An Integrated Analysis Reveals Geniposide Extracted From Gardenia Jasminoides Ellis Regulates Calcium Signaling Pathway Essential for Influenza a Virus Replication

LiRun Zhou
China Academy of Chinese Medical Sciences https://orcid.org/0000-0002-1350-940X
Lei Bao
China Academy of Chinese Medical Sciences
Yaxin Wang
China Academy of Chinese Medical Sciences
Mengping Chen
China Academy of Chinese Medical Sciences
Yingying Zhang
Beijing University of Chinese Medicine Affiliated Dongzhimen Hospital
Zihan Geng
China Academy of Chinese Medical Sciences
Ronghua Zhao
China Academy of Chinese Medical Sciences
Jing Sun
China Academy of Chinese Medical Sciences
Yanyan Bao
China Academy of Chinese Medical Sciences
Yujing Shi
China Academy of Chinese Medical Sciences
Rongmei Yao
China Academy of Chinese Medical Sciences
Shanshan Guo (ssguo@icmm.ac.cn)
China Academy of Chinese Medical Sciences
Xiaolan Cui
China Academy of Chinese Medical Sciences

Research

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Abstract

Background

Geniposide, an iridoid glycoside purified from the fruit of Gardenia jasminoides Ellis, has been reported to possess pleiotropic activity against different diseases. In particular, geniposide possesses a variety of biological activities and exerts good therapeutic effects in the treatment of several strains of the influenza virus. However, the molecular mechanism for the therapeutic effect has not been well defined.

Methods

This study aimed to investigate the mechanism of geniposide on influenza A virus (IAV). The potential targets and signaling pathways of geniposide in the IAV infection were predicted using network pharmacology analysis. According to the result of network pharmacology analysis, we validated the calcium signaling pathway induced by IAV and investigated the effect of geniposide extracted from Gardenia jasminoides Ellis on this pathway.

Results

The primary gene ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways KEGG enrichment analysis indicated that geniposide has a multi-target and multi-pathway inhibitory effect against influenza, and one of the mechanisms involves calcium signaling pathway. In the current study, geniposide treatment greatly decreased the levels of RNA polymerase in HEK-293T cells infected with IAV. knocking down CAMKII in IAV-infected HEK-293T cells enhanced virusRNA (vRNA) production. Geniposide treatment increased CAMKII expression after IAV infection. Meanwhile, the CREB and c-Fos expressions were inhibited by geniposide after IAV infection. The experimental validation data showed that the geniposide were able to alleviated extracellular Ca2+ influx, dramatically decreased neuraminidase activity and suppressed IAV replication in vitro via regulating the calcium signaling pathway.

Conclusions

These anti-IAV effects might be related to the disrupted interplay between IAV RNA polymerase and CAMKII and the regulation of the downstream calcium signaling pathway essential for IAV replication. Taken together, the findings reveal a new facet of the mechanism by which geniposide fights IAV in a way that depends on CAMKII replication.

1. Background

Influenza A viruses (IAV) is common in the all age group population. It has been regarded as a severe public health. It is one of the most common infectious diseases with a high incidence rate and high mortality rate [1]. Although there are 26 licensed inactivated vaccines that are available for the prevention of influenza, but the number of the death caused by epidemics worldwide is still estimated as 300,000–

650,000 every year [2, 3]. As influenza A virus (IAV) rapidly evolves through antigenic drift and shift, new subtype strains continuously emerge [4, 5]. For example, the most recent influenza pandemic caused by the H1N1 pdm09 influenza virus originated from swine, spread rapidly to nearly all countries and territories [6, 7]. Compared to seasonal influenza viruses, the H1N1 pdm09 influenza virus causes more severe disease and deaths among adults aged 18–64 years [8, 9]. Human infection with H7N9 influenza viruses of avian origin emerged in March 2013 in China, and these viruses have continued to spread into populations with unprecedented mortality and morbidity [10, 11]. Obviously, Influenza has the potential for mortality, high mutation rates, and pandemic risk; thus, it is crucial to learn more about the virulence and pathogenicity of influenza and identify the targets for the development of new drugs [12].

Antiviral drugs play a critical role in defending against influenza epidemics and pandemics, especially against antigenically different strains or new subtypes [13]. Currently, the anti-influenza drugs approved by the United States Federal Drug Administration can be divided into two classes: adamantane-based M2 ion channel blockers, which inhibit viral replication by preventing endosome acidification and viral ribonucleoprotein delivery into the cytoplasm; and neuraminidase inhibitors, which inhibit the release of newly formed virus particles from infected cells [14, 15]. However, surveillance research has indicated that almost all circulating human IAVs are resistant to adamantine, and the viruses rapidly develop resistance to neuraminidase inhibitors after their widespread application [16, 17]. Given the inherent limitations of drugs targeting viral proteins, the development of novel drugs targeting cellular components may be a promising strategy [18].

The life cycle of the influenza virus involves host cellular proteins and pathways [19, 20]. The outcomes of influenza pathogenesis depend on the interactions between the virus and the host cellular proteins along with the activation of signal transduction pathways [21, 22]. The host cellular proteins and signaling pathways may facilitate virus replication by hijacking the host molecular machinery required for the viral life cycle or trigger the innate immune defense of the host to inhibit the virus [22]. Ca^{2+} influx may play a crucial role in the regulation of IAV entry and infection. Influenza A infection has been demonstrated to induce Ca^{2+} oscillation in host cells, and infection with influenza A is obviously attenuated by Ca^{2+} chelation [23]. In the early stage of infection, viral glycoprotein hemagglutinin binds to voltage-dependent Ca^{2+} channels on the host cell surface to induce intracellular Ca^{2+} oscillations, mediate the entry of IAV, and subsequently evoke the calcium signaling pathway of the host cell to facilitate infection [24].

Gardenia jasminoides Ellis (Rubiaceae), which is called Zhi-Zi in the Chinese pharmacopoeia, is an important heat-clearing and detoxifying Chinese herb [25] that has been used in the treatment of inflammation, acute febrile disease, ischemia/reperfusion injury, and hepatic disorders for a long time in East Asia [26-29]. Geniposide, a type of iridoid glycoside, is the main bioactive component isolated from *Gardenia jasminoides* Ellis [30]. Geniposide has diverse pharmacological activities, including antioxidant, anti-inflammatory, neuroprotective, and antithrombotic activities [31-34]. Moreover, accumulating evidence has demonstrated the antiviral properties of geniposide, which has been shown to inhibit infection by influenza A (H1N1) pdm09 virus, enterovirus 71 virus, and Epstein–Barr virus both *in vitro*

and *in vivo* [35-37]. Our previous study showed that the iridoid glycoside extracted from *Gardeniae fructus* inhibited extracellular Ca^{2+} influx induced by IAV; however, the precise mechanism of action remains unclear [38].

In the current study, to better understand the antiviral mechanism of geniposide against influenza, we first constructed a protein–protein interaction (PPI) network of influenza virus-related molecules and then mapped geniposide-related genes to the network to reveal the correlation between them. After module identification, the primary gene ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified by enrichment analysis. Based on the results, we confirmed that the calcium signaling pathway is induced by the IAV and investigated the effect of geniposide extracted from *Gardenia jasminoides* Ellis on this pathway. Finally, the neuraminidase activity of IAV and extracellular Ca^{2+} influx was detected in Madin-Darby canine kidney (MDCK) cells, the expressions of proteins involved in calcium signaling [calcium/calmodulin-dependent protein kinase II (CAMKII), cAMP response element-binding protein (CREB), and c-Fos] were evaluated in A549 cells, and the RNA polymerase activity of influenza virus was determined in HEK-293T cells transfected with CAMK II siRNA.

2. Material And Methods

2.1 Biosafety statement

All experiments involving live IAV were carried out in the Animal Biosafety Level 2 Laboratory of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

2.2 Cells and virus stock

MDCK, A549, and HEK-293T cells were provided by the Cell Center of the Institute of Basic Medical Sciences, Peking Union Medical College (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco). For validation studies, the A FM/1/47 (H1N1) influenza virus (ATCC-VR-1754-ATC) was used. The virus was propagated in 10-day-old embryonated chicken eggs (Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China) according to the method previously described [39]. The virus was titrated in MDCK cells and titers (median tissue culture infective dose, TCID50) determined to be 10−4.5 using the Reed–Muench method [40].

2.3 Geniposide extracted from *Gardenia jasminoides* Ellis

Geniposide with a purity of 39.4% was extracted from Gardenia jasminoides Ellis with 70% ethanol. The purity of iridoid glycoside exceeded 90%, as detected by ultraviolet spectrophotometry.

