

Kaempferol inhibits pseudorabies virus replication in vitro through regulation of MAPKs and NF- κ B signaling pathways

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Abstract

Pseudorabies virus (PRV), belonging to the family Herpesviridae, is a pathogen of Aujeszky's disease leading great economic losses to pig industry. Re-outburst of pseudorabies implies that new control measures are urgent needed. The present study provides a candidate drug for PRV infection that kaempferol possesses the ability to inhibit PRV replication in a dose-dependent manner in vitro. Kaempferol at a concentration of 52.40 μM could decrease PRV-induced cell death by 90%. Kaempferol with a IC_{50} of 25.57 μM is more effective than acyclovir (Positive control) with a IC_{50} of 54.97 μM . Mode of action study indicated that kaempferol inhibited viral penetration and replication stages and virus load was decreased by 4-fold and 30-fold, respectively. Addition of kaempferol within 16 hours post infection (hpi) could significantly inhibit virus replication, and the DNA copies were decreased by almost 15-fold when kaempferol was added at 2 hpi. Kaempferol could regulate NF- κB and MAPKs signal pathways involved in PRV infection and change the levels of the target genes of MAPKs (ATF-2 and c-Jun) and NF- κB (IL-1 α , IL-1 β and IL-2) signaling pathways. All the results indicated that kaempferol has the ability to be an alternative control measure for PRV infection.

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Background

Pseudorabies virus (PRV) is a swine herpesvirus of the *Alphaherpesvirinae* subfamily [1]. The genome of PRV consists of double-stranded linear DNA of approximately 150kbp with high content of G/C nucleotides of 73%[2]. PRV infection causes outbreak of Aujeszky's disease, one of the most important infectious diseases of swine [3], which can lead the attack of most mammals and some avian species [4]. Pigs is the only natural host of PRV, infections could induce the damages of propagating system, respiratory system and nervous system in different degrees [3, 5, 6]. With the use of live-attenuated vaccines with the glycoprotein E gene deletion, Aujeszky's disease was controlled well worldwide [7], even has been eradicated from pig farm in some European countries such as USA [8], Germany [9], New Zealand [10]. However, the Aujeszky's disease remains constant threat to pig industry in the developing countries. Since late 2011 the disease re-outbroke in many vaccinated pig farms in China [11], which suggests that the live-attenuated vaccines cannot provide enough protect against PRV infection and new control for PRV infection are needed.

Kaempferol (3, 5, 7 - trihydroxy-2 - (4 - hydroxyphenyl) - 4H - 1 - benzopyran - 4 - one), belonging to flavonoids, is widely distributed in tea, broccoli, apples and beans [12]. It exhibits antioxidant [13–16], anti-inflammatory [17–19], anti-cancer [20–24] and antiviral activities. The previous studies indicated that kaempferol alleviated H9N2 swine influenza virus-induced acute lung injury by inactivation of TLR4/MyD88—mediate NF- κB and MAPKs signaling pathway [25] and inhibited herpes simplex virus and human immunodeficiency virus (HIV) multiplication [26–29]. It had ability to inhibit the genomic RNA synthesis of poliovirus [30]. However, there is little study on antiviral activity of kaempferol against PRV. In the present study, the anti-PRV activity of kaempferol was evaluated to provide a new alternative

measure for Aujeszky's disease. Besides, acyclovir, exhibiting dramatically anti-herpesvirus activity, was selected as a positive control.

Results

Cytotoxicity and antiviral activity of kaempferol and acyclovir

The cytotoxicity of different concentrations of ethyl alcohol and DMSO on PK-15 cells were tested since kaempferol was dissolved in ethyl alcohol and acyclovir was dissolved in DMSO during the anti-PRV activity evaluation. The results indicated that when the final concentrations of ethyl alcohol and DMSO were below 1%, there were no cytotoxicity and no anti-PRV activity on PK-15 cells (data not shown). Therefore, throughout the tests, the concentrations of ethyl alcohol or DMSO were no more than 1%. There was no toxicity for PK-15 cells when the concentration of kaempferol was below 104.81 μM and the CC_{50} was 212.97 μM . The CC_{50} of acyclovir was 348.85 μM . kaempferol at the concentration of 52.40 μM could inhibit PRV-induced cell death by 90% (Fig 1). In contrast, the inhibition rate of acyclovir could reach to 90%, when the concentration is 113.23 μM (Fig 1). The IC_{50} of kaempferol was 25.57 μM , which had a selectivity index (SI) of 8.33. The IC_{50} of acyclovir was 54.97 μM , with a SI of 6.34 (Table 1).

Table 1
Antiviral activities of kaempferol and acyclovir against PRV in PK-15 cells

Compounds	CC_{50} ($\mu\text{g}/\text{mL}$) ^a	IC_{50} ($\mu\text{g}/\text{mL}$) ^b	SI ^c
Kaempferol	212.97 \pm 1.86 μM	25.57 \pm 0.74 μM	8.33
Acyclovir	348.85 \pm 2.23 μM	54.97 \pm 4.34 μM	6.34

^aCytotoxic concentration 50% (CC_{50}), concentration required to reduce PK-15 viability by 50%, was measured by CCK-8 method.

^bInhibition concentration 50% (IC_{50}), concentration required to reduce inhibit 50% cell death caused by PRV-infection.

^cSI: Selectivity index is defined as the ratio of CC_{50} to IC_{50} ($\text{SI} = \text{CC}_{50}/\text{IC}_{50}$).

In order to test whether kaempferol could also show anti-PRV activity when the initial amount of infected virus was increased, PK-15 cells were infected with $\text{MOI} = 1$ PRV/well in the presence or absence of kaempferol or acyclovir. Kaempferol could significantly inhibit PRV replication in the concentrations ranged from 52.40 to 13.10 μM (Fig 2). When the concentration of kaempferol was 52.40 μM , the DNA copies were inhibited by about 50-fold in comparison with untreated group. In contrast, acyclovir could reduce the viral DNA copies by about 6-fold at a concentration of 113.23 μM (Fig 2). Kaempferol exhibited higher anti-PRV activity than acyclovir when the input virus was increased to $\text{MOI} = 1$.

