Data availability statement

Requests for data sharing should be addressed to the corresponding author.

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Figures

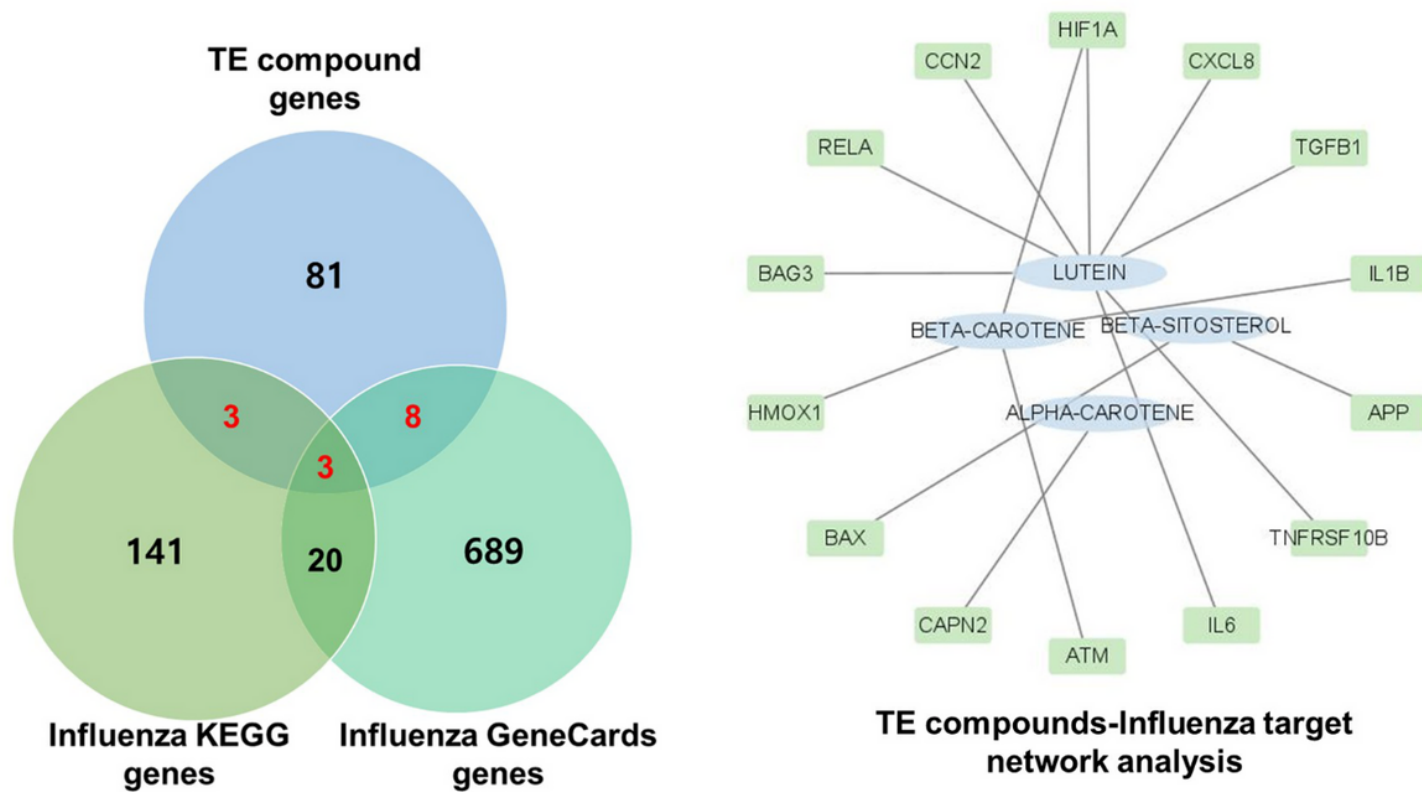


Figure 1

Target prediction. (A) Intersecting active targets. (B) Compound–disease target network analysis.