2.4 Acquisition of genes related to influenza virus and geniposide

Genes related to influenza virus were searched in the OMIM database (https://www.ncbi.nlm.nih.gov/omim/). Geniposide was entered into the STITCH (http://stitch.embl.de/),

GeneCards (https://www.genecards.org/), and CTD (http://ctdbase.org/) databases to search for related genes.

2.5 Network construction and module identification

Influenza virus-related molecules were determined based on the STRING database (Version 9.05). A network of the interactions among virus-related molecules and first-order neighbor proteins was constructed with Homo sapiens as the background. Geniposide-related genes were mapped to this network to understand the correlation between them. We used MCODE (http://baderlab.org/Software/MCODE), a clustering algorithm-based software, to identify gene network modules and the modularity of each network using the following parameters: degree cutoff = 2, node score cutoff = 0.2, max depth = 100, and K-core threshold = 2.

2.6 Functional enrichment analysis

In this study, DAVID 6.7 software (http://david.abcc.ncifcrf.gov/) was used for the functional enrichment analysis of each module, with species restricted to Homo sapiens. As primary GO biological process and KEGG pathway can describe the biological features of modules. Modified Fisher's exact test and the Benjamini–Hochberg method were utilized to calculate and correct the P-value ($P < 0.05$).

2.7 Neuraminidase activity of IAV

The antiviral activity of geniposide against IAV was evaluated by neuraminidase activity assay. MDCK cells were seeded in 96-well plates at a density of 1´105 cells/ml and infected with the FM/1/47 strain of the H1N1 influenza virus (100TCID50, 100 mL/well) for 1 h. The infected cells were then treated with serially diluted geniposide solutions at concentrations of 320, 160, 80, and 40 mg/ml. Ribavirin served as a reference drug at a final concentration of 3.13 mg/ml. After incubation at 37°C for 48 h, the cells were harvested, and neuraminidase activity was determined using a neuraminidase assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

2.8 Confocal microscopy

MDCK cells were cultured on 35-mm glass-bottomed culture dishes (NEST, China) at a concentration of 5 ´105 cells/ml. When the cells grew to 80%–90% confluence, a solution of influenza virus A/FM1/47 (100TCID50) was added to the cultures. After incubation at 37°C for 30 min, the MDCK cells were loaded with 10 mg/ml of fluo-3 AM probe (Invitrogen) for 30 min to detect the intracellular calcium concentration. The cells were then imaged using a laser confocal microscope (Olympus, FV1000, Japan). To measure Ca2+ influx, the samples were excited by an argon laser at 488 nm, and the fluorescence intensity of emission was detected at 530 nm.

2.9 Western blotting assay

A549 cells at 12, 24, 36, and 48 h after infection with IAV/FM1/47 were lysed in RIPA lysate buffer supplemented with phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma). Total protein concentrations were determined using a bicinchoninic acid kit to ensure equal sample loading. Proteins were separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel and then transferred onto a 0.45-μm nitrocellulose membrane. After blocking with non-fat milk, the blots were incubated with the primary antibodies overnight at 4°C: CAMKII and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000, Abcam, UK, CREB, and c-Fos (1:1500, CST, USA). Subsequently, secondary antibody incubation was conducted with goat anti-rabbit IgG antibody (1:20000) for 3 h at room temperature. The blots were visualized by enhanced chemiluminescence, and the density of bands was determined using Image J software (National Institutes of Health, USA).

2.10 Dual-luciferase reporter gene assay

HEK-293T cells were transfected with CAMKII siRNA (forward primer, 5'- CACCACCAUUGAGGAGGAATT -3' and reverse primer, 5'-UUCCUCCUCAAUGGUGGUGTT-3') at 37°C under 5% CO2. After 12 h, the cells were co-transfected with four plasmids containing the cDNA of the RNA-dependent RNA polymerase (RdRP) of influenza virus A/WSN/33H1N1) (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP) or the negative control pFlu-luc) using a lipofectamine 3000 transfection kit (Invitrogen, USA) according to a previous report [41]. After 12 h of transfection, the supernatant was discarded, and the cells were treated with different concentrations of geniposide (320 or 160 μg/ml) for 24 h at 37°C and 5% CO2. The luciferase activity was finally detected using a dual-luciferase reporter assay system (Promega, USA) according to the manufacturer's instructions. The relative luciferase activity was determined by the ratio of the Renilla luciferase value to the firefly luciferase value.

2.11 Statistical analysis

Experimental results are presented as the mean ± standard error of the mean (SEM). Differences were analyzed by one-way analysis of variance using GraphPad Prism 7.0 software. Significant differences were determined by the Bonferroni test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1 Genes related to influenza virus and geniposide

Based on the OMIM database, a total of 77 genes related to influenza viruses were obtained. A total of 35 genes related to geniposide were obtained from searches of the STITCH, GeneCards, and CTD databases.

3.2 Network and module identification

The PPI network constructed from virus-related molecules and first-order neighbors consisted of 2395 nodes and 5574 edges (score ≥ 0.9). Geniposide-related genes were mapped to the PPI network, and 17

were found to be associated with influenza virus (Fig. 1A). After MCODE analysis, we obtained nine modules consisting of more than three nodes from the PPI network, and the modules were sorted according to the scores (Fig. 1B, Table 1).

3.3 Functional enrichment analysis of modules

Based on DAVID 6.7 software (http://david.abcc.ncifcrf.gov/), Module 1 contained 14 pathways ($P < 0.05$): chemokine signaling pathway, neuroactive ligand-receptor interaction, taste transduction, cytokine–cytokine receptor interaction, gap junction, melanogenesis, progesterone-mediated oocyte maturation, intestinal immune network for IgA production, dilated cardiomyopathy, GnRH signaling pathway, complement and coagulation cascades, oocyte meiosis, vascular smooth muscle contraction, and calcium signaling pathway (Table 2). Module 2 contained 11 pathways ($P < 0.05$): toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, cytosolic DNA-sensing pathway, small-cell lung cancer, pathogenic Escherichia coli infection, NOD-like receptor signaling pathway, adipocytokine signaling pathway, pathways in cancer, epithelial cell signaling in Helicobacter pylori infection, pancreatic cancer, chronic myeloid leukemia (Table 3). Module 3 contained one pathway ($P < 0.05$): proteasome (Table 4).

Module 1 is enriched in 326 GO biological processes with 21 biological functional annotations: 52 for cell communication; 48 for metabolism; 40 for transport; 34 for signal transduction; 29 for nucleobase, nucleoside, nucleotide, and nucleic acid metabolism; 19 for biosynthesis; 19 for behavior; 19 for ion transport; 18 for response to external stimulus; 15 for response to stress; 12 for cell–cell signaling; 11 for response to endogenous stimulus; and 10 for protein modification ($P < 0.05$). Module 2 is enriched with 113 GO biological processes ($P < 0.05$), while Module 3 is enriched with 64 GO biological processes ($P < 0.05$; Fig. 2).

To verify the results of the GO analysis, the genes of the calcium signaling pathway in Module 2 were enriched by KEGG pathway analysis (Fig. 3).

3.4 Geniposide inhibits the neuraminidase activity of IAV

Neuraminidase plays a pivotal role during the final stages of influenza virus infection. Neuraminidase facilitates the release of progeny virus and the spread of virus from infected cells to neighboring ones. To explore the inhibitory effect of geniposide on IAV, the neuraminidase activity in MDCK cells was analyzed at 48 h after infection. In the virus control group, the neuraminidase activity increased dramatically compared to the cell control group. Geniposide treatment at concentrations of 320, 160 and 80 μg/ml dramatically decreased the neuraminidase activity (Fig. 4A). These results demonstrate that geniposide significantly suppressed IAV in MDCK cells.

3.5 Geniposide inhibits IAV polymerase activity in HEK-293T cells

Above, we demonstrated the inhibitory effect of geniposide on IAV. CAMKII is a crucial protein in the calcium signaling network. To clarify whether geniposide inhibits virus replication in a calcium-dependent manner, the IAV polymerase activity was determined in HEK-293T cells in which CAMKII protein expression was depleted by siRNA. When CAMKII was knocked down, the RNA polymerase activity was attenuated. Therefore, the activity of RNA polymerase, which is responsible for virus replication and transcription, was inhibited by the knockdown of CAMKII. This indicates that CAMKII may promote viral proliferation in the viral life cycle. Geniposide treatment at concentrations of 320 and 160 μg/ml remarkably decreased IAV polymerase activity; however, IAV polymerase activity was much more robust in CAMKII-deficient 293T cells (Fig. 4B). These results suggest that the inhibition of IAV replication by geniposide might be related to the calcium signaling pathway.

