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Original Research Article

Investigating the Potential Targets and Mechanisms of Action of Chinese Medicine for the Treatment of EIV Based on Network Pharmacology and Molecular Docking as Well as *in vitro*

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Abstract

Background: Equine influenza virus (EIV) can cause acute infections and outbreaks of epidemics in horses and donkeys. It is one of the most economically impactful pathogens in equine respiratory diseases globally, resulting in substantial financial losses within the farming industry. Utilizing targeted anti-viral drugs is an effective strategy.
Objectives: The present study analyzes the potential of herbal medicines for the treatment of equine influenza (EI) based on network pharmacology, molecular docking techniques, and *in vitro* anti-viral studies.
Materials and Methods: The construction of a "traditional Chinese medicine (TCM) component–target–disease" network was performed using Cytoscape 3.9.0. The protein–protein interaction (PPI) network is performed through the STRING system. Bioconductor software was employed to conduct gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) functional enrichment analyses of biological processes (BPs). Molecular docking techniques revealed the degree of binding of core components to key target genes. Characterization of the anti-EI effect of TCM by cytotoxicity and *in vitro* studies.
Results: Consequently, five core TCMs were screened, which had 79 core targets related to EI. PPI network analysis highlighted 10 significant targets. Molecular docking results analysis revealed binding interactions between the main core component, kaempferol, and the targets prostaglandin-endoperoxide synthase 2 (PTGS2), matrix metalloproteinase-9 (MMP9), and estimated glomerular filtration rate (EGFR), with binding energies of −9.1, −8.3, and −8.0 (kcal/mol), respectively. *In vitro* studies have demonstrated that the inhibitory effect of kaempferol on EI is mainly in the initial phase.
Conclusion: Through network pharmacology, molecular docking, and *in vitro* experiments, kaempferol was demonstrated to combat EI through key targets of PTGS2, MMP9, EGFR, AKT, tumor necrosis factor (TNF), and IL-6. This study provides a basis for treating EI with herbal medicine and for later drug development. Future research should integrate network pharmacology with clinical applications, focusing on large-scale clinical trials to evaluate the efficacy and safety of TCM in influenza treatment, thereby enhancing its potential role in treating the disease.

Keywords

Cyberpharmacology, donkey, equine influenza virus, molecular docking, traditional Chinese medicine

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Introduction

Equine influenza (EI) is a highly contagious infectious disease in equine animals caused by the equine influenza virus (EIV), belonging to the influenza virus genus within the Orthomyxovidae family (Ferguson et al., 2016; Timoney, 1996). The virus can be transmitted between animals through various routes, including respiratory droplets, direct contact,

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and mating. Studies have confirmed that the EI A virus (subtype H3N8) has been isolated from dogs and pigs, indicating its potential to cross species barriers (Abente et al., 2016; Solórzano et al., 2015). More recently, a human case of H3N8 avian influenza has been documented, raising concerns about the possibility of H3N8 transmission from birds to humans. Although this does not conclusively demonstrate human susceptibility to the EIV, it highlights the broader potential for cross-species transmission of influenza viruses (Yang et al., 2022). Given the airborne nature of the virus and its high genetic variability, there is increasing apprehension regarding its pandemic potential (Daly et al., 2021; Tellier, 2006). While a vaccine exists for horses, its limited availability for donkeys, combined with the virus's highly contagious and variable nature, signifies the potential for a global outbreak. Hence, controlling the dissemination of the EIV is imperative.

EI primarily results from the H7N7 and H3N8 viruses, identified in 1956 and 1963, respectively (Oxburgh et al., 1994; Webster et al., 1992). Subsequently, the rising incidence of both clinically asymptomatic and infected animals has contributed to the global spread of EIV, with exceptions limited to a few countries (Cullinane & Newton, 2013). Reported outbreaks in China between 1993 and 1994 indicated that mules and horses displayed mild clinical symptoms, whereas donkeys exhibited severe effects, which led to mostly fatal outcomes despite similar exposure conditions to the virus (Shortridge, 1995; Timoney, 1996). Donkeys display heightened susceptibility to EIV infection, and while the clinical presentation of illness in both donkeys and mules resembles that of horses, donkeys typically exhibit more severe symptoms, likely attributed to their increased predisposition to bacterial bronchopneumonia (Yang et al., 2018). During an outbreak caused by the H3N8 sublineage in Shandong, China, in 2017, 300 seropositive animals fell ill, resulting in a 25% mortality rate (Yang et al., 2018). Recently, the city of Liaocheng in China tested 120 unvaccinated donkeys in 2020, resulting in an average seropositivity rate of 32.5%. All of these outbreaks occurred in Chinese provinces, suggesting that EIV poses a significant threat to large donkey farms in China.

China stands as one of the world's largest donkey-breeding nations, boasting a lengthy history of donkey breeding and abundant donkey resources (Seyiti & Kelimu, 2021). In China, donkeys fulfill roles beyond labor, as their skins serve as the primary material for the renowned donkey-hide gelatine (DHG), a well-known animal-derived Chinese herbal medicine recognized for its blood-tonifying and stopping-bleeding effects (Maggs et al., 2023). It has been reported that DHG may be a potential therapeutic agent for inflammatory skin diseases (Lee et al., 2022). Donkey milk is gaining attention as a natural nutritional and medicinal product mainly because of its compositional similarity to breast milk and its potential biological properties, including antioxidant, anti-inflammatory, anti-aging, anti-microbial, and anti-cancer attributes (Li et al., 2022). Donkey milk has also been reported to be used as a substitute for children with cow's milk intolerance, mainly because of its nutritional similarity to human milk (HM) and its excellent taste (Bonelli et al., 2020; Sarti et al., 2019). In addition, donkey meat is consumed in large quantities for its nutritional value and unique flavor (Seyiti & Kelimu, 2021; Sun et al., 2023). Despite the fact that donkeys are abundant in some countries and contribute significantly to local communities and such countries' economies, little attention has been paid to research on infectious diseases affecting laboring donkeys and mules (Câmara et al., 2020; Zhang et al., 2023). Oseltamivir carboxylate and zanamivir have been proposed as effective treatments for most EIVs and may be beneficial for treating EIs in horses (Yamanaka et al., 2006). Additionally, peramivir has demonstrated the ability to inhibit EIV activity *in vitro*. Horses administered a single intravenous dose of peramivir (3,000 mg/600 mL/animal, 7.8–9.3 mg/kg of body weight) showed significantly milder clinical symptoms (fever, runny nose, and cough) (Yamanaka et al., 2012). However, fewer studies have been reported on drugs for the treatment of EI, and the mechanism of action is unclear. There is a serious lack of research on the epidemiology and pathogenesis of viral infections in donkeys, and in the absence of an effective vaccine, control of infectious diseases relies on the detection of positive animals to break the transmission cycle (Câmara et al., 2020; Daly et al., 2021). However, in many cases, donkeys may display drug resistance or low susceptibility when infected, rendering the detection of infected animals challenging and potentially serving as a persistent source of infection for horses and other species, including humans. Therefore, there is an urgent need for a highly effective targeted drug with few side effects to prevent and control virus transmission. Chinese medicines have become a research hotspot for anti-viral therapy because of their abundant resources, low price, and few adverse effects (Liu, Hu et al., 2019). Chinese herbal medicine has been reported to be effective not only in the prevention of diseases but also in the treatment of common equine diseases (Mangan & Xie, 2022; Shmalberg et al., 2011). However, few studies on the treatment of EI with Chinese medicines have been reported so far, and their related mechanisms of action are not clear.

Network pharmacology is a new discipline based on the theory of systems biology, which analyzes networks of biological systems and selects specific signaling nodes for multi-target drug molecular design (Liu, Li et al., 2019; Niu et al., 2019). In addition, molecular docking is a unique computerized tool to aid drug design and discovery. It helps understand how compounds interact with their molecular targets and is commonly used in drug discovery and development (Chen et al., 2023). This study aims to analyze the targets of traditional Chinese medicine (TCM) alongside the disease targets related to EI using network pharmacology. The goal is to identify the common targets where TCM may act upon EI. The initial screening of the TCM library identified the top 10 TCMs with

cross-talk with EI. From these, the top five TCMs were further selected based on their ranking for network analysis, which examined the relationships between drug components, targets, and the disease. A key compound was identified, and molecular docking analysis was conducted to evaluate its binding interactions. The molecular docking and metabolic pathway analyses of the core components of TCM and the disease targets will be conducted to preliminarily determine the molecular-target-pathway mechanism of action of TCM in the treatment of EI, so as to provide a reference for in-depth research and development of new drugs in the later stage of the study.