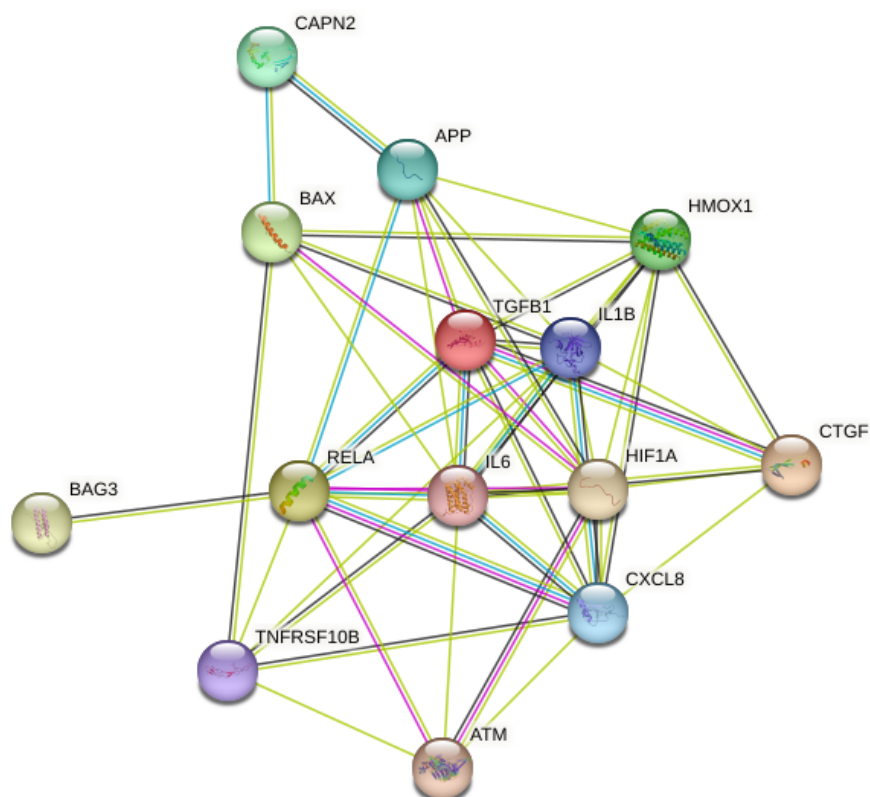


Figure 2

Target protein–protein interaction (PPI) network.