3.6 Inhibitory effect of geniposide on influenza-induced Ca^{2+} influx

Extracellular Ca^{2+} influx plays a pivotal role in IAV entry and subsequently mediates the activation of the calcium signaling pathway and facilitates IAV infection in the host cell. To explore the effect of geniposide on Ca^{2+} influx during the early stage of infection, MDCK cells were stained with fluo-3 AM dye, and the fluorescence intensity was determined by laser scanning confocal microscopy. In MDCK cells infected with IAV, $[Ca^{2+}]i$ increased significantly at 30 min after infection. Geniposide treatment at concentrations of 320, 160, and 80 μg/ml significantly inhibited the elevation in $[Ca^{2+}]i$ at 30 min after infection, demonstrating that Ca^{2+} influx can be prevented by geniposide in the early stage of IAV replication (Figs. 5A and 5B).

3.7 Effect of geniposide on the calcium signaling pathway in A549 cells infected by IAV

PPI network construction and functional enrichment analysis showed that geniposide has a multi-target and multi-pathway inhibitory effect against influenza, and one of the mechanisms involves calcium. To verify the effect of geniposide on the calcium signaling pathway, the protein expressions of CAMKII, CREB, and c-Fos in A549 cells were determined at 24, 36, and 48 h after IAV infection.

CAMKII expression was markedly decreased at 12, 24, 36, and 48 h after IAV infection. In contrast, the CREB and c-Fos expressions were significantly increased at 12, 24, 36, and 48 h after IAV infection. Geniposide treatment increased CAMKII expression in a dose-dependent manner at 12, 24, 36, and 48 h after IAV infection. Meanwhile, the CREB and c-Fos expressions were inhibited by geniposide at 12, 24, 36 and 48 h after IAV infection (Figs. 6A–6D). The results demonstrate that the IAV-induced changes in the calcium signaling pathway were reversed by geniposide treatment.

4. Discussion

Current anti-influenza therapeutics and drugs under development directly target proteins encoded by the virus; for instance, oseltamivir inhibits neuraminidase, adamantanes block the M2 ion channel, and the nucleoside analog favipiravir targets RdRP [42]. Alternative antiviral approaches that target essential host proteins and pathways in the viral life cycle are appealing because they are effective against different virus strains and are less prone to resistance. Therefore, small-molecule inhibitors that target host factors may temporarily block the virus cycle without compromising cellular function.

Unique host genes have been confirmed to be critical for influenza virus replication. For example, host genes ADAMTS7, CPE, DPP3, MST1, and PRSS12 govern inflammation (NF-κB), cAMP/calcium signaling (CRE/CREB), and apoptosis [43]. Host factors BUB3, CCDC56, CLTC, CYC1, NIBP, ZC3H15, C14orf173, CTNNB1, and ANP32B specifically inhibit viral replication and transcription by decreasing the relative activity of viral RNA polymerase without affecting host protein synthesis [44]. A genome-wide CRISPR/Cas9 screen revealed that the host factors SLC35A1, C2CD4C, TRIM23, PIGN, CIC, JAK2, and PIAS3 are critical for the replication of intracellular pathogens [45].

The neuraminidase of the influenza virus is a viral surface glycoprotein that promotes the release of virions by cleaving sialic acid to facilitate viral release and spread in the respiratory tract [46]. To explore the inhibitory effect of geniposide on IAV, the neuraminidase activity in MDCK cells was analyzed at 48 h after infection. Geniposide significantly reduced the increase in neuraminidase activity after virus infection in a dose-dependent manner, providing further evidence that geniposide exerts anti-IAV activity.

Viral RNA-dependent RNA polymerase (vRNPs) consists of vRNA, the RNA polymerase complex (RdRp), and the nuclear protein, which are the smallest units of viral replication. Therefore, RNA polymerase is the key to viral replication [47]. CAMKII has been identified as an antiviral host factor that interacts with IAV polymerase. The interaction between CAMKII and IAV polymerase plays a pivotal role in the outcome of virus infection and antiviral immune response. In the current study, geniposide treatment greatly decreased the levels of RNA polymerase in HEK-293T cells infected with IAV. In a ribonucleoprotein (RNP) reconstitution assay, knocking down CAMKII in IAV-infected HEK-293T cells enhanced vRNA production. These results demonstrate that the inhibition of IAV replication by geniposide might be related to the calcium signaling pathway after virus infection.

PPI network analysis indicated that 17 genes associated with geniposide are also associated with influenza viruses: CTLA4, AKT1, IL6, JUN, RAF1, MAPK14, CASP3, PIK3CG, FOXO1, NGF, MAPK8, BCL2, FASLG, KITLG, PTPN22, AQR, and gallocatechin gallate (GCG). Existing research indicates that the host genes of geniposide, including cytokines TNF-α, IFN-γ, IL-6, IL-4, and IL-10, may be may related to the anti-influenza activity of geniposide. Geniposide can reduce tissue damage by reducing the inflammatory cascade initiated by the cytokines TNF-α, INF-γ, and IL-6 [35]. Moreover, geniposide can enhance the protective anti-inflammation mechanism and immune regulation by enhancing the expressions of IL-4 and IL-10, thereby having an anti-influenza effect [48, 49]. Thus, the mechanism of geniposide's inhibition of viral propagation may be the downregulation of the expression of cleaved caspase3 and the reduction of caspase3 activation. Thus, reduce exporting RNPs from the nucleus, which reduce the RNPs to be

packaged into infectious progeny virions at the cell membrane. Purpose of inhibiting the influenza virus is achieved [50-52]. The following genes are related to influenza virus: CTLA4, AKT1, JUN, RAF1, PIK3CG, FOXO1, BCL2, FASLG (FASL), MAPK8, MAPK14, PTPN22, GCG, NGF, and KITLG. According to the different functions of each gene, their mechanisms of inhibition on influenza virus can be divided into positive feedback and negative feedback.

IAV infection affects apoptosis in the early and late stages of infection. Among the positive feedback genes, Bcl-2 can act as an antagonist to cell death; in late infection, IAV infection pro-apoptotic by decreasing Bcl-2 [53]. Studies have shown that AKT3 induced by IAV can inhibit FoxO1, and the FoxO pathway can inhibit IAV infection by mediating anti-apoptosis and anti-inflammatory reactions [54]. MAPK8 and MAPK14 are important members of the mitogen-activated protein kinase (MAPK) family. MAPKs control a series of cell activities in innate immune response and participate in the regulation of cytokine gene expression and programmed cell death; thus, they are closely related to the prevention and treatment of influenza virus [55]. H1N1 infection induces early and significant NGF upregulation. The overexpression of NGF likely plays a neuroimmunomodulatory role in H1N1 infection. NGF acts on the nociceptive fibers innervating the lower respiratory tract, leading to enhanced neurogenic inflammation in infected lungs [56]. Differential genes such as KITLG, FOXP3, miR-451, IL-2, IL-10, IL-6, and TNF-α are mainly involved in viral infection and the immune inflammatory response. These differential genes might play a role in preventing the host from being infected by viruses and exert immune regulatory effects in the cytoplasm [57]. Neuraminidase is one of two glycoproteins on the surface of influenza virus. Neuraminidase cleaves terminal sialic acid residues and promotes the release of virus from infected cells. Studies have shown that GCG has a strong inhibitory effect on neuraminidase, thereby affecting the spread of influenza virus [58].

Among the negative feedback genes, CTLA-4, PTPN22, and FASL are related to the immune system. CTLA-4, which is a receptor in T cells, plays a key role in the downregulation of antigen-activated immune response and takes charge of the steady state of the immune system through regulatory T cells [59]. Thus, CTLA-4 plays a key role in the pathogenesis of influenza virus and the defense against virus infection [60]. Studies have shown that PTPN22 inhibits immune-induced T cell expansion/activation and the immune amplification of mouse T cells into peptides. PTPN22 encodes lymphoid phosphatase, which may diminish an individual's capacity to generate protective immunity against influenza virus. T cell activation contributes to protective immunity after influenza infection [61, 62]. Therefore, high levels of this gene are not conducive to the prevention and treatment of influenza. The upregulation of Fas expression in virus-infected cells leads to enhanced apoptosis mediated by FasL. Fas-FasL is involved in the apoptosis of lymphocytes induced by human influenza virus H1N1-infected monocytes through direct contact between cells [63].