Materials and Methods

Access to Core Chinese Medicine

The TCM Systems Pharmacology Database and Analysis Platform (TCMSP) (http://lsp.nwu.edu.cn/tcmsp.php) was used to retrieve drug constituents and identify their action targets by MOL.ID number. TCMSP is a specialized pharmacological platform for herbal medicine, providing insights into the relationship between targets, drugs, and diseases. Active ingredients of TCM were selected based on chemical properties meeting the criteria of drug-likeness (DL) ≥ 0.18 and oral bioavailability (OB) $\geq 30\%$ (Tang et al., 2022). The compounds were downloaded from the Universal Protein (UniProt) database (http://www.uniprot.org/) and organized in an Excel Spreadsheet. The UniProt database is a protein database containing protein sequences, functional information, and research article indexes. Next, we optimized the data using the "TRIM" function and matched target gene names using the "VLOOKUP" function. Finally, we annotated and screened the target proteins of the chemical compositions obtained using the UniProt database.

Disease Target Prediction

Gene symbol information corresponding to EI was retrieved by searching the GeneCards (https://www.genecards.org/) and Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/) databases. Preliminary screening was conducted, including entering disease keywords into the OMIM database for further searching, downloading the retrieved results, and obtaining disease-related targets. The final step involved integrating all targets, eliminating duplicate genes, and consolidating them as the disease targets for this study.

Acquisition of Intersection Target of Core Chinese Medicine and EI

Based on the screened core TCMs (*Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba*), the cross-targets shared between the core TCMs and EI were determined. The intersecting targets of active compounds within these TCMs and the disease were identified using Venny software (https://bioinfogp.cnb.csic.es/tools/venny/). These intersecting targets were considered potential key targets for therapeutic intervention.

"Component–Target–Disease" Network Analysis

Cytoscape 3.9.0 software was employed to analyze the network's topology, adjusting the target points' graphics, color, transparency, and size based on their degree. This process facilitated the construction of the network diagram depicting "TCM components–targets–diseases".

Protein–Protein Interaction (PPI) Network Construction and Network Topology Analysis

Protein interactions were obtained by importing intersecting genes through the STRING (https://string-db.org/) platform. Subsequently, Cytoscape 3.9.0 software was utilized to extract network topology parameters. The tab-separated values (TSV) files downloaded were then imported into Cytoscape software to create PPI maps. Finally, the protein interaction network maps were generated by filtering the top 10 core targets based on their degree values.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis

The GO analysis involves three components, specifically annotating the role of target proteins in gene function in biological process (BP), cellular component (CC), and molecular function (MF), respectively, to elucidate the impact of drug treatment on diseases. KEGG analysis is primarily a pathway analysis aimed at elucidating the major signaling pathways involved in the treatment of disease with drugs. The bioinformatics open-source software Bioconductor (http://www.bioconductor.org/) was first utilized to install and run the clusterProfiler, Stringin, and Pathview packages within the R software, which was then analyzed for GO and KEGG functional enrichment of BPs and visualized through the Microbiology Letter platform.

Molecular Docking

Molecular docking was performed using AutoDock Vina 1.1.2 software to assess the binding of kaempferol (PubChem CID: 5280863) with target proteins including AKT1 (UniProt ID: A0A9L0IW99), CASP3 (UniProt ID: A0A8C4MGV3), estimated glomerular filtration rate (EGFR) (UniProt ID: A0A8C4MRZ3), estrogen receptor 1 (ESR1) (UniProt ID: A0A8C4MI61), interleukin-1 beta (IL-1β) (UniProt ID: A0A8C4LCP8), interleukin-6 (IL-6) (UniProt ID: A0A8C4N5H8), matrix metalloproteinase-9 (MMP9) (UniProt ID: A0A8C4N1V2), prostaglandin-endoperoxide

synthase 2 (PTGS2) (UniProt ID: A0A8C4PIG3), tumor necrosis factor (TNF) (UniProt ID: A0A9L0JZG0), and tumor protein 53 (TP53) (UniProt ID: Q29480). The amino acid sequences of the target proteins were obtained from the UniProt database, and full-sequence protein structures were modeled using AlphaFold2. Models with the highest predicted local distance difference test (PLDDT) values were selected for molecular docking. Protein pre-treatment was performed using PyMOL 2.4, which involved removing water molecules and extraneous ligands and adding hydrogen atoms. ChemDraw 20.0 was used for the energy minimization of compounds. AutoDock tools 1.5.6 were used to generate protein data bank, partial charge, and torsion (PDBQT) files for docking simulations. The docking grid box was configured to encompass the entire protein structure, while other parameters were maintained at default settings. The docking conformation with the lowest binding energy and highest clustering frequency is considered to be the most potent binding mode between ligand and protein. Finally, we visualized the docking results using protein–ligand interaction profiler (PLIP) and PyMOL 2.4 software. In this way, we were able to visualize the binding of the ligand to the receptor and further analyze the stability and interactions of the complex (Adasme et al., 2021; Trott & Olson, 2010).

Cell Activity Test

Madin–Darby canine kidney (MDCK) epithelial cells (NBL-2; ECACC No. 84121903, CN) were cultured at 34°C in a humidified atmosphere containing 5% CO_2. The cells were maintained in Eagle's minimum essential medium supplemented with fetal calf serum (FCS) (10% Vol). For virus isolation and quantification, the cells were transferred to a serum-free medium containing 1.25 g of beef pancreas trypsin (Sigma–Aldrich)/mL and incubated for 7 days at 34°C in an atmosphere of 5% CO_2 (Quinlivan et al., 2004).

MDCK cells were inoculated in 96-well cell culture plates. Chinese medicines were diluted to varying concentrations (20, 40, 60, 80, 100, 120, 150, and 200 μmol/L) using serum-free Dulbecco's modified Eagle medium (DMEM). Dimethyl sulfoxide (DMSO) was used as a control. The cell viability assay was performed using a cell counting kit-8 (CCK8) kit. Treated plates were incubated in a 5% CO_2 incubator at 34°C for 48 h. The optical density (OD) at 450 nm was measured using a Tecan Infinite M200 Pro multifunctional microplate reader. The cell survival rate was calculated for each experimental group based on the OD measurements.

Effect of Core Chinese Medicines on EI Replication

The inhibitory effects of core Chinese medicines on the virus were assessed by determining the virus titer in MDCK cells using the 50% tissue culture infective dose ($TCID_{50}$) method. Equal volumes of Chinese medicines at concentrations of 40, 80, 100, and 200 μmol/L and the virus were added to MDCK cells under three treatment conditions: pre-treatment, post-treatment, and co-treatment. The supernatant from infected cells was collected 48 h post-infection, and cytopathic lesions were scored to calculate $TCID_{50}$ values using the Reed-Muench method (Zhang et al., 2024). To determine $TCID_{50}$, viral samples were serially diluted and inoculated into cell cultures. The inoculated plates were incubated under appropriate conditions, and the confirmation of cytopathic effect (CPE) in each well was periodically observed and recorded. The number of wells exhibiting CPE and those without were documented for each dilution. The cumulative number of wells with and without CPE, as well as the proportion of infected wells (percentage of infection), were calculated. The $TCID_{50}$ was determined using the formula: $lgTCID_{50}$ = Logarithm of virus dilutions greater than or equal to 50% + Distance ratio × Logarithm of the dilution factor. The $lgTCID_{50}$ was then converted to a $TCID_{50}$ value, usually expressed as the number of $TCID_{50}$ per millilitre of viral suspension. EI virus expression was then analyzed using $TCID_{50}$ at 24, 48, 72, and 96 h.

Effects of Core Chinese Medicines on Core Targets Induced by EI

Core Chinese medicine was utilized to detect the effects of EI-infected MDCK cells on core targets. MDCK cell monolayers in 6-well plates were washed and infected with EI at a multiplicity of infection (MOI) of 1. The messenger ribonucleic acid (mRNA) levels of these factors were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Bonometti et al., 2019; de Laat et al., 2011) (see Table A1).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software, version 8.0. Significant differences between groups were detected using one-way analysis of variance (ANOVA) or an independent *t*-test. Statistical significance was set at $p < .05$. The levels of significance were denoted as follows: $*p < .05$ indicated a statistically significant difference, $**p < .01$ represented a highly significant difference, and $***p < .001$ denoted an extremely significant difference between groups. A greater number of asterisks corresponded to a higher degree of statistical significance.