Mode of action

For elucidating the mode of action of kaempferol, according to different phases of viral life cycle, five independent experiments were performed. In antiviral tests, kaempferol and acyclovir sometimes directly incubated with the virus during infection. Inactivation was conducted to evaluate whether the observed antiviral effects were due to their inactivation, but no activity was detected (data not shown). In pretreatment assay, the drug was incubated with cells prior to virus infection, which was designed to detect whether the kaempferol could bind to virus receptors on the cell surface involved in the initial virus entry. However, neither kaempferol nor acyclovir exhibited inhibitory activity (data not shown). During virus adsorption, PRV and kaempferol / acyclovir were simultaneously incubated with cells. The results also showed that kaempferol and acyclovir could not inhibit virus adsorption (data not shown). After attachment to cell surface, penetration was triggered. Kaempferol could inhibit PRV penetration and the viral DNA copies were reduced by almost 4-fold (Fig. 3A), while acyclovir exhibited no activity (data not shown). When virus achieved penetration into cells, virus replication began by using host resources. The results showed that both kaempferol (Fig. 3B) and acyclovir (Fig. 3C) could significantly suppress PRV replication in a dose-dependent manner. After treatment with kaempferol (52.40 μM) and acyclovir (113.23 μM), the virus copies were reduced by almost 30-fold and 3-fold in comparison with untreated group, respectively.

Time of addition

Kaempferol was added at different times post infection in order to test the anti-PRV potency of kaempferol after PRV entry into cells. As shown in Fig 4, compared with control group, Addition of kaempferol within 16 hpi significantly inhibited PRV reproduction and viral DNA copies increased gradually from 2 hpi to 16 hpi. Compared with control group, the virus copies were decreased by almost 15-fold when kaempferol was added at 2 hpi.

Effect of kaempferol on PRV activated cell signaling pathways

Virus infection always induces the change of cell signaling pathway to regulate immunity and apoptosis [31, 32]. Therefore, the effect of kaempferol on the classical signaling pathways (NF- κB and MAPKs) involved in virus infection on PRV-infected cells were test (Fig 5). At 4dpi, compared with blank control, the expressions of P65, p-P65, ERK1/2, p-ERK1/2, P38, p-P38 and p-ERK1/2/ERK1/2 were significantly increased and the expressions of p-P65/P65 and p-P38/P38 were significantly decreased in PRV-infected cells without treatment; compared with PRV-infected cells without treatment, expressions of P65, p-ERK1/2, P38, p-P38, p-P65/P65 and p-P38/P38 were significantly enhanced and the expression of p-ERK1/2/ERK1/2 was significantly reduced in PRV-infected cells with kaempferol treatment. At 8 hpi, PRV infection decreased the expressions of P65, p-P65, ERK1/2, p-ERK1/2, P38, p-P38, p-P65/P65, p-

ERK1/2/ERK1/2, and p-P38/P38 compared with blank control. After kaempferol treatment, the protein levels of P65, p-P65 and p-P65/P65 were decreased and the expressions of ERK1/2, p-ERK1/2, P38, p-P38, p-ERK1/2/ERK1/2, and P-P38/P38 were significantly increased in PRV-infected cells. At 12 hpi, compared with blank control, PRV infection significantly up-regulated the expressions of P38, p-P38 and P-P38/P38 and significantly down-regulated the expressions of P65, p-P65, ERK1/2, p-ERK1/2, P-P65/P65 and p-ERK1/2 /ERK1/2, while kaempferol-treatment significantly up-regulated the expressions of P65, p-P65, ERK1/2, p-ERK1/2, P38, p-P38, p-P65/P65, p-ERK1/2/ERK1/2, and p-P38/P38.

Effect of kaempferol on PRV-induced antiviral gene expression

Based on the results of PRV-activated cell signaling pathways, the effect of kaempferol on the target genes of the signal pathways were determined (Fig 6). PRV infection inhibited the activation of P65, while the activation of P65 was enhanced after kaempferol treatment. As the target genes of P65, the expressions of IL-1 α , IL-1 β and IL-2 were tested. The result indicated that PRV infection significantly up-regulated the expressions of IL-1 α , IL-1 β and IL-2. After kaempferol treatment, the levels of IL-1 β and IL-2 were significantly decreased. However, after kaempferol treatment, the expression of IL-1 α on PRV-infected cells was increased significantly in comparison with PRV-infected cells without treatment. PRV infection inhibited the activation of ERK1/2 and enhanced the activation of P38; after kaempferol treatment, the activation of ERK1/2 and P38 were up-regulated. As the common target genes of ERK1/2 and P38, the levels of ATF-2, c-Fos, c-Jun, c-Myc and STAT1 were determined. As shown in Fig 6, the levels of ATF-2, c-Fos, c-Jun and c-Myc were significantly increased, the level of STAT1 was slightly increased by PRV infection. After treatment with kaempferol, the levels of ATF-2 and c-Jun were decreased significantly, while the levels of c-Fos, c-Myc and STAT1 were augmented significantly. MEF2A is the target gene of P38. PRV infection significantly down-regulated the level of MEF2A, after kaempferol treatment, the level of MEF2A was dramatically decreased.

Discussion

PRV infection is a constant threat for many countries [33, 34], especially occur of the emerging PRV variant. However, there is no specific medicine for PRV infection but vaccine which cannot provide full protection against variants [11]. Herein an alternative natural compound kaempferol is demonstrated to possess potent anti-PRV activity though regulating the NF- κ B and MAPKs signaling pathways, which exhibits the potential for control of PRV infection.

As the common indexes to evaluate the antiviral activity of compounds [35], CC₅₀ and IC₅₀ were always tested in antiviral study. The CC₅₀ of kaempferol is lower than that of acyclovir, indicating that acyclovir had higher safe level to PK-15 cells. The IC₅₀ and SI of kaempferol were higher than those of acyclovir, which suggested that kaempferol exhibited a higher anti-PRV activity on PK-15 cells than acyclovir. For

further confirmation of anti-PRV efficacy, the initially amount of infected virus were increased, kaempferol decreased virus reproduction by an order of magnitude.