Other negative feedback genes that are not related to the immune system include AKT1, JUN, RAF1, and PIK3CG. IAV can activate Akt, trigger the intracellular PI3K/Akt signaling pathway, and promote cell entry, virus protein synthesis, and virus replication [64, 65]. C-jun can be activated (phosphorylated) in the early stage of IAV infection [66]. The downregulation of C-jun significantly suppresses viral replication and

mitigates the subsequent expression of inflammatory cytokines. Therefore, C-jun plays an important role in virus infection and replication [51]. Based on the analysis of RNA synthesis in vitro, RAF-1 (RNA polymerase activator 1) and RAF-2 have been identified as host factors that can stimulate RNA synthesis in the influenza virus [67]. Therefore, reducing RAF-1 can inhibit the influenza virus from the early stage.

With the maturation of gene research, the above genes have been shown to have some anti-influenza functions. By increasing the levels of positive feedback genes and reducing the level of negative feedback genes, the influenza virus can be prevented and treated.

But a keyword search did not reveal evidence that AQR is related to the influenza virus by Pubmed.

The primary GO biological processes and KEGG pathways were identified by module enrichment analysis. The primary GO biological processes identified were related to cell communication (14.53%), metabolism (13.41%), transport (11.17%), signal transduction (9.5%), response external stimulus (5.03%), and the viral life cycle (0.84%); these may be the main mechanisms of the anti-influenza activity of geniposide. Nine identified GO biological processes were related to the functions of calcium ion and the associated signaling pathway: cellular calcium ion homeostasis; calcium ion homeostasis; cytosolic calcium ion homeostasis; the elevation of cytosolic calcium ion concentration; regulation of calcium ion transport; elevation of cytosolic calcium ion concentration during G-protein signaling; negative regulation of calcium ion transport via voltage-gated calcium channel activity; regulation of calcium ion transport via voltage-gated calcium channel activity; and negative regulation of calcium ion transport 3. These processes have functions in cell homeostasis, cell communication, transport, ion transport, and signal transduction. The results indicate that calcium ions might play a pivotal role in the inhibition of influenza virus by geniposide.

CAMKII is a crucial protein in the calcium signaling network. Calcium signaling, which is activated by Ca^{2+}, is a key regulator of IAV internalization and infection, and IAV has been shown to cause Ca^{2+}influx. Calcium signaling is a key regulator of IAV internalization which is a ubiquitously expressed calcium sensor that regulates diverse cellular functions. Functional enrichment analysis identified the calcium signaling pathway in Module 1; thus, geniposide may exert an anti-influenza effect by inhibiting calcium influx. Genome-wide RNAi screens confirmed that CAMKII is involved in the post-entry steps of influenza virus replication. A recent study highlighted that diltiazem, a calcium channel blocker, significantly inhibited viral production in human lung epithelial A549 cells (in vitro) and a reconstituted human airway epithelium model (ex vivo). Moreover, diltiazem treatment remarkably prevented mortality and reduced weight loss in mice infected with influenza A(H1N1) pdm09; thus, diltiazem is a promising candidate for the treatment of influenza infections [68]. Diltiazem repurposing of drugs as Novel Influenza Inhibitors from Clinical Gene Expression Infection Signatures.

To clarify whether geniposide inhibits virus replication in a calcium-dependent manner, the activity of RNA polymerase, which is responsible for virus replication and transcription, was inhibited by the knockdown of CAMKII. The results indicated that CAMKII may promote viral proliferation during the viral life cycle.

Geniposide treatment remarkably decreased IAV polymerase activity; however, IAV polymerase activity was remarkably more robust in CAMKII-defi2cient 293T cells. The results suggest that the inhibition of IAV replication by geniposide might be related to the calcium signaling pathway.

Extracellular Ca^{2+} influx plays a key role in the invasion of IAV by mediating the activation of the calcium signaling pathway and promoting IAV infection in host cells. To further investigate whether geniposide suppresses IAV replication by decreasing extracellular Ca^{2+} influx, MDCK cells were stained with fluo-3 AM dye, and the fluorescence intensity was determined by laser scanning confocal microscopy. Geniposide significantly inhibited the increase in $[Ca^{2+}]i$ in MDCK cells infected with IAV at 30 min after infection. Thus, geniposide exerted an antiviral effect by preventing Ca^{2+} influx at the early stage of the IAV replication cycle.

The CAMK cascade is important for many normal physiological processes. The misregulation of the CAMK cascade can lead to a variety of disease states of cell proliferation and apoptosis [69]. Numerous studies have linked the transcription factor CREB to the modulation of various inflammatory mediators. CREB signaling is an important cellular process that serves a variety of functions. Most notably, with respect to influenza infection, CREB signaling has been shown to activate protein kinase A (PKA) and thus play a role in protein synthesis [43, 70]. Because c-Fos is a CREB downstream expression factor, it also plays an important role in viral infection. Studies have shown that c-Fos expression is significantly increased after viral infection. Investigations of the underlying mechanisms demonstrated that c-Fos transcriptional activating protein can activate the IL-6 and IL-8 promoters, so as to exert anti-IAV function[71, 72]. CAMKII induces the expression of the downstream factor CREB, and c-Fos plays a crucial role in Ca^{2+} influx after IAV infection. CAMKII activation leads to the inhibition of the downstream factor CREB and c-Fos activity, which leads to the inhibition of virus replication. This process is facilitated by the repression of IAV polymerase, which is an efficient way to suppress virus replication. The effect of geniposide on the Ca^{2+} signal transduction pathway may be related to the inhibition of the CAMKII downstream factor CREB and c-Fos activity.

To verify the inhibition of CaMKII and the activation of CREB and c-Fos induced by IAV infection, we evaluated the expressions of CAMKII, CREB, and c-Fos in infected A549 cells at 12, 24, 36, and 48 h after infection. Geniposide treatment markedly increased CAMKII expression and inhibited CREB and c-Fox expression at these four post-infection time points. These results provide evidence that geniposide exerts anti-IAV activity by ameliorating the changes to the calcium signaling pathway induced by IAV. These results also verify the related findings of the module enrichment analysis.

Collectively, the data from the current study are consistent with the results of the PPI network analysis. The findings indicate that geniposide suppresses IAV replication in vitro. These anti-IAV effects may be directly related to the inhibition of viral proliferation by host factors. Geniposide inhibits virus replication in a CAMKII-dependent manner, which prevents the over-activation of IAV polymerase induced by IAV infection. Taken together, our findings reveal a new facet of the mechanism by which geniposide inhibits IAV; geniposide inhibits IAV replication by disrupting the interplay between IAV RNA polymerase and

CAMKII and regulating the changes in the calcium signaling pathway essential for IAV replication. The results of this study may pave the way for the development of new antiviral agents against influenza.

Conclusion

At present surveillance research has indicated that almost all circulating human IAVs are resistant to relative drugs. The outcomes of influenza pathogenesis depend on the interactions between the virus and the host cellular proteins along with the activation of signal transduction pathways. So the development of novel drugs targeting cellular components may be a promising strategy. Geniposide has diverse pharmacological activities. Accumulating evidence has demonstrated the antiviral properties of geniposide, which has been shown to inhibit infection by influenza A (H1N1) pdm09 virus and enterovirus 71 virus both in vitro and in vivo. And our previous study showed that the iridoid glycoside extracted from Gardeniae fructus inhibited extracellular Ca2+ influx induced by IAV; however, the precise mechanism of action remains unclear. There is no relevant report on "geniposide regulates calcium signaling pathway essential for Influenza A Virus Replication" at present. This study aimed to investigate the mechanism of geniposide on influenza A virus (IAV). Our conclusion shows that the potential targets and signaling pathways of geniposide in the IAV infection were predicted by network pharmacology analysis. According to the result of network pharmacology analysis, we validated the calcium signaling pathway induced by influenza A virus (IAV) and investigated the effect of geniposide extracted from Gardenia jasminoides Ellis on this pathway. These anti-IAV effects might be related to the disrupted interplay between IAV RNA polymerase and CAMKII and the regulation of the downstream calcium signaling pathway essential for IAV replication. Taken together, the findings reveal a new facet of the mechanism by which geniposide fights IAV in a way that depends on CAMKII replication. We finding the highlights of the new mechanism of the interactions between the virus and the host cellular proteins along with the activation of signal transduction pathways. The results of this study may pave the way for the development of new antiviral agents against influenza.

Abbreviations

IAV	influenza A virus
GO	primary gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
vRNA	virusRNA
PPI	protein–protein interaction
MDCK	Madin-Darby canine kidney
CAMKII	calcium/calmodulin-dependent protein kinase II
CREB	cAMP response element-binding protein
CRE/CREB	cAMP/calcium signaling
vRNPs	Viral RNA-dependent RNA polymerase
RdRp	RNA polymerase complex
RNP	ribonucleoprotein
GCG	gallocatechin gallate
MAPK	mitogen-activated protein kinase
RAF-1	RNA polymerase activator 1
GO-BP	Gene ontology biological processes
SEM	standard error of the mean

Declarations

Ethics approval and consent to participate

“Not applicable” in this section.