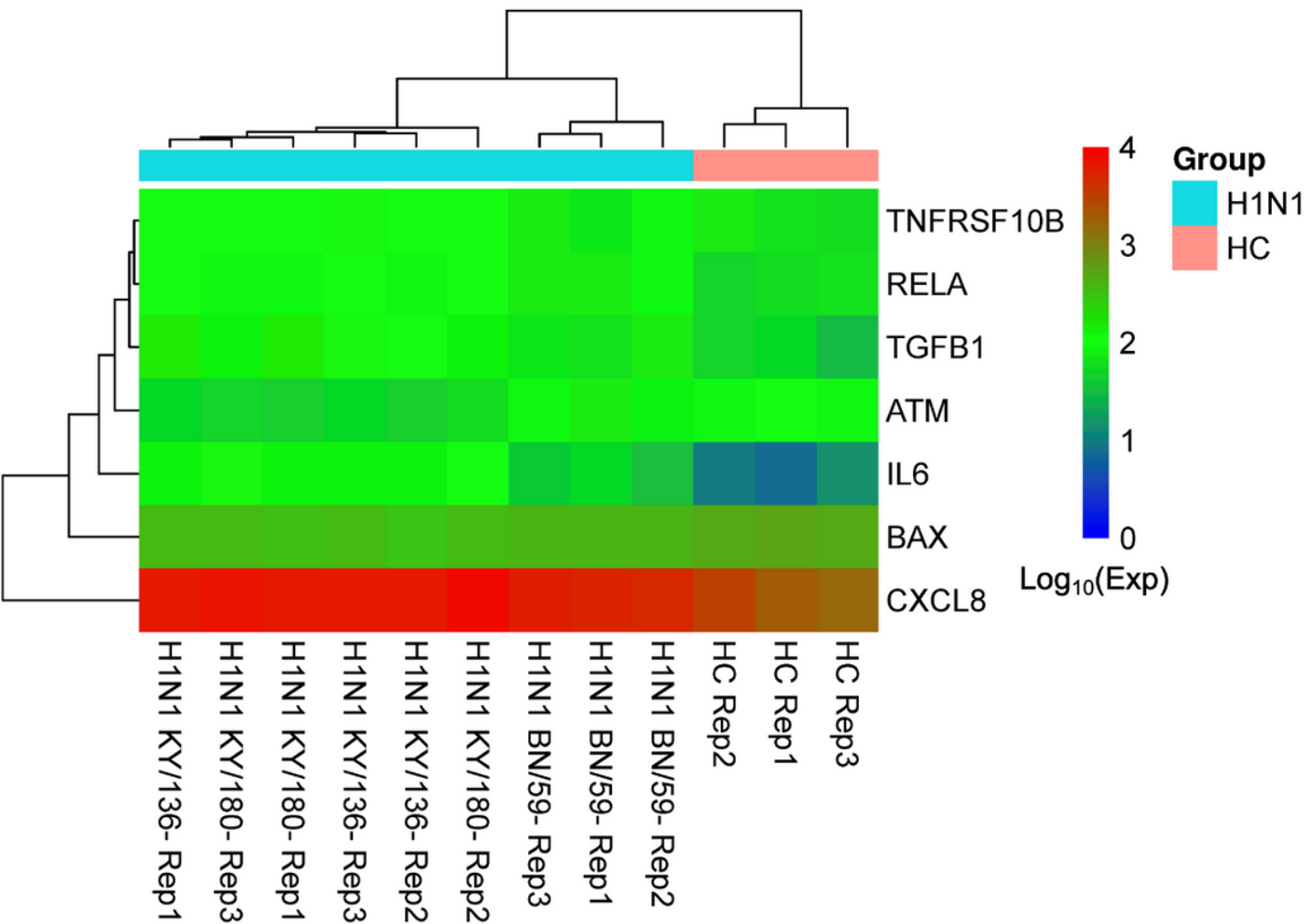