Just as herpesvirus infection is initiated by attachment of virions to target cells and fusions of the virion envelope and the cellular cytoplasmic membrane [36], the similar processes were taken in the PRV infection of cells. The PRV attachment is made by interaction of virion gC with heparin sulfate proteoglycans at the cell surface, which forms a primary, relatively labile interaction, followed by interaction of gD with its cellular receptor which mediates the conversion of the labile interaction into the stable binding [37]. A tight contact is needed between the cellular cytoplasmic membranes and the viral envelop to complete their fusion which at least requires four viral glycoproteins: gB, gH, gL and gD [36]. Nucleocapsid is translocated into the cytoplasm of the cell, then transported to the nuclear membrane and locates next to nuclear pores. Then a cascade-like fashion started with the expression of only one immediate-early gene to regulate PRV transcription [38]. In the present study, the capability of kaempferol to reduce virus titer, a basic index to evaluate the antiviral activity of samples [39], at different infection stages was tested. The study confirmed that kaempferol had ability to decrease the virus titer, especially at penetration and replication stages, which implied that kaempferol could inhibit the function of gB, gH, gL or gD [36] and the cascade-like fashion of PRV transcription [40]. Time of addition study implied that the inhibition of kaempferol on PRV occurs at early stage of PRV replication cycle. Therefore, kaempferol exhibited the possibilities to inhibit the transcription of immediate-early and early genes of PRV. Acyclovir only inhibited replication stage of PRV. The previous studies implied that acyclovir is targeted at thymidine kinase, whose activity could be activated by a HSV- or VZV-specified TK [41]. Besides, acyclovir is similar with deoxynucleoside triphosphate and has ability to compete with deoxyguanosine triphosphate for the viral DNA polymerase [41]. That's why the anti-PRV activity of acyclovir mainly occurred at replication stage. However, antiviral activity of kaempferol was higher dramatically than that of acyclovir, which is consistent with the values of IC_{50} and SI.

NF- κ B and MAPKs signaling pathways take part in innate immunity [42, 43], which plays an important role in virus infection [44]. The acute response against virus infection should be a general and natural biological effect and NF- κ B was activated by PRV infection at 4hpi by a luciferase reporter assay [45]. In the present study, at 4hpi, as the early stage of the PRV infectious cycle [45], the levels of P65 and P-P65 were increased significantly. However, at 8 hpi and 12 hpi the levels of P65 and P-P65 were decreased significantly. Besides, after kaempferol treatment, the levels of P65 were increased significantly, which indicated that NF- κ B mediates PRV-induced immunity, but kaempferol was not able to inhibit the activation of NF- κ B. Virus infection changes the expression of ERK1/2. The previous study implied that KSHV induces ERK1/2 very early in infection and the authors speculated that the activation probably allowed it to overcome the restriction on viral gene transcription imposed by the host cell and facilitated the viral gene expression and thus the establishment of infection [46]. The study by zhao [et.al](#) indicated that PRV infection activated ERK1/2 [35]. In the present study, ERK1/2 was activated by PRV infection at early stage (4hpi) and inhibited at 8hpi and 12hpi, and kaempferol treatment was able to recover the level of ERK1/2 changed by PRV infection. The activation of P38 signaling takes part in PRV-induced

apoptosis [47]. Compared with the levels of P38 and P-P38 on blank control cells, those were enhanced after PRV infection at 4hpi and 12hpi, while those were decreased at 8hpi. The levels of P38 and P-P38 changed by PRV infection were recovered with kaempferol treatment. Perhaps the anti-PRV activity of kaempferol on PK-15 cells generated by the effect on MAPKs rather than NF- κ B. Based on the previous studies [48], the PRV-induced apoptosis in cultural cells was clarified. NF- κ B and MAPK signal pathways also mediate cell apoptosis [45, 49, 50], which perhaps is one of the reasons that PRV induced the changes of NF- κ B and MAPKs signal pathways.

Pro-inflammatory cytokines were always increased by virus infection [51]. In the study, the expressions of IL-1 α [52], IL-1 β [53] and IL-2 [54, 55], as the target genes of NF- κ B, were detected to further confirm the NF- κ B signal pathway that was regulated by kaempferol. The results implied that the levels of IL-1 α , IL-1 β and IL-2 were up-regulated and kaempferol treatment down-regulated significantly the levels of IL-1 β and IL-2, which is consistent with the previous study [35]. Virus infection always changes the target genes of MAPKs signal pathway [46]. In the study, the expressions of c-Jun, c-Fos, ATF-2, MEF2A, c-Myc and STAT1, as the target genes of MAPKs signal pathway [46], were changed by PRV infection, which is consistent with the previous study [56]. Kaempferol treatment decreased significantly the expressions of ATF-2 and c-Jun which were increased by PRV infection, but did not recover the expressions of c-Fos, MEF2A, c-Myc and STAT1. The c-Jun, as the most extensively studied protein of activator protein-1 (AP-1) complex, takes part in variety cell activities, including proliferation, apoptosis, survival, tumorigenesis and tissue morphogenesis [57]. In the study, the expression of c-Jun was increased by PRV infection, which possibly due to c-Jun mediated the apoptosis of cells induced by PRV infection, and kaempferol possesses the ability to inhibit the effects induced by c-Jun.

The study on the anti-cancer activity of kaempferol disclosed that kaempferol exerts protective effects for non-mutated cells and triggers apoptosis for mutated cells [20]. In the present study, the regulation effects of kaempferol were also different on normal cells and PRV-infected cells. Based on the innate immunity and anti-apoptosis effects of NF- κ B and MAPKs signal pathways, we speculated that kaempferol exhibited anti-PRV activity by regulating innate immunity and protecting cells from PRV-induced apoptosis, which probably attributed to the antioxidant effects of kaempferol on inhibition of ROS generation and lipid peroxidation, then protecting the cells from damaged in a broad-spectrum activity [58].

Conclusion

In conclusion, kaempferol exhibited anti-PRV activity in PK-15 cells, which is better than acyclovir. Kaempferol mainly inhibited PRV replication through regulation of NF- κ B and MAPKs signal pathways and their target genes (Fig 7). Further study should be conducted to evaluate antiviral activity of kaempferol against PRV *in vivo*.

Materials And Methods

Compounds

Kaempferol and acyclovir were bought from Sigma with a purity of 98% and kept at 4°C from light. For antiviral assays, kaempferol was dissolved in 95% ethanol at the concentration of 349.36µM and acyclovir was dissolved in dimethyl sulfoxide (DMSO) at concentration of 444.05µM as the stored solutions. The stored solutions were diluted by at least 100-fold to different concentrations in Dulbecco's modified eagle medium (DMEM, HyClone) for antiviral assays.

Cell and virus

PK-15 cell was preserved in Natural Medicine Research Center Sichuan Agricultural University (Chengdu, China) and grown in DMEM (high glucose) supplemented with 10% (v/v) fetal calf serum (Hyclone), 100U/mL penicillin and 100µg/mL streptomycin. For maintenance medium (MM), the serum concentration was reduced to 2%.

PRV (RA strain) was bought from China Veterinary Culture Collection Center (Beijing, China) and propagated in PK-15 cells. The 50% tissue culture infective dose (TCID₅₀) was determined as 10^{-6.1}/mL.