Results

Data Construction and Comparative Analysis of Core Chinese Medicines and EI Targets

The ten herbs exhibiting the strongest correlations with the disease targets were initially identified: *Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae*

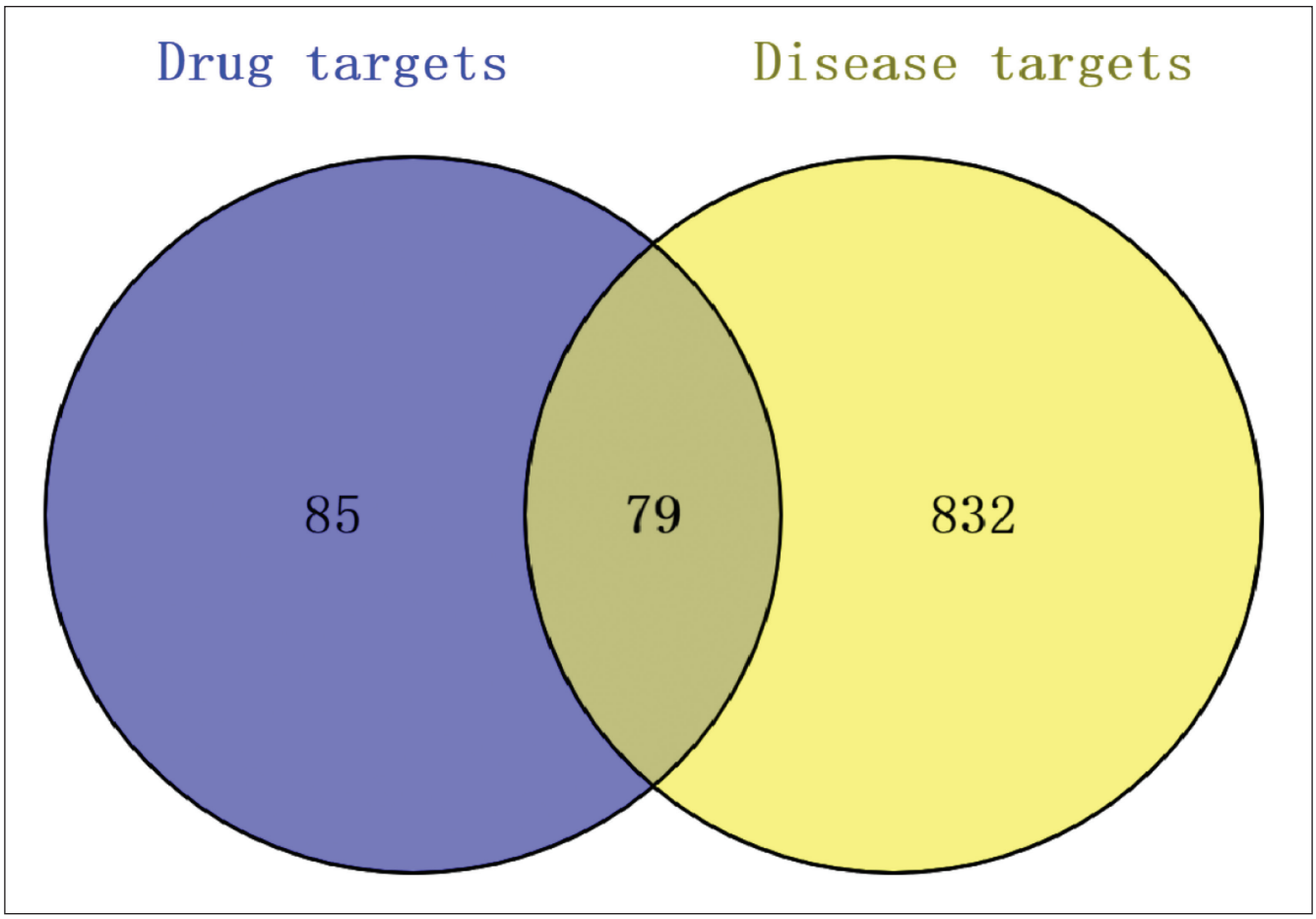


Figure 1. Core Component Targets of Action and Equine Influenza (EI)-related Pathogenesis Targets Wayne's Chart.

Rhizoma, Menthae Haplocalycis Herba, Piperis Longi Fructus, Xanthii Fructus, Aconiti Kusnezoffii Folium, Cicadae Periostracum, and *Folium Clerodendri Trichotomi*. Among these, *Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba* were selected for further analysis based on intersecting targets, yielding 85 drug-associated targets. Then, the EI-related target genes were analyzed, and 832 related targets were obtained. The intersection of drug-associated targets and EI-related targets was determined using a Wayne diagram, revealing 79 overlapping targets (Figure 1).

Network Analysis Results for Components, Targets, and Diseases

The network topology analysis using Cytoscape 3.9.0 revealed the construction of a "component–target–disease" network diagram based on the degree value. It demonstrated that the core herb—*Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba*—acted through multiple components and targets in their action against EI. Notably, kaempferol demonstrated the highest number of target interactions with EI, totaling 20 targets. Consequently, kaempferol was prioritized for subsequent experimental validation (Figure 2).

PPI Network Construction and Network Topology Analysis Results

Protein interactions were obtained by importing intersecting genes through STRING, and subsequent network topology parameters were obtained using Cytoscape 3.9.0 software. Core targets were then screened based on their degree values, leading to the identification of key targets, including MMP9, PTGS2, EGFR, AKT1, TNF, interleukin 1 beta (IL-1β), and IL-6 (Figure 3).

GO and KEGG Pathway Enrichment Analyses

To further understand the functions of the core targets screened above and their roles in signaling pathways, GO and KEGG functional enrichment analyses of BPs were performed using the bioinformatics open-source software Bioconductor. The resultant GO functional enrichment analysis yielded 4715 GO entries, of which 3304 were BPs. The major BPs enriched included response to lipopolysaccharide,