Consent for publication

“Not applicable” in this section.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Conflict of Interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

SG and XC designed the study; LZ, LB, YW, MC, and ZG performed the antiviral experiments; YZ conducted the network pharmacology analysis; RZ, JS, YB, YS, and RY analyzed and interpreted the data; SG and LZ wrote the manuscript.

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"Not applicable" in this section.

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Tables

Table 1 Modules from the PPI network

Module	Score	Node	Edge	Nodes Name
1	2.973	223	663	TAS2R60, CCR7, CCL4, CXCL8, CXCR5, CXCL10, CCR1, CCR3, CXCL9, PF4, CCR2, CXCL1, CXCL12, CXCR3, CXCR4, CXCL11, CXCL2, CCL4L1, CCL13, CCL20, CCL27, CXCL5, CX3CL1, PPBP, CCL25, CCR4, CXCR2, CXCL3, CCL1, CXCR1, CXCL6, CX3CR1, CCR10, CXCL16, CXCR6, C3AR1, CCR9, CCR8, CCL16, GNAI2, APLN, ANXA1, APP, GPR183, AGT, AGTR2, ACKR3, S1PR1, C5AR1, GNAI1, GNAI3, CCL28, FPR3, ADORA3, SAA1, FPR2, C3, S1PR3, CASR, FPR1, S1PR2, GNAT3, OPRM1, PTGDR2, S1PR4, KNG1, P2RY12, GNGT2, CNR1, S1PR5, SST, C5, OPRL1, POMC, P2RY13, APLNR, CNR2, NPY, GNB3, CORT, NPY1R, NPY2R, DRD2, DRD3, OPRK1, HCAR2, PTGER3, HRH4, GPR18, ADORA1, OPRD1, GABBR1, GRM8, GNB4, GNG11, GNB2, GNB5, GNG4, GNG2, TAS1R3, GNB1, GRM2, GNG7, P2RY14, PNOC, GNG10, GPR31, GNG5, HCAR3, CHRM2, GNG12, GNGT1, GNG8, GNG13, GNG3, PMCH, GRM6, GPSM3, GRM3, ADCY4, SUCNR1, LPAR5, LPAR2, GRM4, GPR55, HCAR1, PENK, HTR1B, ADCY7, ADRA2A, LPAR3, DRD4, OXER1, GRM7, GABBR2, PYY, LPAR1, BDKRB1, TAS2R7, ADRA2C, GAL, ADRA2B, GPER1, PDYN, ADCY8, ADCY3, SSTR3, HRH3, ADCY5, ADCY1, ADCY6, ADCY2, NMUR1, OXGR1, GPR17, ADCY9, HTR1D, PSAP, NPBWR1, BDKRB2, MCHR1, MCHR2, HTR1A, CHRM4, TAS2R39, TAS1R1, TAS2R41, TAS2R38, GPR37, TAS2R46, NPY5R, NPY4R, NPBWR2, NMUR2, P2RY4, PCP2, SSTR5, SSTR2, SSTR4, HTR1F, MTNR1A, GALR2, TAS2R31, TAS2R1, TAS1R2, TAS2R16, HTR5A, TAS2R14, HTR1E, GPSM2, NMS, NMU, TAS2R42, TAS2R19, TAS2R10, TAS2R30, RXFP4, TAS2R40, INSL5, MTNR1B, TAS2R50, RXFP3, TAS2R4, TAS2R3, SSTR1, TAS2R5, GALR3, TAS2R20, TAS2R13, TAS2R9, NPB, GPR37L1, PPY, HEBP1, TAS2R8, GALR1, GPSM1, TAS2R43, CCR5, NPW, CCL5, RLN3, CCL19
2	2.923	26	76	IKBKE, OASL, ISG15, IFIT1, OAS1, RSAD2, STAT1, TRIM25, IFIT3, MX2, IFIT2, LY96, TRAF6, TRAF3, TBK1, IKBKG, PTPN11, UBE2N, TICAM2, TLR4, TANK, DDX58, CHUK, IRF7, UBE2V1, CD14
3	1.5	6	9	CFTR, PSMB9, NFKBIB, ITCH, NFKBIA, PSME3
4	1.429	7	10	LCK, B2M, PTAFR, FCGR1A, PIK3R2, PLCG1, HLA-DPA1
5	1.4	5	7	PYCARD, NLRC4, NLRP3, IL1B, CASP1
6	1.2	5	6	JAK3, IL7, IL2RG, IL2, MAPK1
7	1	3	3	IL17RC, IL17RA, IL17F
8	1	4	4	CSF1, IL10, TIMP1, DNAJC3
9	1	3	3	NCR2, NCR1, NCR3

Table 2 pathway of module 1.

Pathway	Gene	*P*-value
Chemokine signaling pathway	ADCY3, ADCY4, ADCY1, ADCY2, ADCY7, ADCY8, ADCY5, ADCY6, CXCR1, CXCR2, CXCR3, CXCL11, CXCL12, CXCL10, GNG8, CXCR5, CXCR4, CCR10, CXCR6, GNG2, GNG3, GNG4, GNG5, GNG7, CCL4L1, CCR9, CCR8, CCR7, PPBP, CCR5, GNB2, CCR4, GNB1, CCR3, CCR2, CX3CR1, GNB5, GNB4, GNB3, CXCL1, CCL1, GNAI3, CXCL5, GNAI2, GNAI1, CCR1, CXCL3, CXCL2, CXCL9, GNG13, GNG11, PF4, CXCL6, CX3CL1, GNG12, CCL5, CCL28, CCL4, CCL27, CCL25, CCL20, CCL19, CCL16, GNGT1, GNGT2, CCL13, ADCY9, CXCL16, GNG10	1.657958766912765E-55
Neuroactive ligand-receptor interaction	OPRM1, MCHR1, MCHR2, ADORA3, GABBR1, LPAR3, LPAR2, GABBR2, LPAR1, ADORA1, S1PR2, S1PR3, AGTR2, HTR1B, HTR1A, S1PR1, GALR1, NMUR1, NMUR2, GALR3, S1PR4, GALR2, S1PR5, HTR1D, HTR1F, HTR5A, HTR1E, PTGER3, C5AR1, NPBWR1, NPBWR2, SSTR4, SSTR5, GRM4, GRM3, SSTR2, GRM2, SSTR3, CHRM4, SSTR1, GRM8, CHRM2, GRM7, GRM6, C3AR1, DRD3, DRD2, OPRK1, NPY2R, DRD4, FPR1, FPR3, BDKRB1, FPR2, BDKRB2, APLNR, HRH3, P2RY4, CNR1, CNR2, HRH4, ADRA2A, ADRA2C, ADRA2B, OPRL1, NPY1R, NPY5R, P2RY13, P2RY14, MTNR1B, MTNR1A, OPRD1	9.657764704734148E-49
Taste transduction	ADCY4, TAS2R1, TAS2R4, ADCY8, TAS2R5, ADCY6, TAS2R3, GNG13, TAS1R3, TAS1R1, TAS1R2, TAS2R60, TAS2R46, TAS2R9, TAS2R42, TAS2R43, TAS2R8, TAS2R7, GNG3, TAS2R20, TAS2R40, TAS2R41, GNAT3, TAS2R16, GRM4, TAS2R13, TAS2R39, TAS2R50, TAS2R14, TAS2R19, GNB1, TAS2R38, TAS2R31, GNB3, TAS2R10	2.0267715150108459E-38
Cytokine-cytokine receptor interaction	CXCL1, CCL1, CXCL5, CXCL3, CCR1, CXCL2, CXCL9, CXCR1, PF4, CXCR2, CXCL6, CXCR3, CX3CL1, CCL5, CXCL11, CCL4, CXCL12, CCL28, CCL27, CXCL10, CCL25, CXCR5, CCL20, CXCR4, CXCR6, CCR10, CCL4L1, CCL19, CCL16, CCR9, CCR8, CCL13, CCR7, PPBP, CCR5, CCR4, CCR3, CXCL16, CCR2, CX3CR1	1.4758873776336994E-15
Gap junction	ADCY3, ADCY4, ADCY1, ADCY2, GNAI3, ADCY7, GNAI2, DRD2, ADCY8, GNAI1, ADCY5, ADCY6, LPAR1, ADCY9	9.270005860103292E-6
Melanogenesis	ADCY3, ADCY4, ADCY1, ADCY2, GNAI3, GNAI2, ADCY7, ADCY8, GNAI1, ADCY5, ADCY6, POMC, ADCY9	1.356035255661388E-4
Progesterone-mediated oocyte maturation	ADCY3, ADCY4, ADCY1, ADCY2, GNAI3, ADCY7, GNAI2, ADCY9, GNAI1, ADCY8, ADCY5, ADCY6	1.5948731269833238E-4
Intestinal immune network for IgA production	CCR9, CCL25, CXCR4, CCR10, CCL28, CXCL12, CCL27	0.006412361067992899