50% cytotoxic concentration assay

The cytotoxicity of kaempferol and acyclovir was evaluated by determination of cell viability with cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay according to the manufacturer's instructions. PK-15 monolayers grown in 96-well plates were incubated with MM containing two-fold dilutions of kaempferol or acyclovir. After incubation at 37°C for 48 h, 10 µL of the CCK-8 solution was added to each well of the plate. The plates were re-incubated for 30 min at 37 °C, followed by measurement of absorbance values in a microplate reader (Bio-Rad) at 450 nm. The 50% cytotoxic concentration (CC₅₀), the concentration of kaempferol or acyclovir required to reduce cell viability by 50%, was calculated through the Reed-Muench method [59].

50% inhibition concentration assay

The PK-15 monolayer cells in 96-well plates were infected with 200TCID₅₀ PRV/well with or without a series of two-fold dilutions of kaempferol or acyclovir. After incubation at 37°C for 1 h, the medium was removed and MM containing a corresponding dose of kaempferol or acyclovir was added. The virus-infected cells with ethanol treatment (1%, 0.5% and 0.25%) or DMSO treatment (1%, 0.5% and 0.25%) were used as solvent controls. When cells in control groups showed approximately 80% cytopathic effect (CPE), CCK-8 assays was performed as described above. The 50% inhibitory concentration (IC₅₀), the concentration of kaempferol or acyclovir required to inhibit 50% cell death caused by viral infection [60], was calculated through the Reed-Muench method [59].

MOI assay

The PK-15 cells in 6-well plates were infected with MOI = 1 PRV at present or absent of different concentrations of kaempferol or acyclovir for 1h at 37 °C, then the medium were removed and the plates were washed thrice with PBS. The different concentrations of kaempferol or acyclovir were added again and MM were added to cells of control groups. When cells in control groups showed approximately 80% CPE, the sample were subjected to twice freezing and thawing, total DNA was extracted from each well by using DNAiso Reagent (D305; Takara, China) according to the manufacturer's instructions. For determination of viral gene copies, fluorescent quantitative polymerase chain reaction (FQ-PCR) was performed by using a Bio-Rad CFX96 Connect™ real-time PCR detection system (CA, USA) according to the method described by Zhao et al., 2017 [35].

Inhibitory action assay

Pre-treatment assay. Kaempferol and acyclovir were added to cell monolayers and the plates were incubated at 37°C for 1h. Then the medium were removed and the monolayers were incubated with 200 TCID₅₀ PRV for 1h at 37°C. After incubation for 48h, the total DNA was extracted from PRV-infected cells and the virus titers in each well were measured by FQ-PCR method as described above.

Adsorption assay. A PK-15 monolayer grown in 6-well plates was infected with 200TCID₅₀ PRV/well in the presence of dilutions of kaempferol or acyclovir. After incubation at 4 °C for 1h, the cells was washed thrice with cold PBS to remove unadsorbed viruses and then incubated with MM at 37°C. After incubation for 48h, the virus titers in each well were measured by FQ-PCR method as described above.

Penetration assay. A PK-15 monolayer cells grown in 6-well plates was infected with 200TCID₅₀ PRV/well and incubated at 4 °C for 1h. The medium was aspirated and monolayers were washed twice with cold PBS to remove unadsorbed viruses. Dilutions of kaempferol were then added to each well and acyclovir were added as positive group, then plates were incubated at 37 °C for 1h to allow penetration. After removal of medium, the monolayer was washed with a citrate buffer (pH = 3.0) to inactivate virion that had not penetrated the cells. MM was added and the plates were incubated at 37 °C for 48h. The virus titers in each well were measured by FQ-PCR method as described above.

Replication assay. 6-well plates containing PK-15 monolayers were infected with 200TCID₅₀ PRV/well and incubated at 37 °C for 1h to allow adsorption and penetration. After washing, MM containing dilutions of kaempferol or acyclovir were then added to each well and plates were incubated at 37 °C. After incubation for 48h, the virus titers in each well were measured by FQ-PCR method as described above.

Inactivation assay. MM containing dilutions of kaempferol or acyclovir were incubated with an equal volume of concentrated virus suspension (2×10^6 TCID₅₀ PRV) at 37 °C for 1 h. The solution was then

diluted 10^4 times and then added into PK-15 monolayer to allow infection of the residual infectious virus. After incubation for 48h, the virus titers in each well were measured by FQ-PCR method as described above.

Time of addition assay. The PK-15 monolayers in 6-well plates were infected with 1 mL/well 200TCID_{50} PRV for 1h at 37°C , then the plates were washed with PBS for 3 times. The MM were added and the plates were re-incubated. Then kaempferol was added at 2, 4, 8, and 16 hours post infection (hpi) at a final concentration of $52.40\mu\text{M}$. After incubation for 48h, the virus titers in each well were measured by FQ-PCR method as described above.

Western blotting

NF- κB (P65) and MAPKs (P38, p-P38, ERK1/2, p-ERK1/2) signaling pathways were examined by western blotting. The PK-15 cells were infected with or without PRV (MOI = 1) for 1h. Then cells were washed with PBS for 3 times and MM with or without kaempferol at a concentration of $52.40\mu\text{M}$ were added. The total proteins were extracted with a commercial kit (BOSTER, Wuhan, China) at 4, 8 and 12hpi, respectively. Total cell lysates were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for 100 min and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Then the membranes were blocked with 5% BSA for 90 min (Sigma, USA) at room temperature and proteins were stained using primary antibodies directed against P65 (CST, USA, 1:1000), p-P65 (CST, USA, 1:1000), P38 (CST, USA, 1:1000), p-P38 (CST, USA, 1:1000), ERK1/2 (CST, USA, 1:1000), p-ERK1/2 (CST, USA, 1:1000), β -actin (Boster, Wuhan, 1:1000) at 4°C overnight. The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) for 4 times and incubated with horseradish peroxidase-conjugated secondary antibody (CST, USA, 1:5000) for 1 h at room temperature and washed for 4 times, then the proteins were visualized by using enzymatic chemiluminescence (ECL) reagents (Bio-Rad). The expression in total proteins were normalized according to expression of β -actin and ratios of protein band intensity were obtained with ImageJ software.

Real time PCR assay

Real time PCR (RT-PCR) were performed to reveal the effect of kaempferol on express of target genes of the proteins in PRV-infected cells. Briefly, the PK-15 cells in 6-well plates infected with PRV or without PRV in the presence or absence of kaempferol ($52.40\mu\text{M}$). The total RNA was extracted by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and the quality were determined by measuring the A260/A280 ratio. RNA of each sample was reverse-transcribed by Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, USA). cDNA of each sample were used for RT-PCR with SYBR Green Supermix kit (Bio-Rad, USA) and primers used in the study were listed in Table 2. The PCR cycling was 3min at 95°C , then 40 cycles of 10 s at 95°C , 30 s at 59.8°C , and 55°C for 5 s. At the end of the cycling a

melt curve analysis was done. Expression of β -actin was used to normalize the differences in total mRNA expression in each sample. Data analysis was performed using Bio-Rad CFX Manger software.