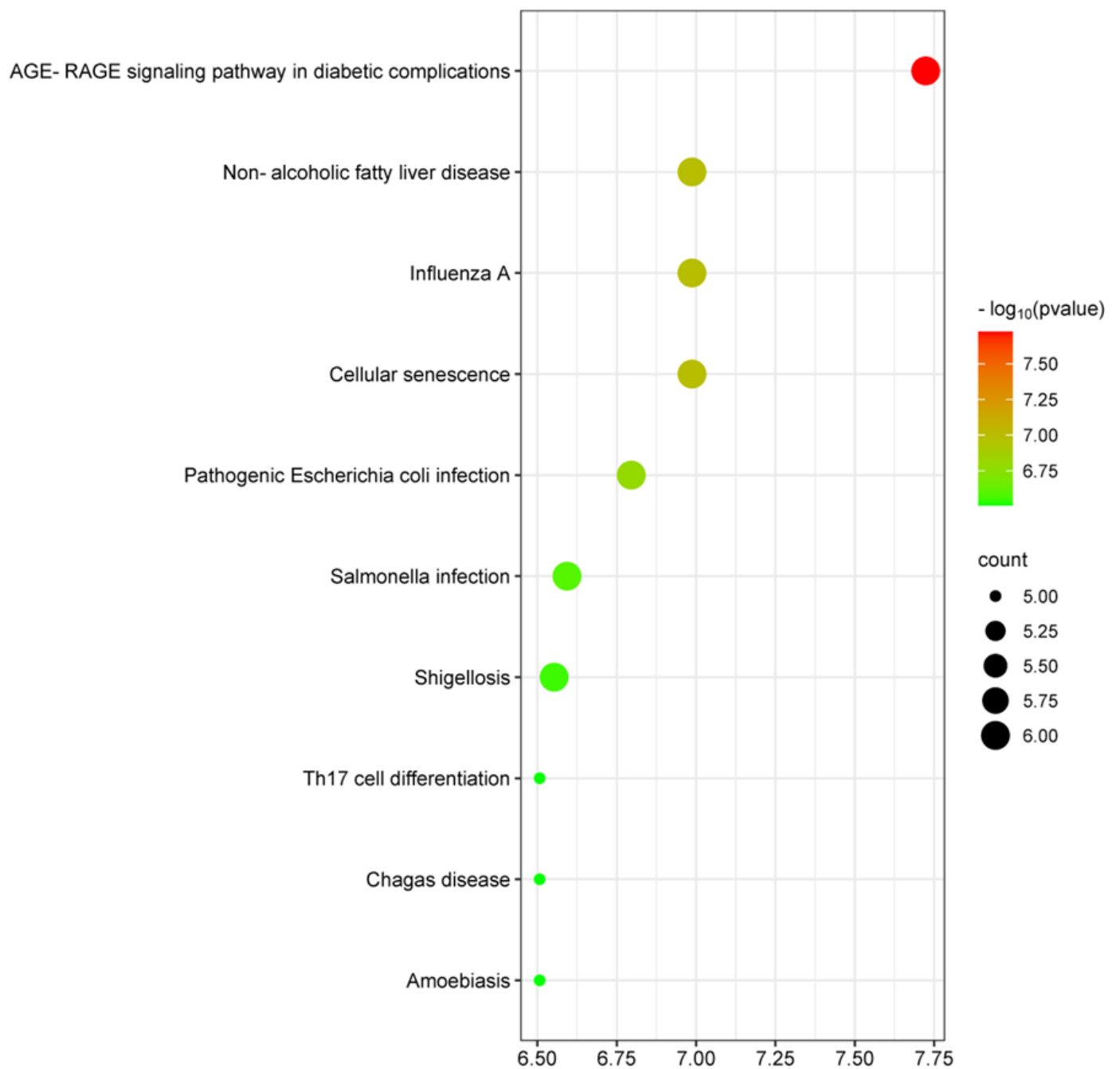