Dilated cardiomyopathy	ADCY3, ADCY4, ADCY1, ADCY2, ADCY7, ADCY9, ADCY8, ADCY5, ADCY6	0.013743836429085802
GnRH signaling pathway	ADCY3, ADCY4, ADCY1, ADCY2, ADCY7, ADCY9, ADCY8, ADCY5, ADCY6	0.019520336471638235
Complement and coagulation cascades	KNG1, C3AR1, C5AR1, C3, C5, BDKRB1, BDKRB2	0.031244059596336946
Oocyte meiosis	ADCY3, ADCY4, ADCY1, ADCY2, ADCY7, ADCY9, ADCY8, ADCY5, ADCY6	0.03592138311782549
Vascular smooth muscle contraction	ADCY3, ADCY4, ADCY1, ADCY2, ADCY7, ADCY9, ADCY8, ADCY5, ADCY6	0.039354698598733834
Calcium signaling pathway	ADCY3, ADCY4, ADCY1, ADCY2, PTGER3, ADCY7, ADCY9, ADCY8, CHRM2, BDKRB1, BDKRB2, HTR5A	0.040643127061523385

Table 3 Pathway of module 2

Pathway	Gene	*P*-value
Toll-like receptor signaling pathway	IKBKE, TBK1, LY96, IRF7, IKBKG, TICAM2, TLR4, TRAF6, STAT1, CHUK, CD14, TRAF3	1.2128632952757494E-15
RIG-I-like receptor signaling pathway	DDX58, IKBKE, ISG15, TBK1, IRF7, IKBKG, TRIM25, TRAF6, CHUK, TANK, TRAF3	2.6132796236858782E-15
Cytosolic DNA-sensing pathway	DDX58, IKBKE, TBK1, IRF7, IKBKG, CHUK	6.896843891896822E-7
Small-cell lung cancer	IKBKG, TRAF6, CHUK, TRAF3	0.0025018429161348032
Pathogenic *Escherichia coli* infection	LY96, TLR4, CD14	0.015072927052996406
NOD-like receptor signaling pathway	IKBKG, TRAF6, CHUK	0.017685026048156277
Adipocytokine signaling pathway	IKBKG, CHUK, PTPN11	0.020476455840164474
Pathways in cancer	IKBKG, TRAF6, STAT1, CHUK, TRAF3	0.020691625131975113
Epithelial cell signaling in *Helicobacter pylori* infection	IKBKG, CHUK, PTPN11	0.021055736236424673
Pancreatic cancer	IKBKG, STAT1, CHUK	0.023441273355369472
Chronic myeloid leukemia	IKBKG, CHUK, PTPN11	0.02530096564417107

Table 4 Pathway of module 3

Pathway	Gene	*P*-value
Proteasome	PSME3, PSMB9	0.045385433295782915

Figures

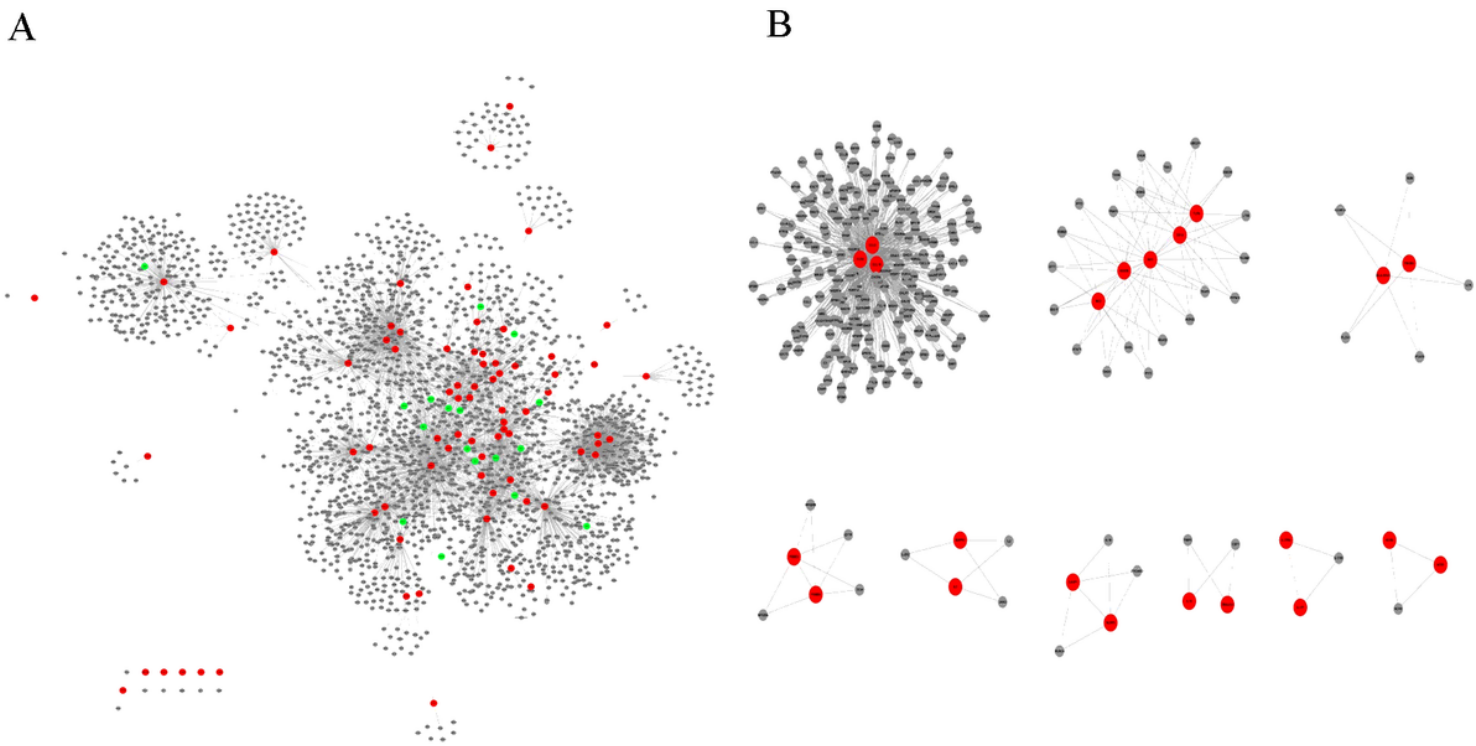

Figure 1

The network of " molecules-disease" of geniposide. Red nodes represent influenza virus-related molecules, gray nodes represent first-order neighbors of the influenza virus-related molecules, and green nodes represent geniposide-related molecules.

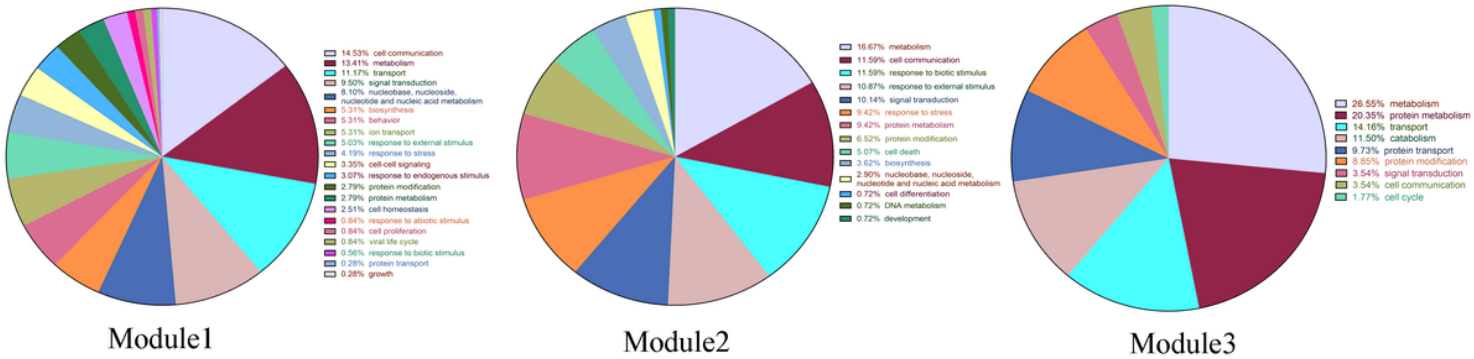

Figure 2

Gene ontology biological processes (GO-BP) classification chart of the three modules.