Table 2
Primer sequences used in Real time PCR^a

Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
β -actin	GGACTTCGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG
IL-1 α	AGAATCTCAGAAACCCGACTGTTT	TTCAGCAACACGGGTTCGT
IL-1 β	GCCCTGTACCCCAACTGGTA	CCAGGAAGACGGGCTTTTG
IL-2	TGCAGCTCTTGTGTTGCATTG	CTTGAAGTAGGTGCACCGTTTG
ATF-2	TCAGCTATTGTTTCGTCCAG	TGAATAATTACGCTTGAGTCAGA
c-Myc	GAAACAGATCAGCAACAACCGAAA	CATTGTGTGTCCGCCTCTTGTC
c-Fos	TGCAGACTGAGATCGCCAACC	CCACTCAGATCAAGGGAAGCCACA
c-Jun	CGAGCGCCTGATAATCCAGTCCA	AGCCCTCCTGCTCGTCAGTCAC
STAT1	TATAAAGTCATGGCCGCTGA	GTTCCCTTTAGGGCCGTCA
MEF2A	ATTCAGATTACGAGGATTATGGAT	GCGATCTCACAGTCACAC

^aThis table shows the list of primer pairs used for analysis of target genes of NF- κ B and MAPKs signal pathway.

Statistical analysis

The data are presented as the mean \pm standard deviation and all of the data were analyzed with SPSS 22 statistical software (IBM, NY, USA). Statistical significance of the data was compared by one way analysis of variance (ANOVA). P \leq 0.05 was considered statistically significant.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SX, XC, YQC, ZQY designed and conducted this study. RW, HYH, XXL collected the samples. CLH, LZY, WZ, GY, YFZ, CL analyzed and interpreted the data. LXL, GZY, HQT, LZ, RYJ performed the histological examination. All authors read and approved the final manuscript.

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Figures

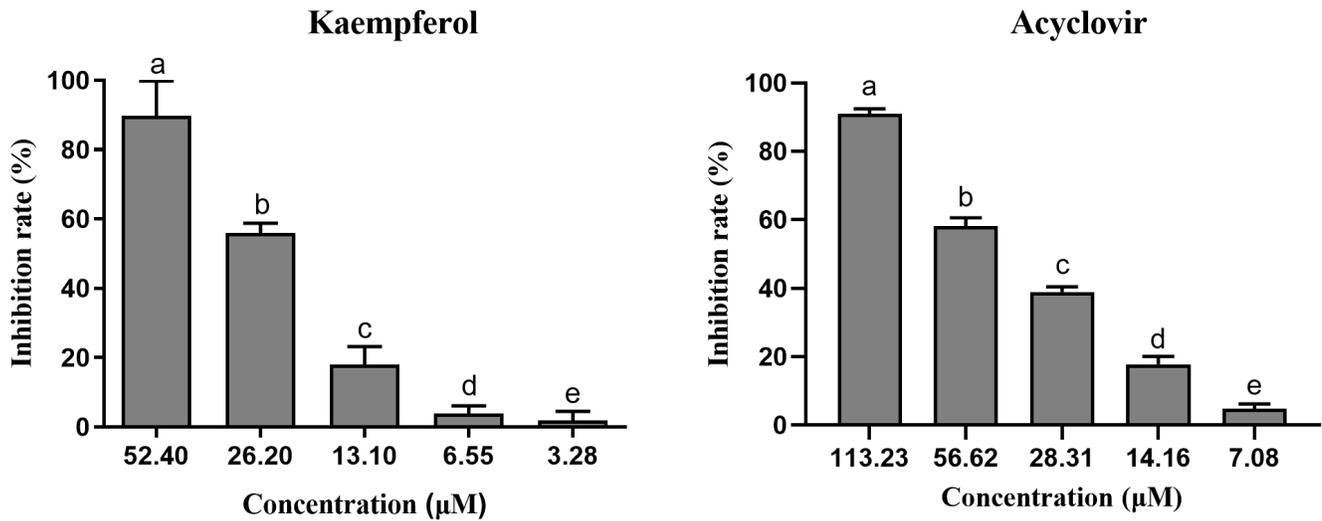


Figure 1

The inhibition rates of different concentrations of Kaempferol and acyclovir against PRV. The PK-15 monolayers in 96-well plates were infected with 200TCID₅₀ PRV/well, followed by treatment with Kaempferol or acyclovir for 48h. The cell viability was determined by CCK-8 method. The inhibition rate were determined as percent of cell viability in treated cells compared with untreated cells. The different letters on a column differ significantly ($P < 0.05$).