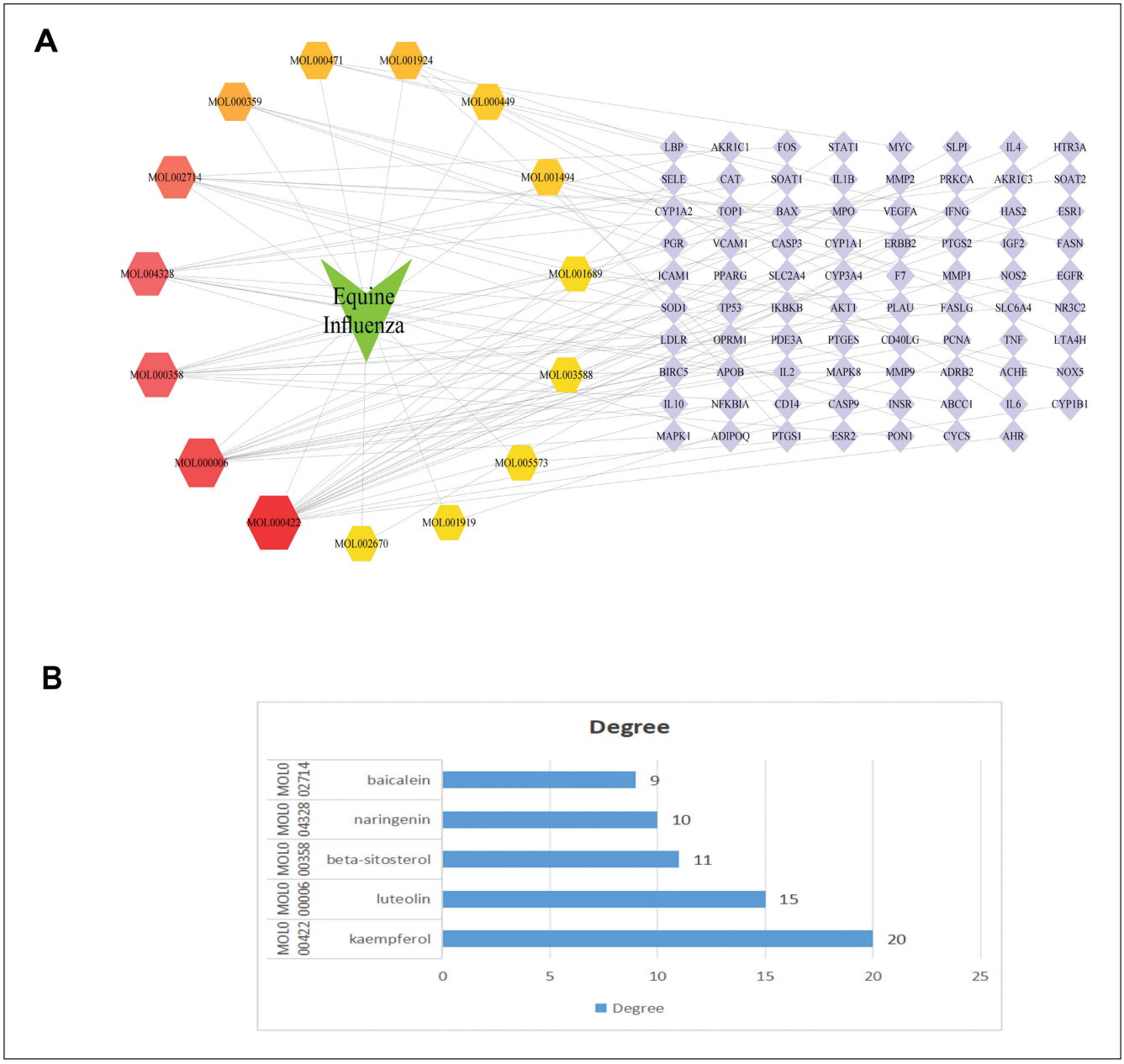


Figure 2. Component–Target–Disease Network Diagram.

Notes: The arrows, hexagons, and quadrilaterals represent diseases, components, and targets, respectively. The links represent the interactions between the three.

response to molecule of bacterial origin, cellular response to chemical stress, response to drug, response to oxidative stress, response to reactive oxygen species, reactive oxygen species metabolic process, cellular response to oxidative stress, steroid metabolic process, and reactive oxygen species biosynthetic process. There were 218 entries for cellular composition (CC), including membrane raft, membrane microdomain, membrane region, plasma membrane raft, vesicle lumen, caveola, external side of plasma membrane, dendrite cytoplasm, cytoplasmic vesicle lumen, and plasma lipoprotein particle. There were 401 entries in the MF category. The major MFs included heme binding, tetrapyrrole binding, steroid binding, amide binding, cytokine receptor binding, cytokine activity, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, peptide binding, nuclear receptor activity, and ligand-activated transcription factor activity. KEGG pathway enrichment screening yielded 273 signaling pathways, mainly involving the TNF signaling pathway, IL-17 signaling pathway, and others (Figure 4).

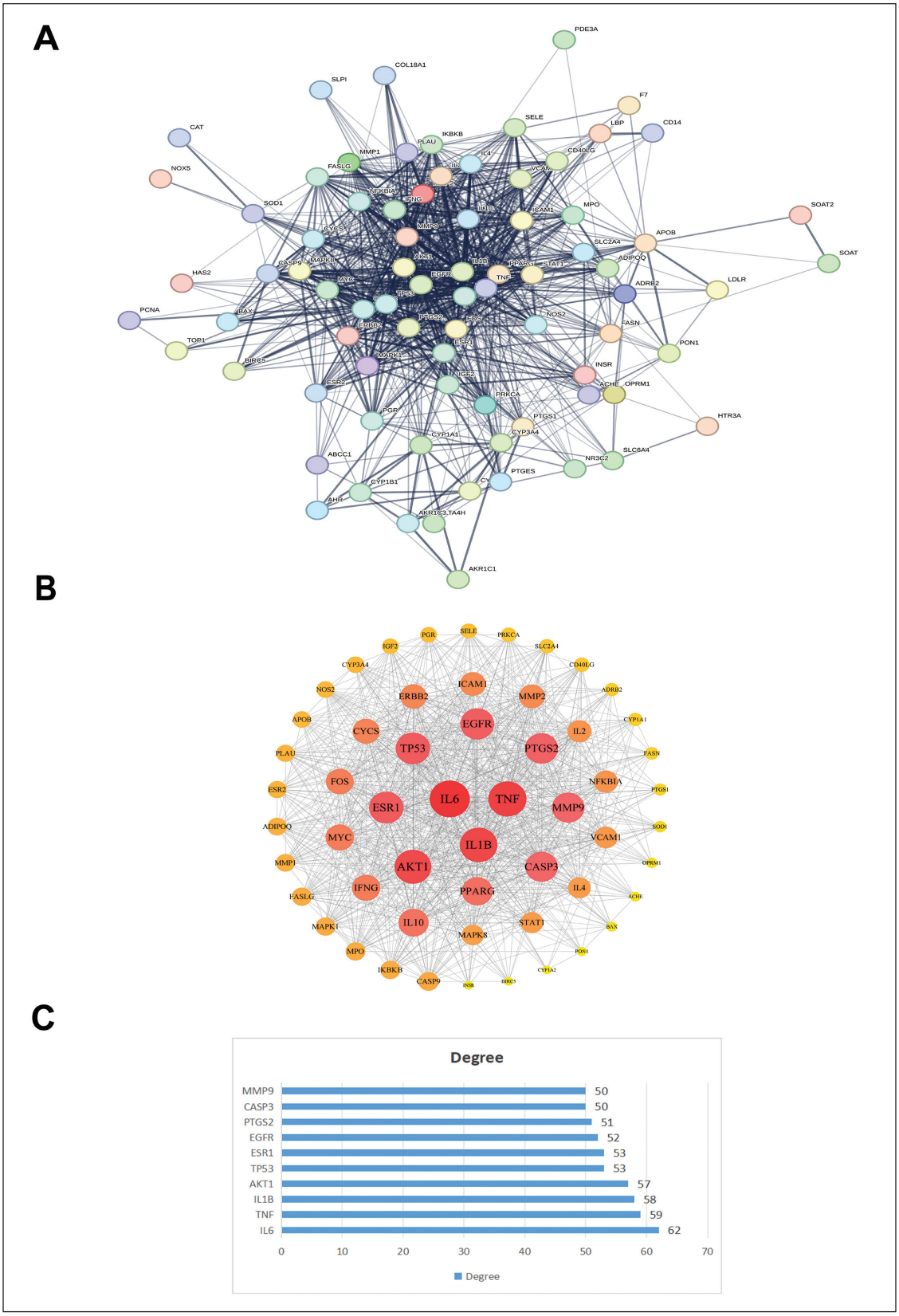


Figure 3. Protein Interaction Network Topology Screening.

Notes: The size and color of the nodes are related to the degree value. The color and size of the nodes are adjusted according to the degree value. The darker the color, the larger the node and the larger the degree value.

