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Inhibition of African swine fever virus infection by genkwanin

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ABSTRACT

African swine fever virus (ASFV) is the causative agent of an economically important disease of pigs for which no effective vaccines or antiviral drugs are available. Recent outbreaks in EU countries and China have highlighted the critical role of antiviral research in combating this disease. We have previously shown that apigenin, a naturally occurring plant flavone, possesses significant anti-ASFV activity. However, apigenin is practically insoluble in highly polar solvents and it occurs typically in derivative forms in plants. Here we screened several commercially available apigenin derivatives for their ability to inhibit ASFV Ba71V strain in Vero cells. Among them, genkwanin showed significant inhibition of ASFV, reducing viral titer from 6.5 ± 0.1 to 4.75 ± 0.25 log TCID/ml in a dose-dependent manner (IC_{50} = 2.9 μM and SI = 205.2). Genkwanin reduced the levels of ASFV early and late proteins, as well as viral DNA synthesis. Our further experiments indicated that genkwanin is able to inhibit ASFV infection at entry and egress stages. Finally, genkwanin displayed potent antiviral activity against highly virulent ASFV isolate currently circulating in Europe and China, emphasizing its value as candidate for antiviral drug development.

African swine fever virus (ASFV) is a double-stranded DNA virus and is the only member of the *Asfarviridae* family. It is the causative agent of an economically important disease of pigs characterized by hemorrhagic fever and high mortality rates reaching up to 100% in affected herds (Sánchez-Cordón et al., 2018). Due to the lack of vaccines and effective control measures, ASFV remains a serious threat to the global pork production (Zakaryan and Revilla, 2016; Arias et al., 2017). Recent outbreaks reported in EU countries and in Transcaucasian and Asiatic countries have highlighted the urgent need for effective antiviral agents against ASFV, which can provide an alternative tool for combating this disease.

We have recently reported that apigenin, a plant-derived flavonoid, possesses a potent, dose-dependent anti-ASFV activity *in vitro* (Hakobyan et al., 2016). This compound is known for its biological activities, including antiviral effects against hepatitis C virus, enterovirus-71 and foot-and-mouth disease virus (Shibata et al., 2014; Zhang et al., 2014; Qian et al., 2015). However, apigenin is chemically unstable and practically insoluble in highly polar solvents such as water. In plants, it occurs typically in derivative forms such as apigenin glycosides (Patel et al., 2007).

In the present study, we aimed to assess the antiviral activity of commercially available apigenin derivatives, glucosylated or methylated, against ASFV. For this purpose, we tested six compounds: acacetin, apigetrin, genkwanin, rhoifolin, vitexin, and vitexin 2-O-rhamnoside (all purchased from Santa Cruz Biotechnology, USA) (Fig. 1A). Acacetin and genkwanin have methylations on hydroxyl groups, whereas the remaining four compounds contain monosaccharides or disaccharides. All compounds were diluted in dimethyl sulfoxide (DMSO) and in MEM at the time of use. DMSO concentration in working solutions remained < 1%.

We first screened the cytotoxicity of apigenin derivatives in Vero cells, the permissive cells for ASFV infection *in vitro*, by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (Arabyan et al., 2018). As shown in Fig. 1B, the compounds can be divided into two groups based on their cytotoxicity profiles. Since, rhoifolin and apigetrin exhibited high cytotoxicity,

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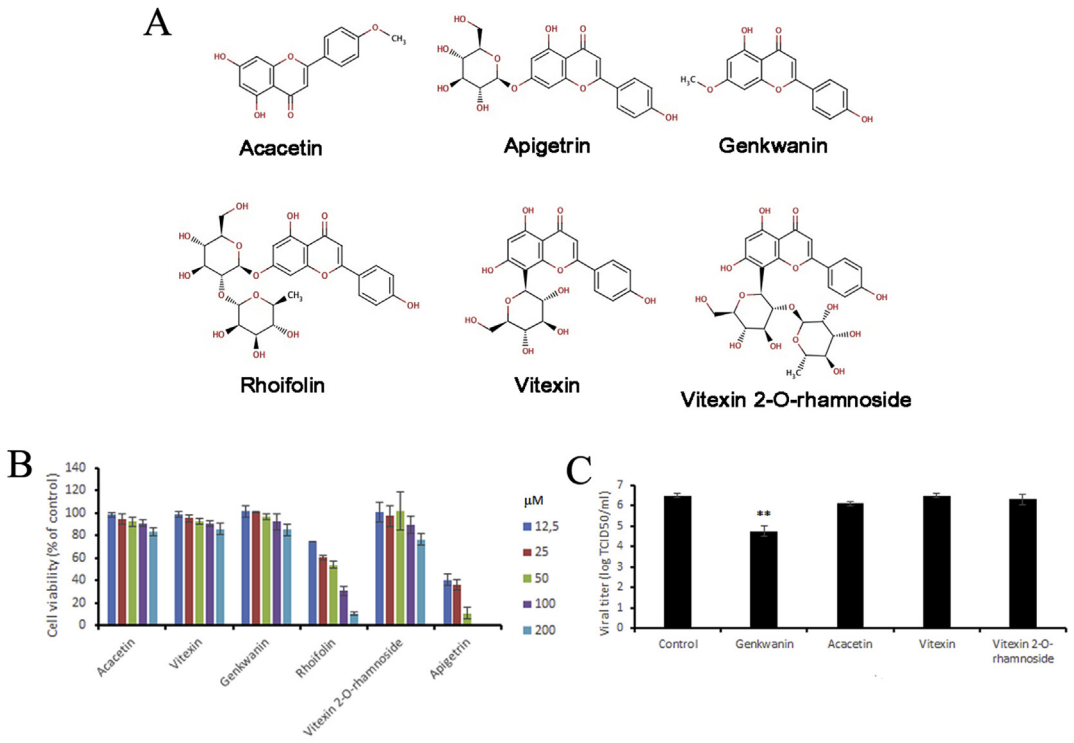