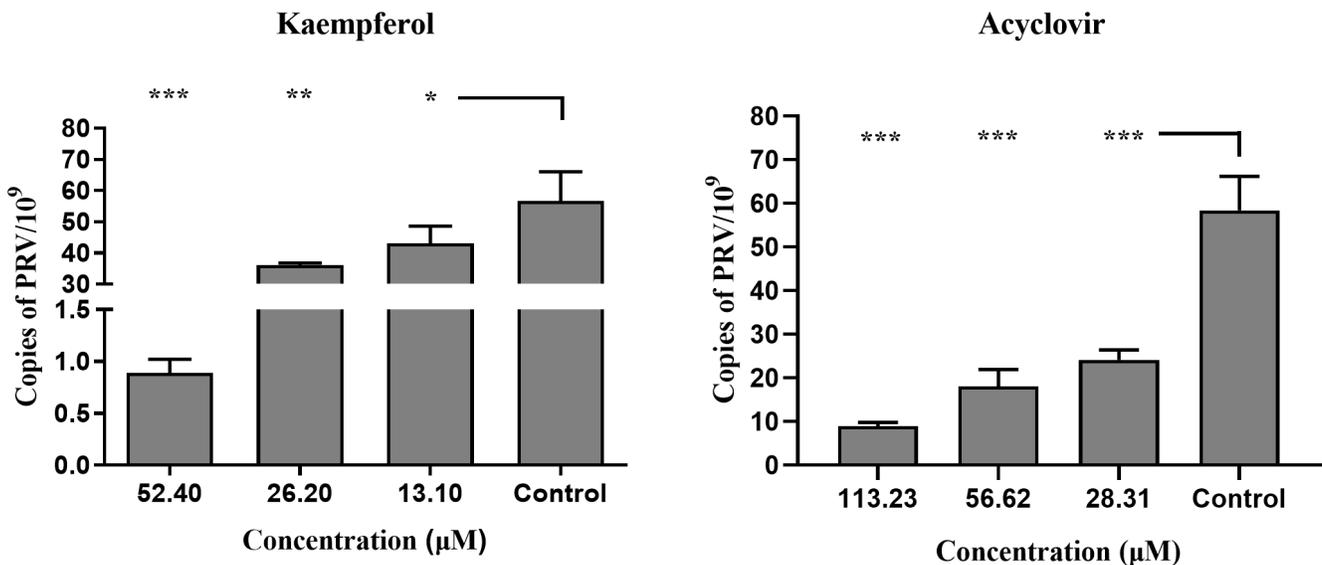


Figure 2

The DNA copies of PRV-infected cells treated with different concentrations of kaempferol and acyclovir. The PK-15 cells were infected with MOI=1 PRV, followed by treatment with kaempferol and acyclovir for

24h. The viral DNA copies were determined by FQ-PCR. Control, the PRV-infected group without any treatment. ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$ vs Control.

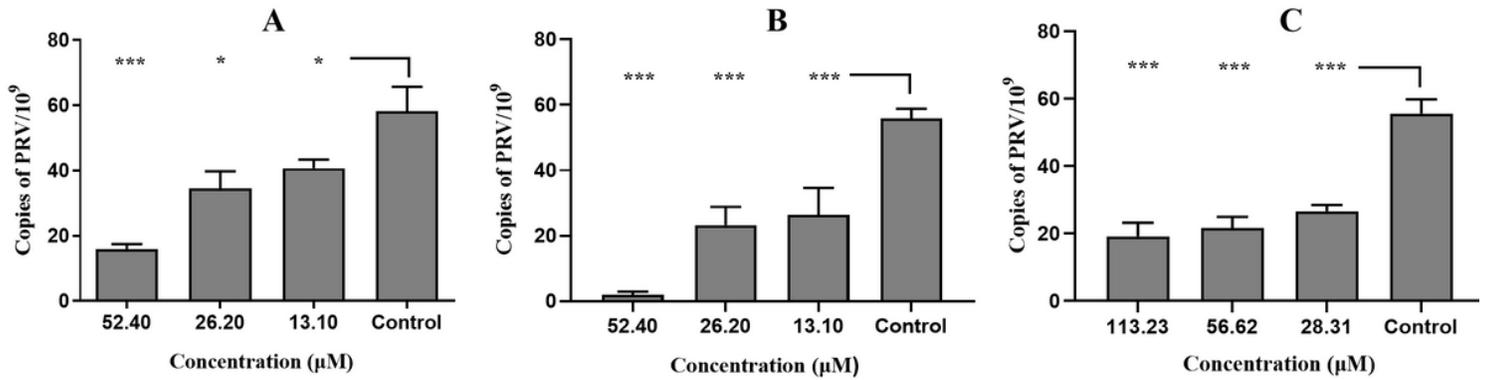


Figure 3

Mode of action of Kaempferol and acyclovir. A, inhibitory effects of kaempferol on PRV penetration; B, inhibitory effects of kaempferol on PRV replication; C, inhibition effects of acyclovir on PRV replication. Control, the PRV-infected group without any treatment. ***, $P < 0.001$; *, $P < 0.05$ vs Control.

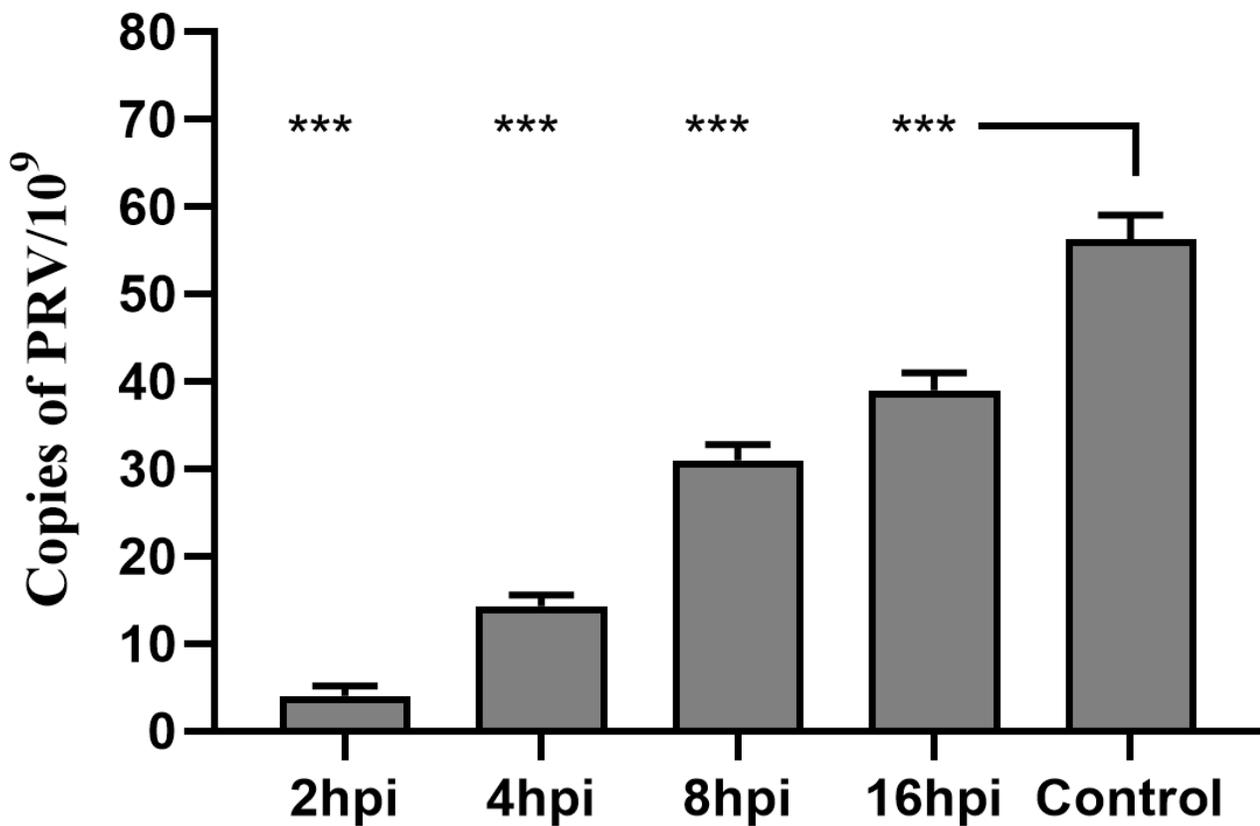


Figure 4

Anti-PRV activity of Kaempferol at different addition times. PK-15 cells were infected with 200TCID50 PRV/mL. Then kaempferol (52.40 μ M) was added at 2hpi, 4hpi, 8hpi and 16hpi, respectively. The viral DNA copies of each sample was tested by FQ-PCR. Control, the PRV-infected group without any treatment. ***, $P < 0.001$ vs Control.