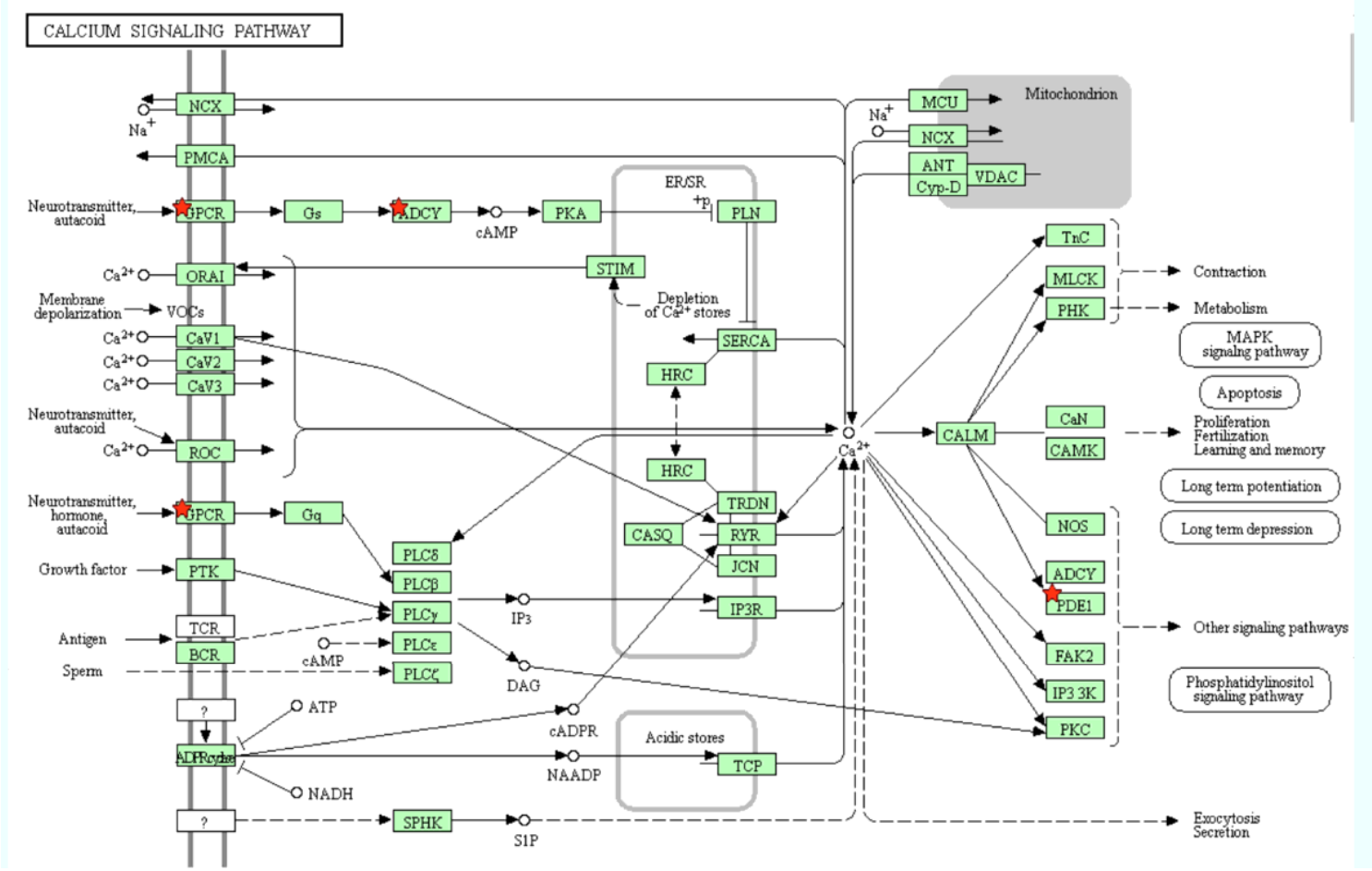

Figure 3

Overview of the calcium signaling pathway. The red stars are the molecules in the module that hit on the signaling pathway.

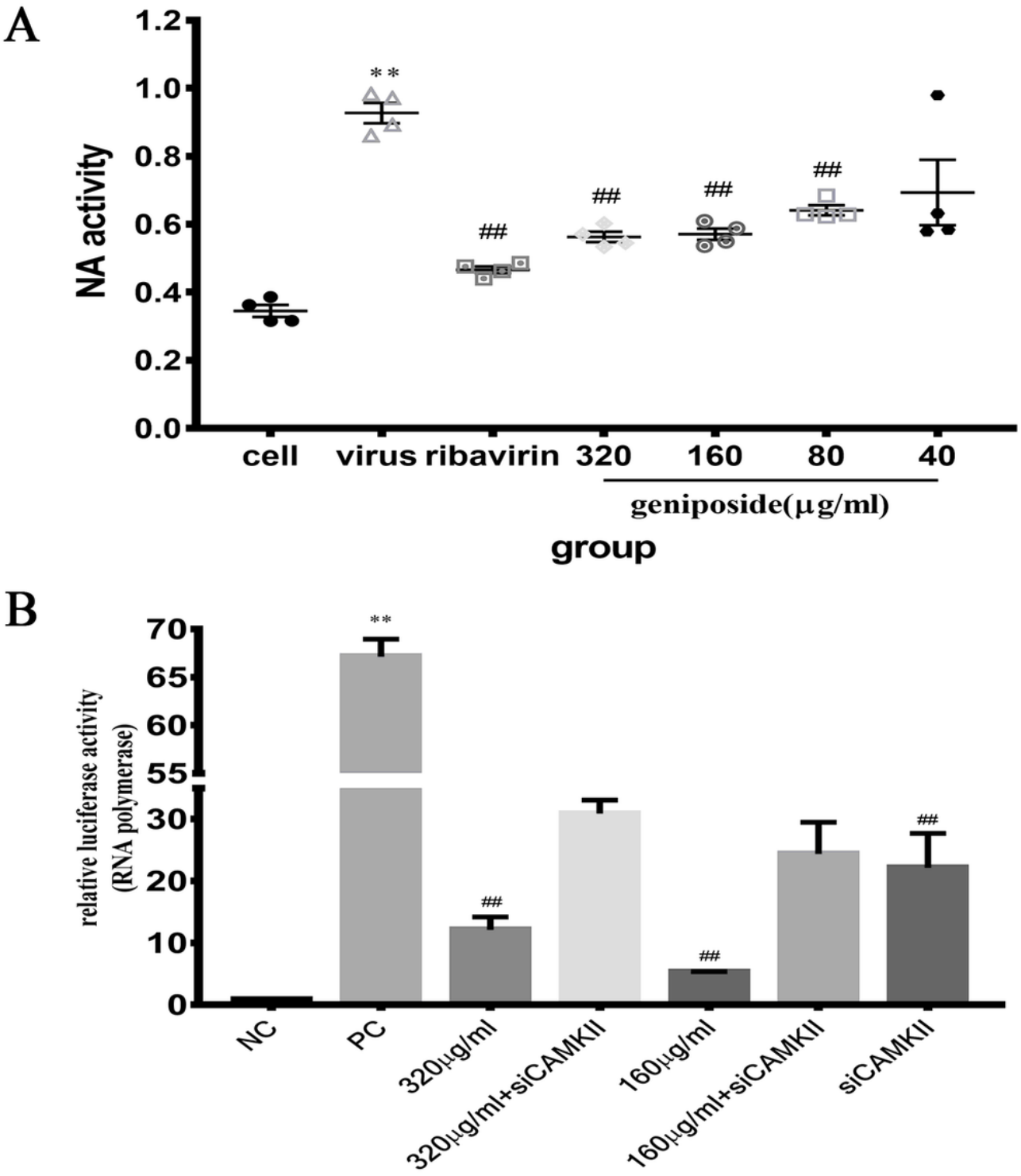

Figure 4

(A) Effect of geniposide on neuraminidase activity in MDCK cells infected with IAV. Values are expressed as mean ± SEM (n = 4). **P < 0.01 compared to the normal control group; ##P < 0.01 compared to the virus control group. (B) Relative luciferase activity of IAV polymerase in HEK-293T cells. Relative luciferase activity was detected using a dual-luciferase reporter assay system. Values are expressed as

mean ± SEM (n = 4). **P < 0.01 compared to the negative control group; ##P < 0.01 compared to the positive control group.