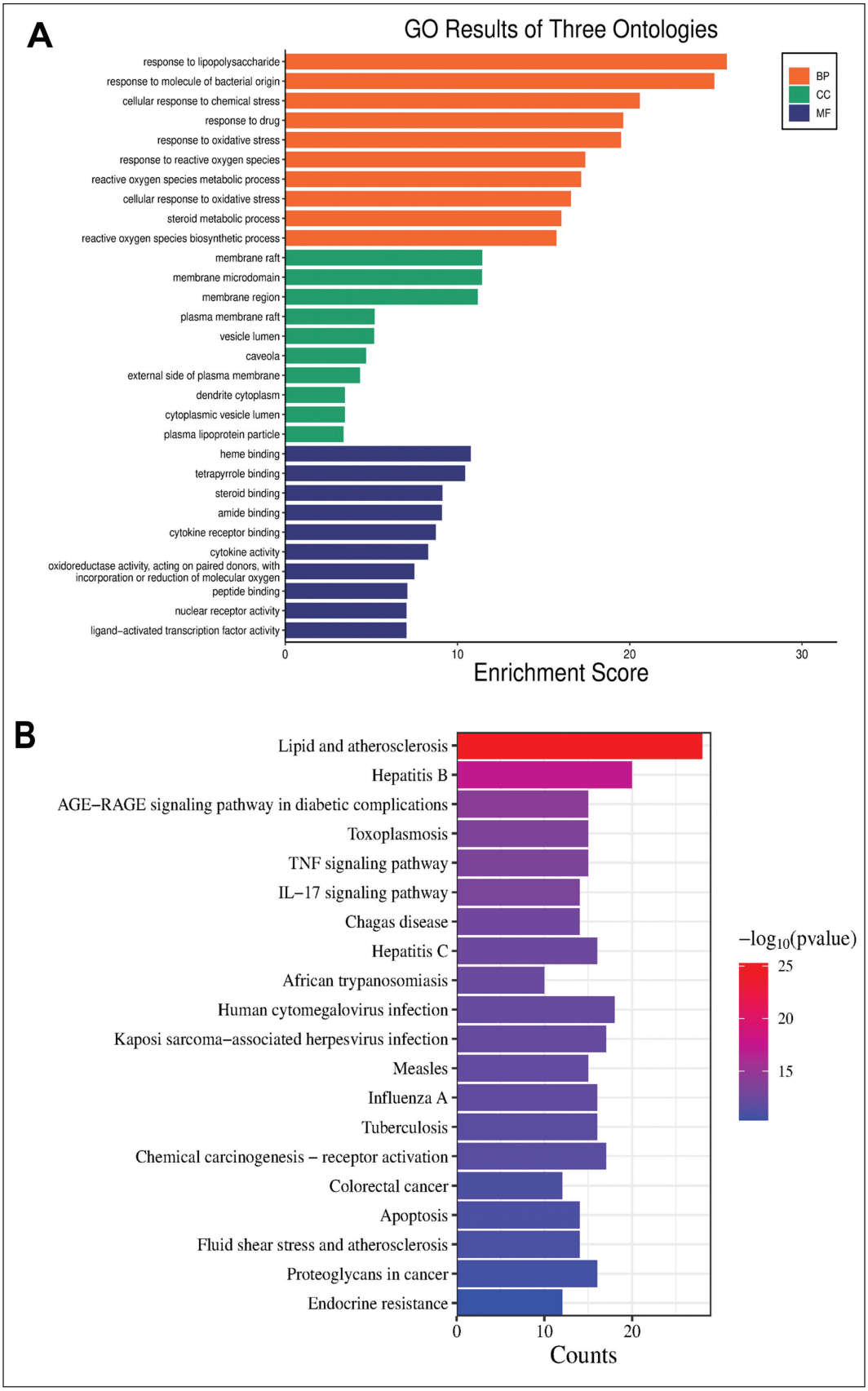


Figure 4. Gene Ontology (GO) (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (B) Pathway Enrichment Analysis.

Molecular Docking Results

Molecular docking analysis was performed to further determine the interaction between the core target and the core component, kaempferol. The more stable the conformation in which the ligand binds to the receptor, the lower the energy and the greater the likelihood of action occurring. The docking results provided data on binding energy, interaction force, and bond length (Table 1). As can be seen from the results, the core component, kaempferol, has good binding activity with PPI core protein macromolecules (PTGS2, MMP9, and EGFR) receptors, with the best binding energy for PTGS2. The respective binding energies for PTGS2, MMP9, and EGFR were −9.1, −8.3, and −8.0 kcal/mol (Table 1, Figure 5). Two-dimensional interaction analysis revealed that kaempferol's hydrophobic carbon chain closely interacted with adjacent amino acids via van der Waals forces. Specific active pocket compositions were identified for the core proteins: the EGFR active pocket included Met, Leu, Thr, Gly, Ala, and Lys; the MMP9 active pocket comprised Ser, Thr, Phe, Tyr, and Leu; and the PTGS2 active pocket contained Ala, Thr, Phe, Tyr, Trp, and Gln residues. Hydrophobic interactions and hydrogen bonding play an important role in the binding of kaempferol to target proteins. Additionally, kaempferol was able to undergo π-cation/stacking interactions with IL-6 and PTGS2. These interactions were able to stabilize the binding of kaempferol to the target proteins and demonstrated the high specificity of the interactions.

Results of Kaempferol Cytotoxicity Assay on MDCK

Kaempferol has no major effect on cell survival across the tested concentrations of 20, 40, 60, 80, 100, 120, 150, and 200 μmol/L, confirming its non-toxic nature for MDCK cells. Based on findings from prior studies, a concentration of 100 μmol/L was determined to optimize anti-viral efficacy while maintaining cellular viability for specific viruses and cell models. For example, an *in vitro* study on dengue virus demonstrated that 100 μmol/L of ribavirin reduced viral load by over 3 log10, while the inhibitory effect was not significant at lower concentrations (Botta et al., 2018). Therefore, 100 μmol/L of kaempferol was selected for subsequent experiments (Figure 6).

Effect of Kaempferol on the Replication of EI Virus

Comparing the viral suppression effects of pre-treatment, co-treatment, and post-treatment can help us understand the strength of the drug's action at different stages. In the pre-treatment model, the drug is administered before viral exposure. Significant inhibition under this condition suggests that the drug may either prevent viral binding to host cells or reduce cellular susceptibility to infection. In the co-treatment model, the drug is introduced simultaneously with viral infection, allowing evaluation of its direct impact on the infection process. Post-treatment involves administering the drug after viral entry and assessing its potential to mitigate established infections. The results of this study showed that kaempferol exhibited the most effective inhibition of the EI virus group infection during the pre-treatment phase. This indicates that kaempferol primarily exerts its anti-viral action during the early stages of viral infection, likely interfering with viral entry or cell susceptibility mechanisms. The observed differences were statistically significant ($p < .05$) (Figure 7A). The virus showed a dose-dependent decrease with increasing kaempferol concentration. A concentration of 100 μmol/L was identified as optimal and selected for subsequent experiments (Figure 7A–7C). Then, the effect of kaempferol on the EI-virus replication in MDCK cells at different times was investigated. The results showed that the inhibition of viral RNA copies was most effective at 48 h ($p < .05$) (Figure 7D).

Effects of Kaempferol on Core Targets Induced by EI

The effect of kaempferol on PTGS2, MMP9, and EGFR, a major target of EI induction, was further investigated based on molecular docking and KEGG results (Table A1). The results showed that kaempferol inhibited the expression of PTGS2, EGFR, and MMP9 ($p < .05$) (Figure A1).

Discussion

Donkeys (*Equus asinus*) and mules make up about 50% of the world's entire livestock population and play a vital role in the lives of thousands of people, especially in developing countries. However, donkeys exhibit varying susceptibility and distinct clinical manifestations in response to specific infectious agents when compared to horses, yet the understanding of the associated pathogenesis, immune response, and pathology in donkeys remains limited, with most available knowledge stemming from veterinary clinical experience (Burden & Thiemann, 2015). More importantly, some viruses may cross the species barrier and affect humans (Câmara et al., 2020), posing an imminent risk to public health. Therefore, this study aimed to analyze the potential of herbal medicines for the treatment of EI through network pharmacology and molecular docking techniques and to lay the foundation for the future development of donkey anti-viral drugs.

The top 10 herbs with the highest relevance to EI targets were first screened from the TCM library. They were *Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma, Menthae Haplocalycis Herba, Piperis Longi Fructus, Xanthii Fructus, Aconiti Kusnezoffii Folium, Cicadae Periostracum, Folium Clerodendri Trichotomi*. From these, the top-ranked *Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba* were selected for the network pharmacological analysis, which

Table 1. Interaction of Kaempferol with Protein Macromolecules.