Figure 3

Gene difference heatmap of 7 targets.



KEGG pathway analysis

Figure 4

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

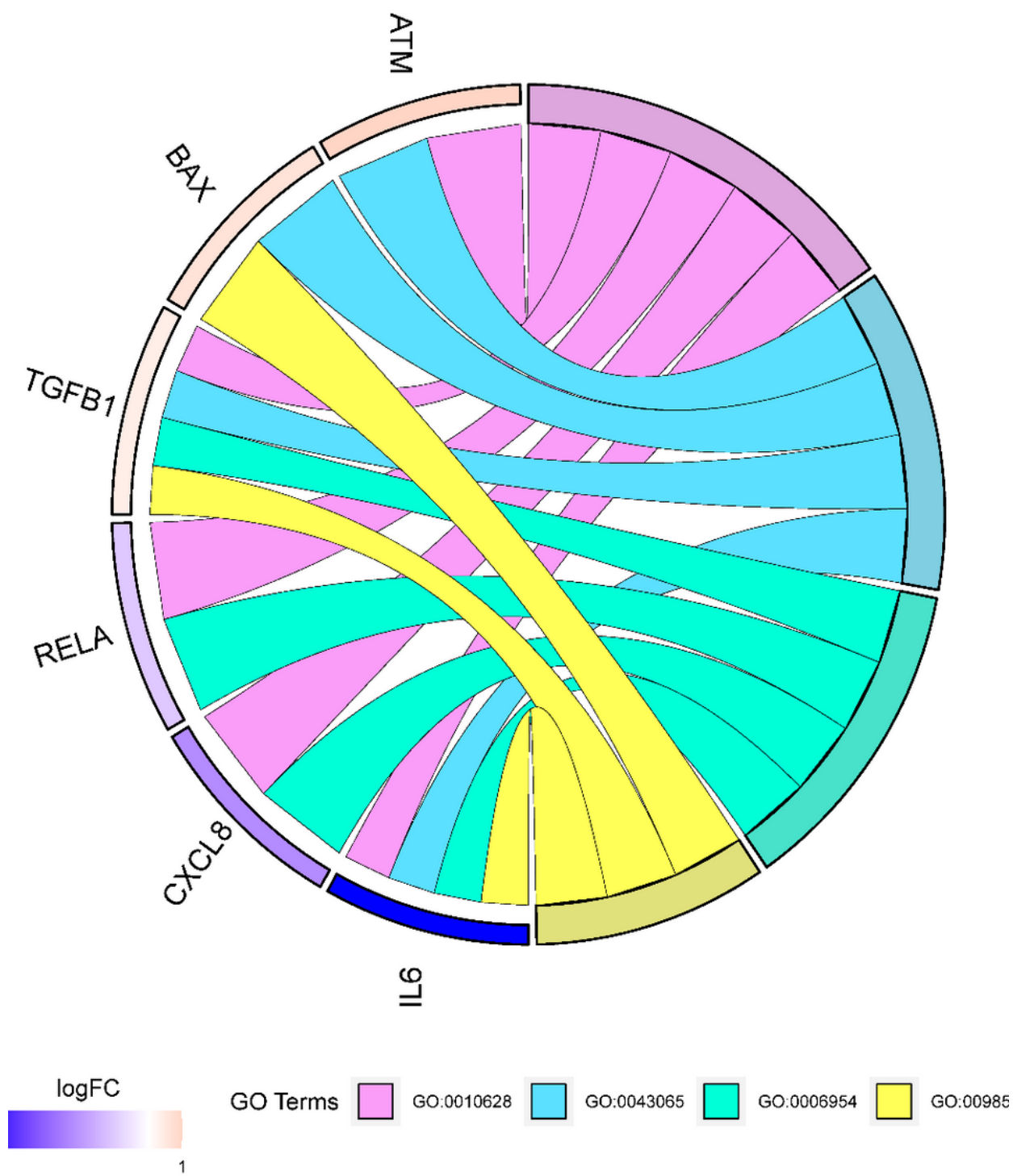


Figure 5

Gene ontology (GO) biological process analysis

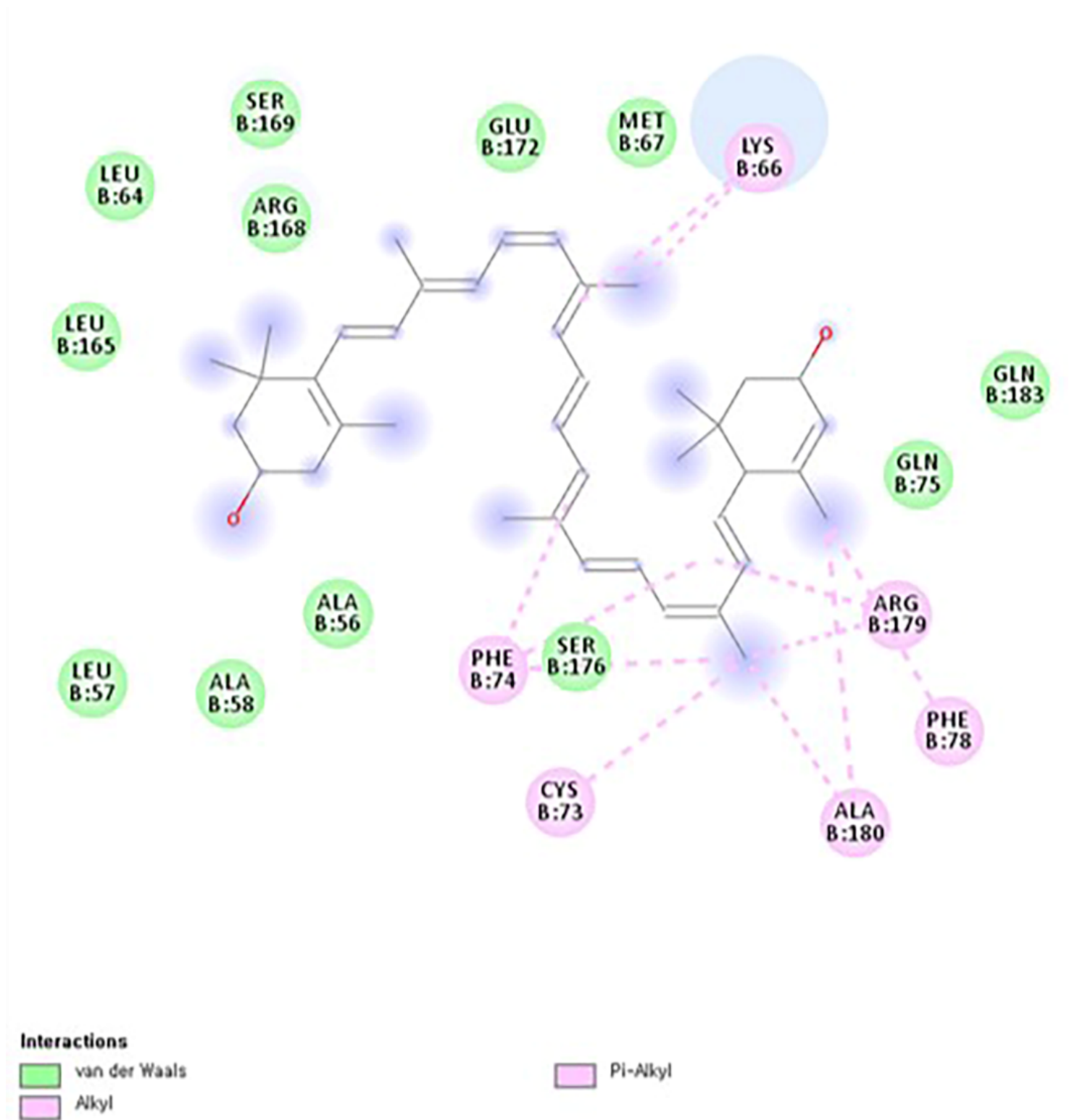


Figure 6

The 2D Ligand-Target interaction of lutein and IL-6

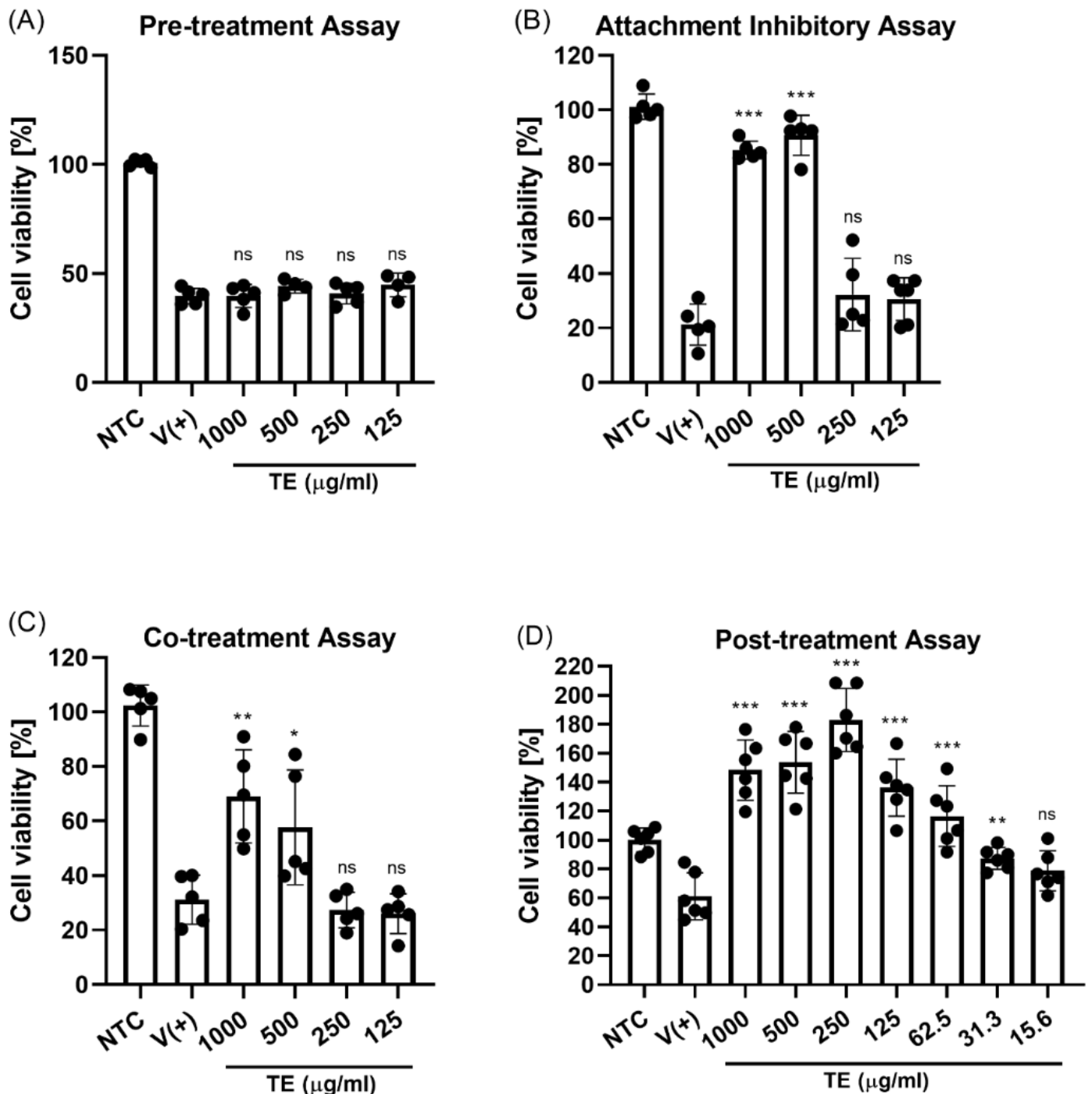


Figure 7

Evaluation of the antiviral properties of the TE extract. (A) Pre-treatment: MDCK cells were treated with different concentrations of the TE extract for 1 h at 37 °C, infected with the influenza virus (100 50% tissue culture infectious dose (TCID₅₀)/well), and incubated with the viral inoculum for 1 h at 37 °C. (B) Blockage of influenza H1N1 virus attachment: MDCK cells were infected with the influenza virus (100 TCID₅₀/well) and co-treated with different concentrations of the TE extract for 1 h at 4 °C. (C) Co-

treatment: MDCK cells were co-incubated with the influenza virus (100 TCID₅₀/well) and different concentrations of the TE extract for 1 h at 37 °C. (D) Post-treatment: MDCK cells were infected with the influenza virus (100 TCID₅₀/well) and incubated for 1 h at 37 °C, after which the virus was removed and cells were treated with different concentrations of the TE extract. NTC: not-infected and not-treated control group; V (+): infected and not-treated group; ***P<0.001, **P<0.01, and *P<0.05; *ns*: not significant (compared with the virus-only control).