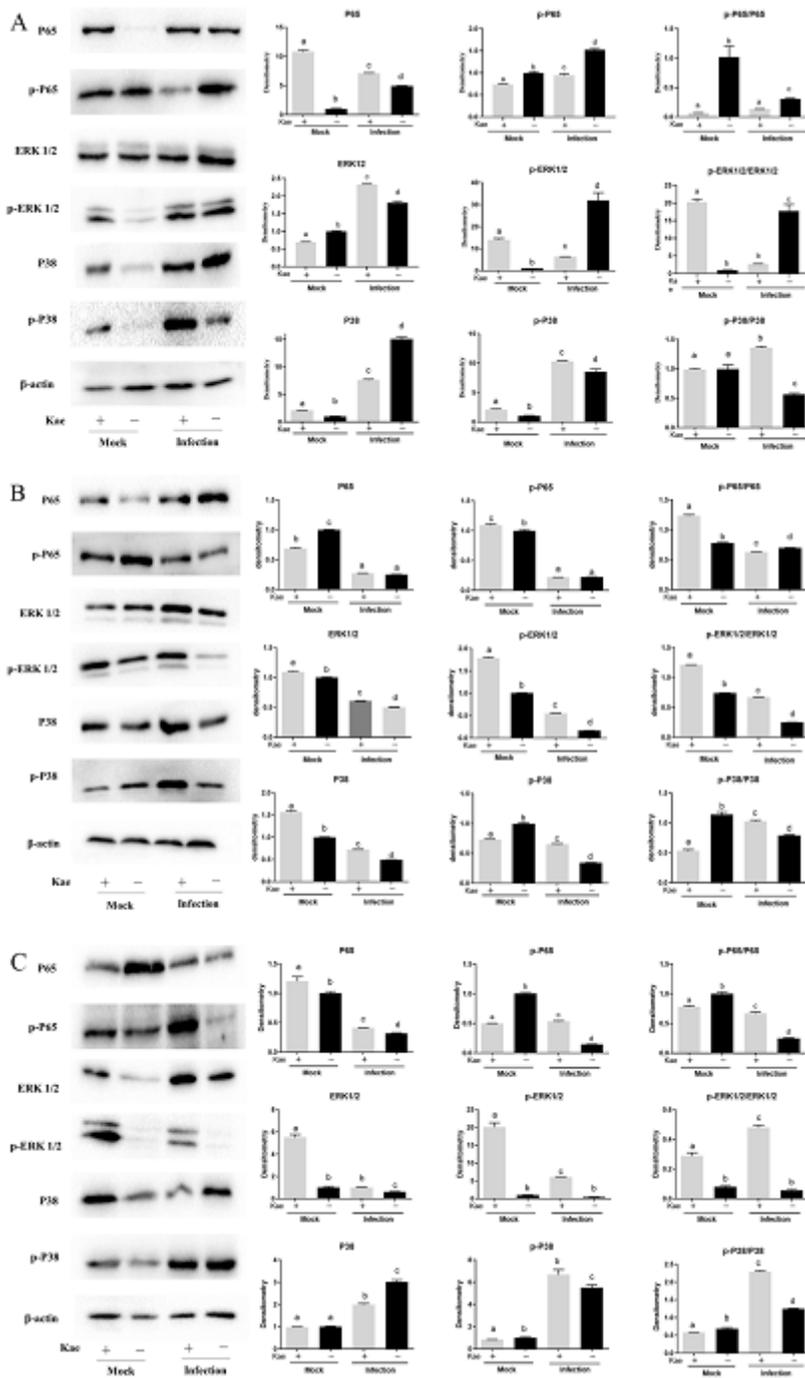


Figure 5

Effect of Kaempferol on PRV-activated cell signaling pathways at 4 (A), 8 (B) and 12 (C) hpi. PK-15 cells were infected with or without MOI=1 PRV in the presence or absence of Kaempferol (52.40 μ M). Expression of P65, p-P65, ERK1/2, p-ERK1/2, P38, and p-P38 were determined by western blotting. The different letters on a column differ significantly ($P < 0.05$).