Macromolecule	Binding Energy (kcal/mol)	Hydrogen Bonds	Hydrophobic Interaction	π-Stacking	π-Cation Interaction
AKT1	−7.9	Gly 302A (2.75 Å, 4.02 Å), Asp 324A (3.89 Å), Arg 327A (4.10 Å, 2.80 Å), Gly 392A (2.67 Å)	Arg 327A (3.74 Å), Pro 387A (3.55 Å)		
CASP3	−6.5	Ile 164A (3.21 Å, 2.70 Å), Gly 174A (3.11 Å), Ser 176A (3.95 Å)	Thr 163A (3.86 Å), Ile 168A (3.72 Å, 3.74 Å)		
EGFR	−8.0	Met 793A (3.01 Å, 2.91 Å), Leu 718A (3.21 Å), Thr 790A (3.02 Å), Gly 796A (3.98 Å)	Leu 718A (3.98 Å), Ala 743A (3.40 Å), Lys 745A (3.23 Å)		
ESR1	−7.6	Glu 330A (2.80 Å), Asp 332A (4.03 Å), Asn 348A (3.36 Å), Tyr 537A (2.70 Å)	Leu 345A (3.35 Å, 3.90 Å), Tyr 331A (3.89 Å), Asp 332A (3.67 Å), Arg 335A (3.62 Å)		
IL-1β	−7.3	Thr 98A (3.34 Å, 3.34 Å), Lys 94A (2.97 Å), Tyr 99A (3.11 Å), Glu 100A (3.26 Å), Arg 194A (3.25 Å)			
IL-6	−7.8	Ser 73A (2.80 Å), Ser 78A (4.06 Å), Asn 184A (3.06 Å), Thr 185A (3.81 Å)	Lys 74A (3.67 Å, 3.57 Å), Phe 72A (3.54 Å), Val 81A (3.91 Å), His 188A (3.76 Å)	Phe 72A (5.00 Å)	Lys 74A (5.45 Å, 5.33 Å)
MMP9	−8.3	Ser 276A (3.86 Å), Thr 187A (3.25 Å)	Phe 290A (3.88 Å, 3.79 Å), Tyr 182A (3.37 Å, 3.69 Å), Leu 195A (3.94 Å)		
PTGS2	−9.1	Ala 247A (3.97 Å), Thr 254A (3.10 Å), Phe 258A (4.02 Å), Tyr 433A (3.31 Å)	Gln 251A (3.97 Å), Trp 435A (3.70 Å)		His 255A (5.73 Å, 5.69 Å), His 434A (4.32 Å)
TNF	−6.2	Ala 114A (3.26 Å), Ala 129A (3.97 Å), Gly 244A (4.02 Å), Val 246A (2.94 Å)	Val 113A (3.68 Å), Pro 116A (3.98 Å), Arg 128A (3.76 Å), Ala 129A (3.61 Å)		
TP53	−6.7	Gln 11A (3.26 Å), Thr 15A (3.81 Å), Arg 158A (2.83 Å), Asp 200A (3.15 Å)	Pro 154A (3.73 Å)		

Note: EGFR: Estimated glomerular filtration rate; ESR1: Estrogen receptor 1; IL-6: Interleukin-6; IL-1β: Interleukin-1 beta; MMP9: Matrix metalloproteinase-9; PTGS2: Prostaglandin-endoperoxide synthase 2; TNF: Tumor necrosis factor; TP53: Tumor protein 53.

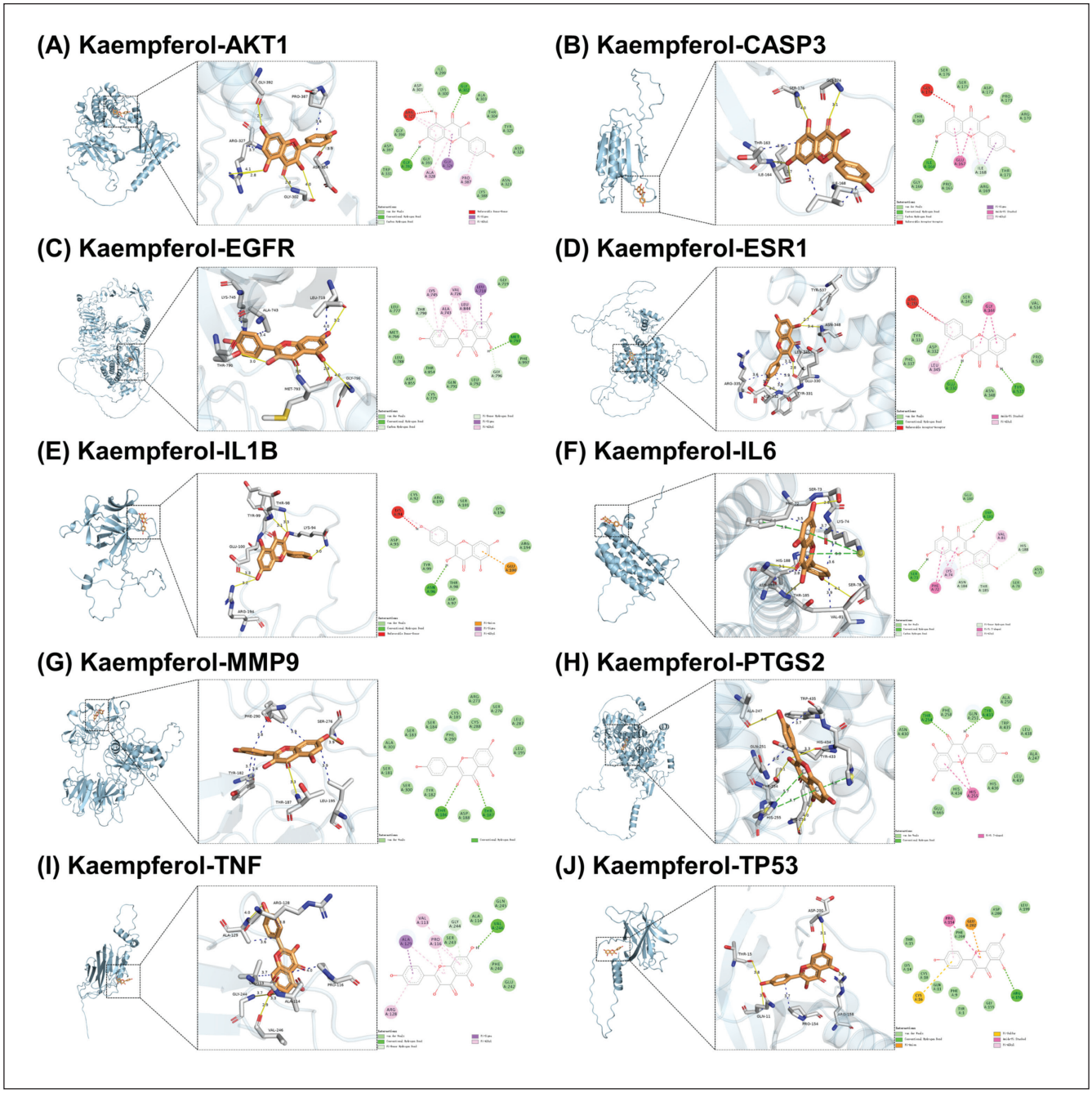


Figure 5. Verification of Docking Between Small Molecules and Key Targets.

Notes: (A) Kaempferol-AKT1; (B) kaempferol-CASP3; (C) kaempferol-estimated glomerular filtration rate (EGFR); (D) kaempferol-estrogen receptor 1 (ESR1); (E) kaempferol-interleukin (IL)-1β; (F) kaempferol-IL-6; (G) kaempferol-matrix metalloproteinase-9 (MMP9); (H) kaempferol-prostaglandin-endoperoxide synthase 2 (PTGS2); (I) kaempferol-tumor necrosis factor (TNF); (J) kaempferol-tumor protein 53 (TP53). The receptor was chosen as the target protein, with kaempferol selected as the ligand. Hydrogen bonds, hydrophobic interactions, and π-cation/stacking interactions are depicted in yellow, blue, and green, respectively.

yielded 79 intersecting targets. Then, a TCM component and target disease network screening were performed with the aim of identifying the major drug components that are against EI. As a result, the major core components were kaempferol, luteolin, β-sitosterol, naringenin, and baicalein. Kaempferol demonstrated the most extensive involvement, interacting with 20 distinct cross-targets. Kaempferol is classified as an organic compound within the flavonoid family. Studies have indicated that kaempferol demonstrates potential antioxidant, anti-mutagenic, anti-fungal, and anti-viral activities, attributed to mechanisms involving apoptosis, cell cycle arrest, cysteine asparaginase activation, modulation of human

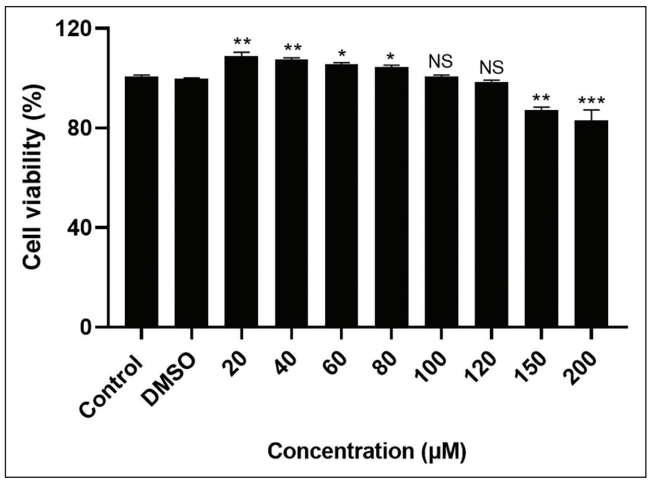


Figure 6. Cytotoxicity Results of Chinese Medicines on Madin–Darby Canine Kidney (MDCK) Cells.