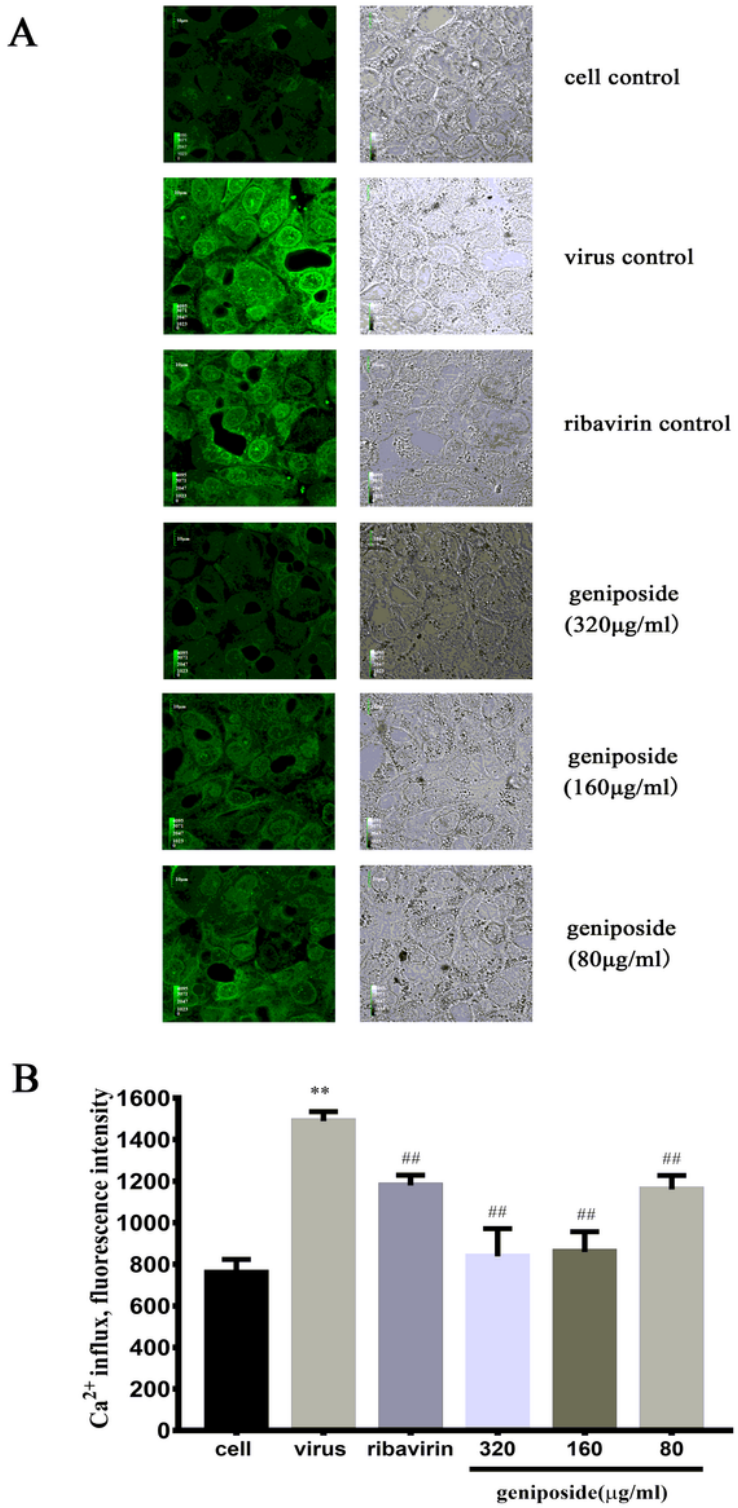

Figure 5

Effect of geniposide on [Ca2+]i levels in MDCK cells infected with IAV. Values are expressed as the mean fluorescence intensity of fluo-3 from four samples in each group. Eight randomly selected fields were analyzed in each sample. (A) Confocal images of MDCK cells stained with fluo-3 AM probe at 1 h after

infection. Green images are fluorescence images taken at the emission wavelength of 530 nm. Grayscale images are control images of MDCK cells without fluo-3 AM staining. (B) The concentration of [Ca2+]i is represented by the mean fluorescence intensity (n = 4). **P < 0.01 compared to cell control group; ##P < 0.01 compared to infection control group. (A color version of this figure is available in the online journal.)

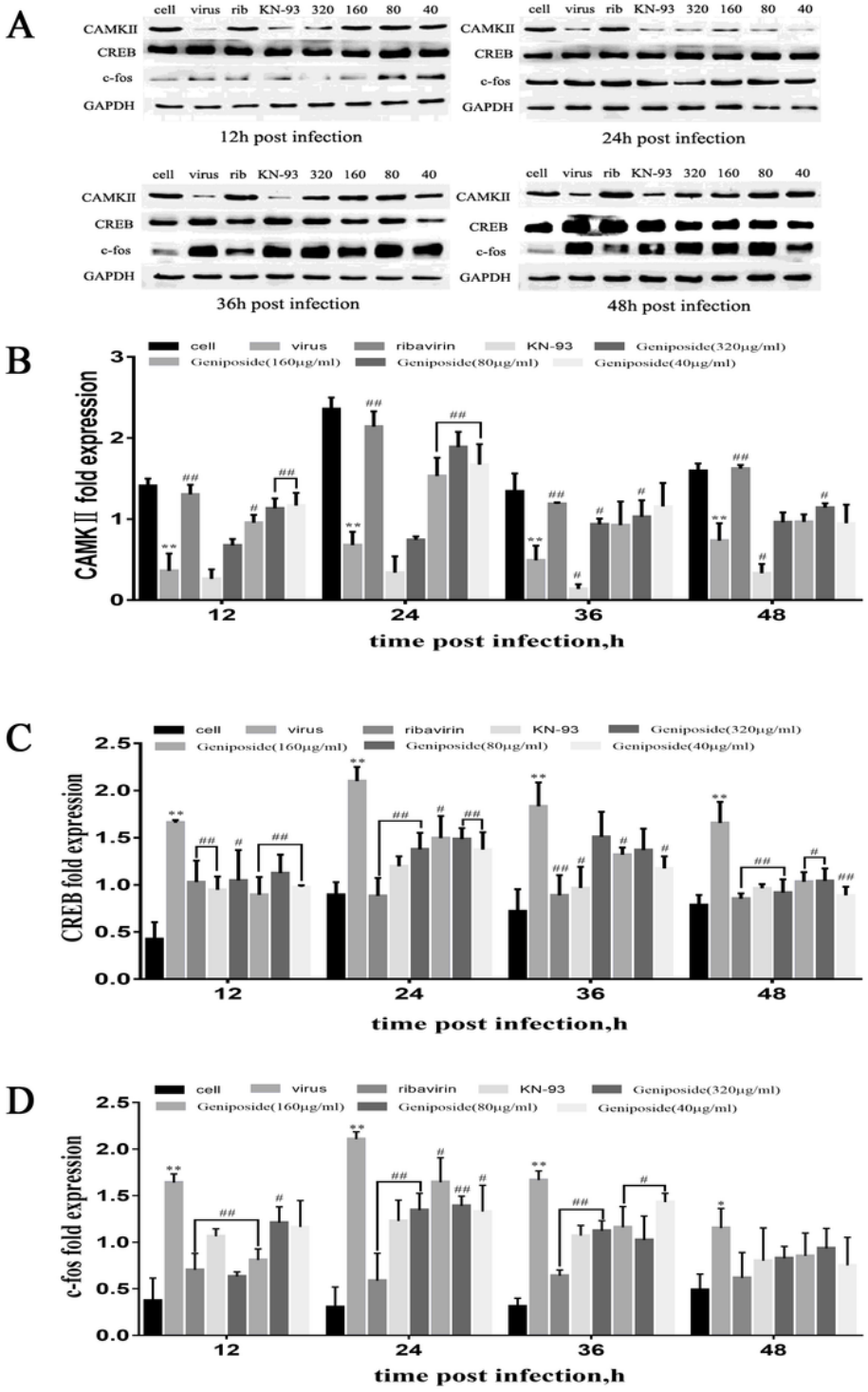

Figure 6

Effects of geniposide on the expressions of CAMKII, CREB, and c-Fos activated by IAV in A549 cells. Values are expressed as the mean ± SEM (n = 4). **P < 0.01 and *P < 0.05 compared to the normal control group; ##P < 0.01 and #P < 0.05 compared to the virus control group. (A) CAMKII, CREB, and c-Fos protein levels expressions were detected by western blotting in A549 cells at 12, 24, 36, and 48 h after infection. (B–D) WB quantification of CAMKII, CREB, and c-Fos. The relative expressions of CAMKII, CREB, and c-Fos were calculated as the ratios of CAMKII, CREB, and c-Fos to GAPDH.