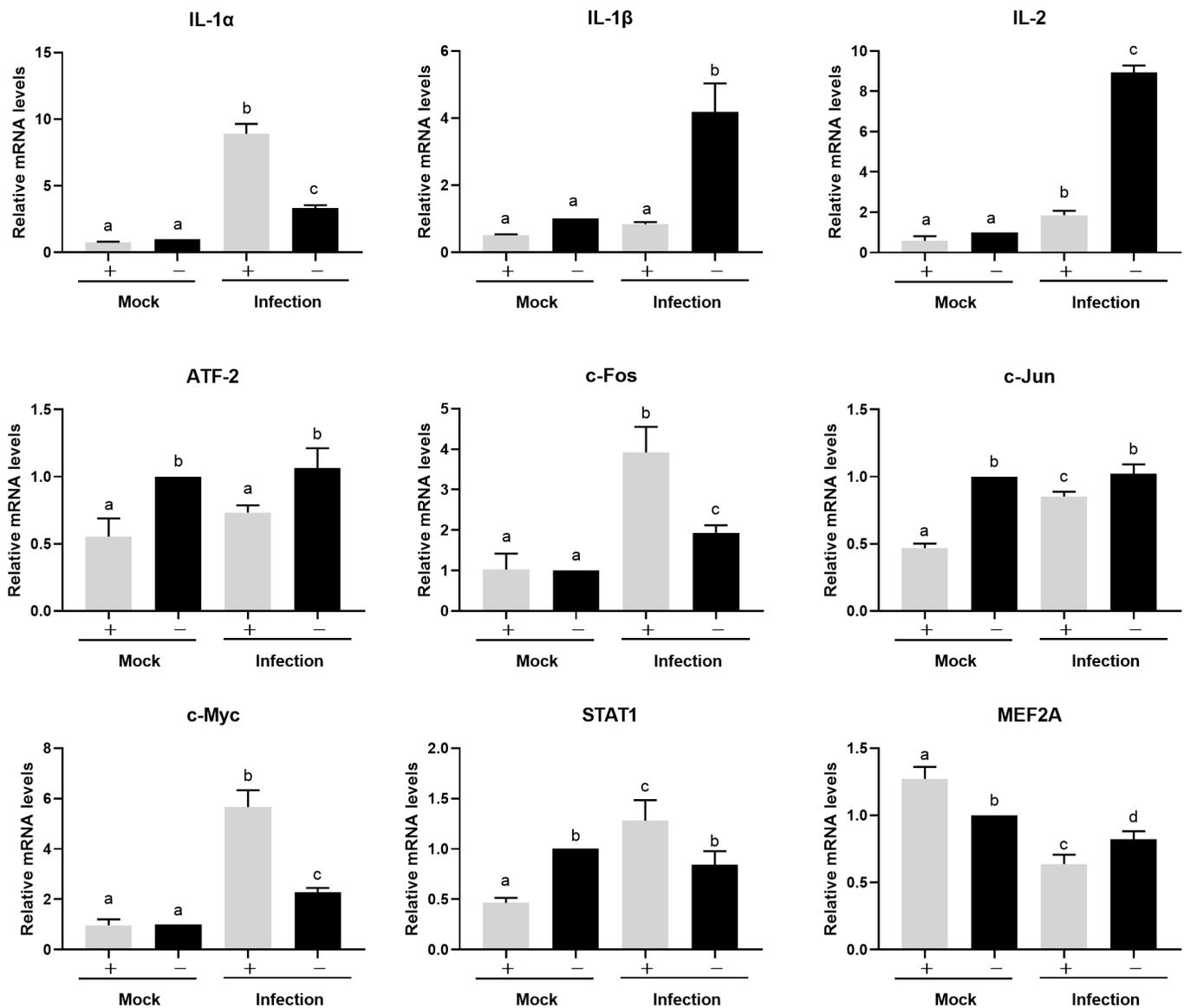


Figure 6

Effect of Kaempferol on gene expressions at 12 hpi. PK-15 cells were infected with or without MOI=1 PRV in the presence or absence of kaempferol (52.40 μ M). The mRNA of each sample was extracted and was subjected to real time PCR to test the expression of the target genes of NF- κ B pathway (IL-1 α , IL-1 β and IL-2) and MAPKs pathway (ATF-2, c-Fos, c-Jun, c-Myc, STAT1 and MEF2A). The different letters on a column differ significantly (P<0.05).

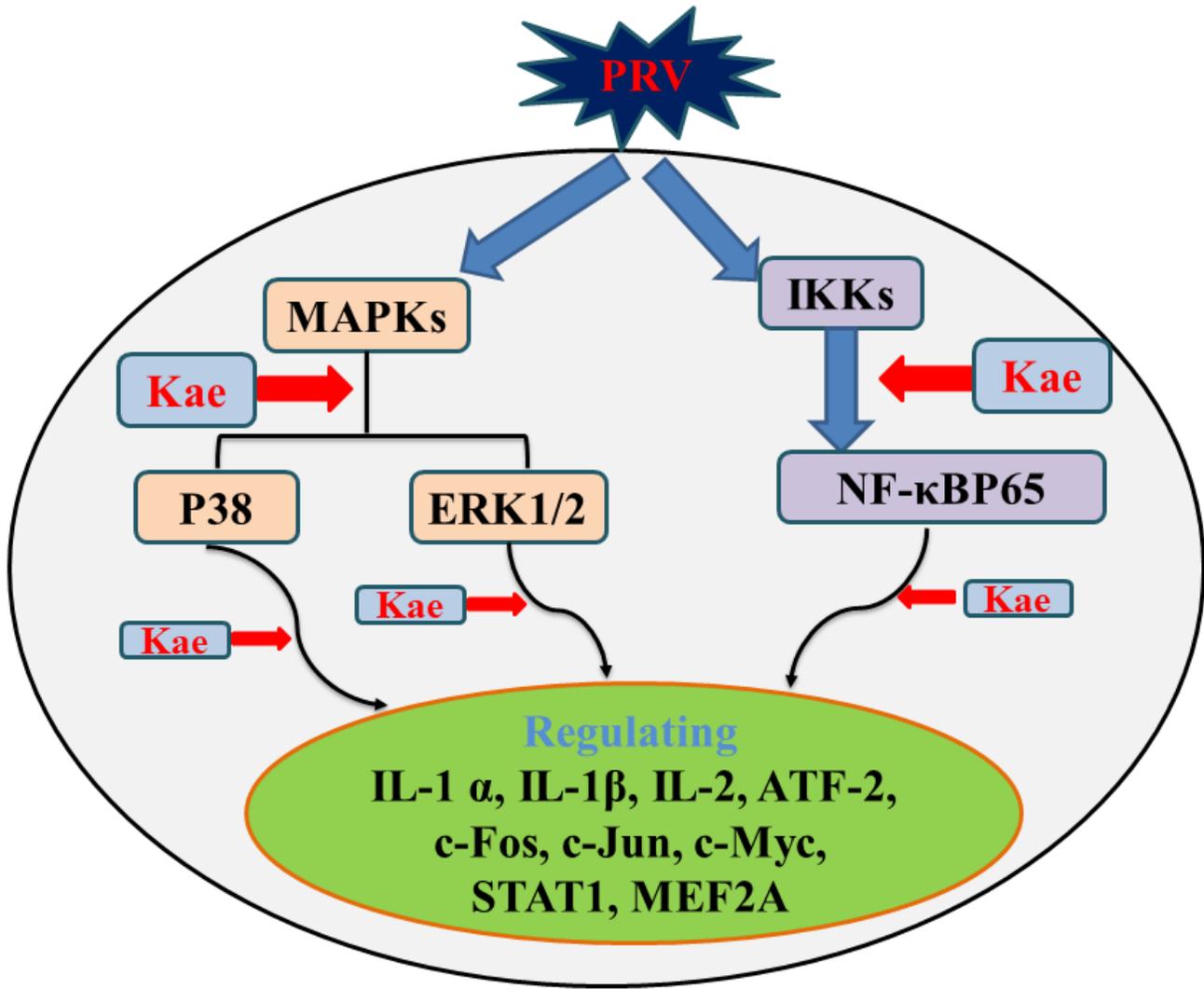


Figure 7

Schematic presentation of possible signaling cascades regulated by kaempferol. Kaempferol exhibit anti-PRV activity through regulation of NF-κB and MAPKs signaling pathways and their target genes. Kae, kaempferol.