Notes: Control: Normal cells. ***$p < .001$, **$p < .01$, *$p < .05$, $n = 3$. Data were presented as mean ± SD.

telomerase reverse transcriptase genes, down-regulation of epithelial-mesenchymal transition (EMT)-related markers, and interference with phosphoinositide 3-kinase/protein kinase B signaling pathways, suggesting its prospective use in disease prevention (Imran et al., 2019; Sengupta et al., 2022). Additionally, kaempferol, an active compound in Lianhua Qingwen capsules (LQC), demonstrates potential therapeutic effects by acting on Akt1 and has implications for COVID-19 (Xia et al., 2020). Care et al. (2020) evaluated the effect of kaempferol on the Japanese encephalitis virus (JEV), observing a significant inhibition of JEV infection (Care et al., 2020). Kaempferol, found in the TCM *Scutellaria baicalensis*, has been reported to exhibit anti-influenza A (H1N1) activity (Pirali et al., 2023). Similarly, kaempferol is also the primary active ingredient in *Paeoniae Radix Alba*. Therefore, it is of great reference value to study the mechanism of action of kaempferol as a core component against EIV. For this reason, kaempferol was selected for further studies.

To better understand how Chinese medicines combat EIV, a PPI network analysis was conducted to identify shared cross-targets between the core components of the top five TCMs and EIV. The analysis revealed key targets such as PTGS2, EGFR, MMP9, AKT1, TNF, IL-1β, and IL-6. Kaempferol, identified as a core active compound, was further analyzed for its interaction with these viral proteins using molecular docking techniques. It was found that the PTGS2 has the lowest binding energy. Prostaglandin G/H synthase 2 (COX2/PTGS2) is prostaglandin peroxidase synthase 2, also known as cyclooxygenase-2 (COX-2). PTGS2 not only plays a role in maintaining normal physiological function but also plays an important role in tumor development, nerve damage, and inflammatory diseases. Curcumin was found to have antiviral and anti-inflammatory activity against JEV by inhibiting the expression of NS5 protein, IL-6, AKT1, TNF-α, and PTGS2 (Maurya et al., 2023). EGFR dimerization can activate its intracellular kinase pathway, which can guide downstream phosphorylation, including MAPK, Akt, and JNK pathways, and induce cell proliferation. The researchers found that influenza virus and rhinovirus (RV)-induced EGFR activation suppressed interferon lambda (IFN-λ) production induced by IFN regulatory factor (IRF) 1 and increased viral infection (Ueki et al., 2013). Studies have shown that luteolin activates the PI3K/Akt signaling pathway by inhibiting MMP9, improving cell viability, and down-regulating cell apoptosis (Luo et al., 2019). The study's findings demonstrated that kaempferol exhibited significant anti-EIV activity. The strongest anti-viral effect was observed when kaempferol was applied prior to viral infection, suggesting that it may protect host cells by preventing viral entry during the early stages of infection. This effect is likely mediated by kaempferol's interactions with specific viral and cellular targets, including PTGS2, EGFR, and MMP9.

The results of GO analysis showed that the core herbs (*Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba*) were mainly involved in the organism's BPs, contributing to the treatment of EI. From the KEGG pathway screening, a total of 273 signaling pathways were identified, primarily encompassing pathways like the TNF signaling pathway and IL-17 signaling pathway, among others. TNF, mainly produced by activated monocytes/macrophages, is an important inflammatory factor and is involved in the pathological damage of certain autoimmune diseases. TNF is classified into two types, TNF-α and TNF-β, and is one of the most potent biologically active factors, with the most potent direct tumor-killing effect discovered so far. Paeoniae Radix Rubra Extract (PRRE) has been observed to hinder iron death and trigger autophagy via the PI3K/Akt signaling pathway, reducing cerebral ischemic injury while also effectively suppressing TNF-α-induced MUC5AC mucin and inflammatory cytokines expression, potentially linked to the ERK pathway (Han et al., 2023; Zhao et al., 2023). Paeonol (Pae) is an active ingredient isolated from *Paeonia lactiflora* extract with anti-apoptotic and anti-inflammatory effects. Studies have reported that Pae reduces the concentration and mRNA levels of TNF-α, IL-6, and IFN-γ, thereby inhibiting the inflammatory response of the placenta in the preeclampsia (PE) mouse model (Wang et al., 2013, 2022). The main core components associated with EI targets in this study were kaempferol, luteolin, β-sitosterol, naringenin, and baicalein, with kaempferol being involved in the most targets. In addition, molecular docking analysis further revealed that kaempferol exhibited strong binding affinity to key EI-associated proteins. Notably, Paeoniae Radix Alba was found to contain kaempferol and β-sitosterol as its primary active components. Based on the results of KEGG pathway analysis, the anti-EI mechanism of kaempferol may be to block the virus at the pre-infection stage and to achieve anti-viral effects by

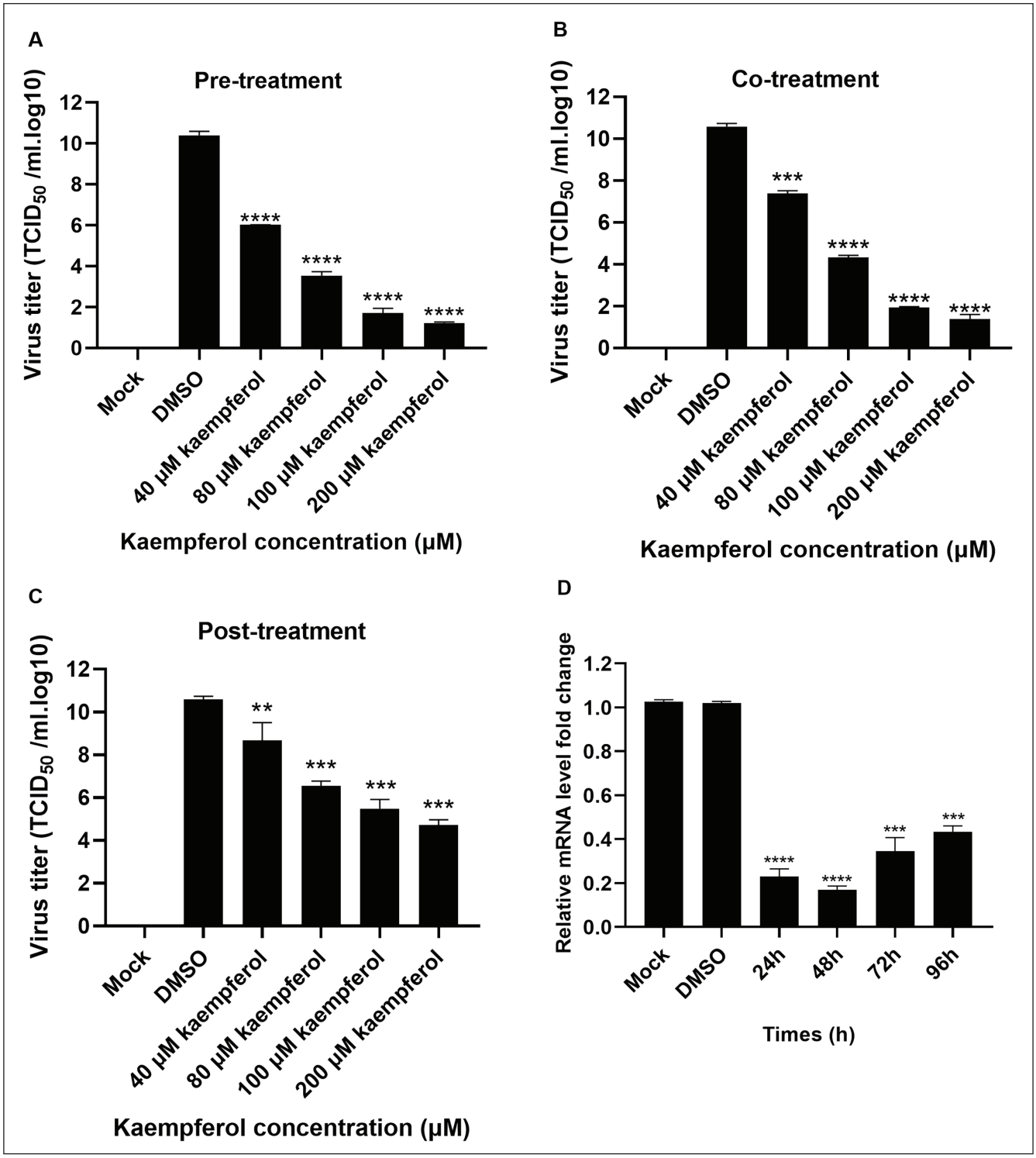


Figure 7. Results of Chinese Medicines on Equine Influenza (EI) Virus Replication.