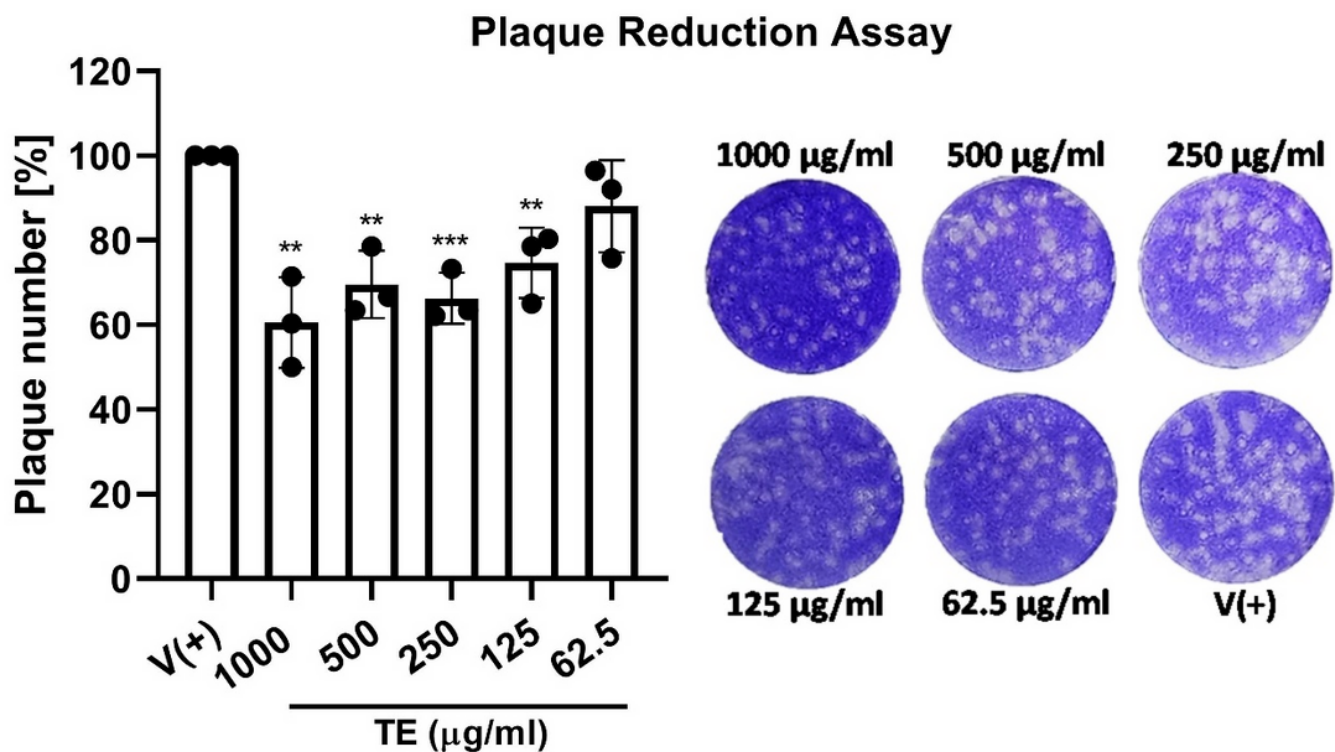


Figure 8

Plaque reduction assay. MDCK cells were infected with the influenza H1N1 virus (50 plaque-forming unit [PFU]/well) and incubated for 1 h at 37 °C. Next, cells were treated with different concentrations of the TE extract and incubated at the same conditions for 1 h. Then, compound mixtures were removed, and 3 mL of the overlay medium was added to each well. After two days, cells were stained with a crystal violet staining solution and plaques were calculated. Plaque reduction was determined by comparing with the untreated infected wells (n=3). **P<0.01 (compared with the virus-only control).