Notes: (A) Pre-treatment; (B) co-treatment; (C) post-treatment; (D) effect of kaempferol on EI inhibition at different times of action. NS: The difference was not significant. ****$p < .001$, ***$p < .01$, **$p < .05$, $n = 3$. Data were presented as mean ± SD.

increasing autoimmunity, as well as lymphocyte viability, increasing the rate of cell division, and affecting the gene expression of cytokines PTGS2, EGFR, MMP9, AKT1, TNF, IL-1β, and IL-6. Overall, Paeoniae Radix Alba exhibits multi-selectivity in both targets and pathways in its action against EI, offering valuable insights for future investigations into the mechanisms of Chinese medicine against this viral infection. For future applications, *Paeoniae Radix Alba* can

be prepared as a decoction and administered orally to animals using tools such as a pill dispenser. Alternatively, it can be processed into a powder and incorporated into animal feed for convenient consumption. Another potential application involves combining *Paeoniae Radix Alba* with other drugs to create compound TCM formulations. In summary, the growing emphasis on health has led to an increasing diversity in the demand for Chinese medicine products. Network screening can help identify Chinese medicines with market potential and provide a basis for the Chinese medicine industry to develop new products. Furthermore, integrating network pharmacology with clinical applications offers a promising approach to analyzing therapeutic targets and signaling pathways. By using network pharmacology to address clinical challenges, potential targets for drug intervention can be identified, facilitating the development of novel drug combinations or therapeutic strategies to support clinical decision-making.

Conclusion

In this study, the potential of herbal medicines for the treatment of EI was analyzed using network pharmacology and molecular docking techniques. The core herbs were identified as *Typhonii Rhizoma, Paeoniae Radix Alba, Angelicae Dahuricae Radix, Pinelliae Rhizoma*, and *Menthae Haplocalycis Herba*. The main active compound, Kaempferol, facilitates the treatment of EI by targeting core factors like PTGS2, EGFR, MMP9, AKT1, TNF, IL-1β, and IL-6, setting the foundation for the next in-depth study of the mechanism of action of TCM against EI. This study demonstrated that network pharmacology and molecular docking are reliable approaches; however, it is important to note that the findings are based on *in vitro* experiments. Further validation through clinical and animal studies is required to confirm these results.

Abbreviations

BP: Biological process; CC: Cellular component; COX-2: Cyclooxygenase-2; DL: Drug-likeness; EHV: Equine herpesvirus; EIV: Equine influenza virus; EIA: Equine infectious anemia; EMT: Epithelial-mesenchymal transition; EVA: Equine viral arteritis; GO: Gene ontology; IRF: IFN regulatory factor; JE: Japanese encephalitis; JEV: Japanese encephalitis virus; JNK: c-Jun N-terminal kinase; KEGG: Kyoto Encyclopedia of Genes and Genomes; KMP: Kaempferol; LQC: Lianhua Qingwen capsules; MAPK: Mitogen-activated protein kinases; MF: Molecular function; OB: Oral bioavailability; OMIM: Online Mendelian Inheritance In Man; PE: Preeclampsia; PI3K: Phosphatidylinositol 3-kinase; PPI: Protein–protein interaction network; RV: Rhinovirus; SymMap: Symptom mapping; TCM: Traditional Chinese medicine; TCMSP: Traditional Chinese Medicine Systems Pharmacology; TNF: Tumor necrosis factor; UniProt: Universal protein; WNF: West Nile fever; WSSV: White spot syndrome virus.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The ethical approval has been obtained from the Institutional Ethics Committee for the study.

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Informed Consent

Not applicable.

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Appendix A

Table A1. Primers for Core Targets.

Number	Target Gene	Primer Sequence (5′-3′)
1	PTGS2	F: GCTTGTTCCAGACGAGCAGG
		R: GGGATGCCAGTGATAGAGCG
2	MMP9	F: CAGGCGTGGGCCAGTTC
		R: GTCGCGCGGCAAGTCT
3	EGFR	F: TTCTGGACTGATATGGGGATT
		R: GGCATCGCACCAATACAACTT
4	GAPDH	F: CCACCCCTAACGTGTCAGTC
		R: AATCGCAGGAGACAACCTGG

Note: EGFR: Estimated glomerular filtration rate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MMP9: Matrix metalloproteinase-9; PTGS2: Prostaglandin-endoperoxide synthase 2.

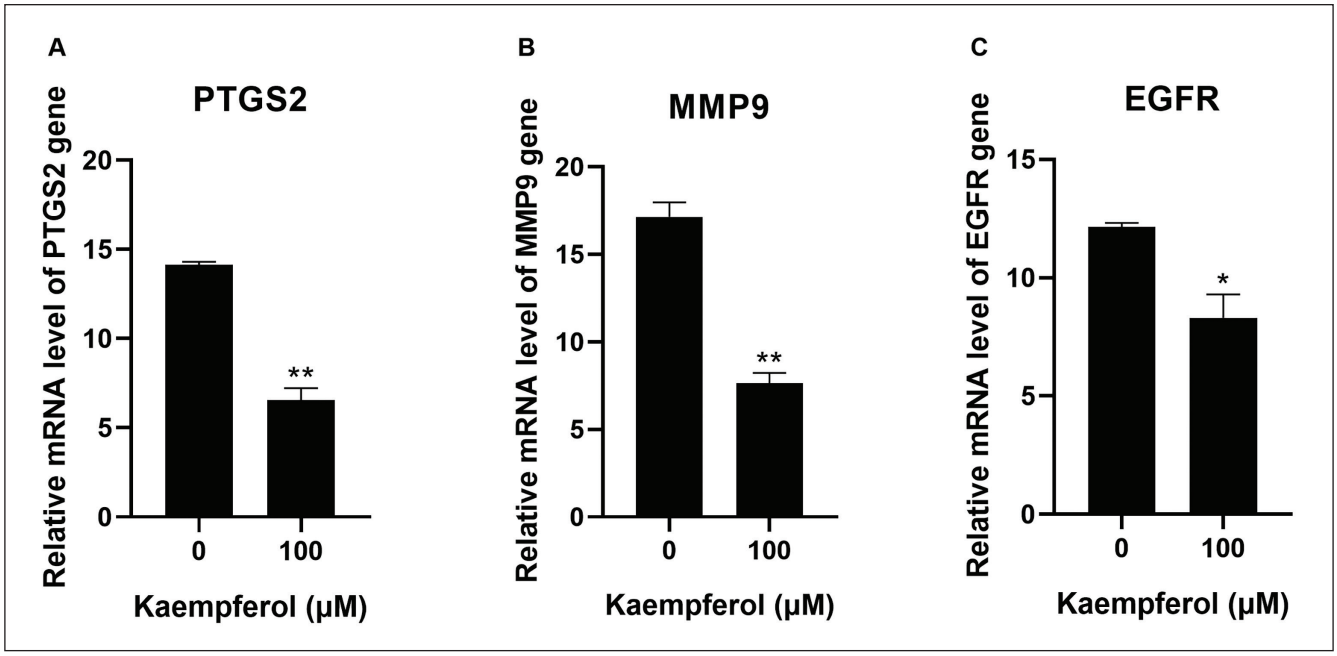


Figure A1. Effects of Kaempferol on Core Targets Induced by Equine Influenza (EI).

Notes: ** $p < .01$, $n = 3$. Data were presented as mean ± SD.