Fig. 1. Chemical structure (A), cytotoxicity (B) and anti-ASFV activity (C) of apigenin derivatives. Values represent mean and standard deviation results from three independent experiments. Data were analyzed by Student's *t*-test. Significant differences compared to control are denoted by **($P < 0.02$).

suggesting that the carbohydrate moiety at the C7-position may increase the *in vitro* toxicity of apigenin derivatives, the antiviral activity of both compounds was not evaluated. In contrast, the antiviral effects of the remaining four compounds were tested, with genkwanin (595.1 ± 45.5 μM) and vitexin 2-O-hamnoside (409.1 ± 50.2 μM) showing to be slightly more toxic than acacetin (659.1 ± 25.9 μM) and vitexin (783.5 ± 64.5 μM) (Fig. 1B). As expected, no cytotoxicity was observed for DMSO concentrations (< 1%) in working solutions prepared in MEM.

To test whether apigenin derivatives have antiviral activity against ASFV, Vero cells grown in a 96-well culture plate (4×10^4 cell/well, MEM supplemented with 3% fetal bovine serum (FBS)) were infected with ASFV/Ba71V strain at MOI of 0.2 $TCID_{50}$/cell (tissue culture infective dose) and treated with apigenin derivatives (six wells per compound), at the following concentrations: 20 μM for acacetin and vitexin, and 40 μM for genkwanin and vitexin 2-O-rhamnoside. The infection was allowed to proceed for 96 h, until the total cytopathic effect (CPE) occurred in mock-treated cells. Supernatant was collected and titrated by CPE-based assay. The titer was calculated by Spearman-Kärber endpoint method and expressed as TCID50/ml. Of the four selected compounds, only genkwanin significantly reduced viral titer from 6.5 ± 0.1 to 4.75 ± 0.25 log TCID/ml (Fig. 1C), in a dose-dependent manner (data not shown) and showing a IC50 (the half maximal inhibitory concentration) = 2.9 μM and SI (selectivity index) = 205.2.

Since previous reports showed that some flavonoids exhibit direct virucidal activity against DNA and RNA viruses (Zandi et al., 2012; Johari et al., 2012; Pradhan and Nguyen, 2018), we first studied the effect of genkwanin on extracellular ASFV particles, as previously described (Hakobyan et al., 2018). As shown in Fig. 2A, the differences in viral titers between treated and untreated Vero cells were statistically insignificant, indicating that genkwanin is not able to impair extracellular viral particles. Having excluded the virucidal activity of genkwanin before cell entry, we performed time-of-addition experiments in an attempt to identify specific time points in the ASFV replication cycle, at which, genkwanin exerts its antiviral properties. For that, confluent Vero cell monolayers grown in 24-well cell culture plate were subjected to genkwanin treatment at 2 h before infection, during the adsorption period and, at 2, 4, 8 and 16 h after infection (0.2 $TCID_{50}$/cell). Supernatant from each treatment group was collected 96 h after addition of the compound (four wells per time-point) and titrated by CPE-based assay. Results showed that 40 μM of genkwanin inhibited ASFV replication when added pre-, during or post-infection except when added at 8 h post-infection (Fig. 2B). The most significant decrease in viral titer was detected at early and late stages of infection suggesting that genkwanin may affect the viral entry and exit processes. Indeed, the flavonoid's ability to block viral entry has been observed in different virus infections. For instance, tangeretin which belongs to the same class of flavonoids as genkwanin inhibits the entry of viral hemorrhagic fever-causing arenaviruses at the fusion step (Tang et al., 2018). Moreover, sorbifolin and pedalitin, two flavonoids extracted from *Pterogyne nitens*, also inhibit HCV entry up to 45.0% and 78.7%, respectively (Shimizu et al., 2017). These and many other examples prompted us to conduct virus attachment and internalization assays to explore the effect of genkwanin on these steps of ASFV life cycle.

For the attachment assay, Vero cells grown in 24-well cell culture plate (four wells per experiment) were incubated with ASFV/Ba71V strain (0.2 $TCID_{50}$/cell) and exposed to genkwanin for 1 h, at 4 °C, to allow virus attachment and to study potential inhibitory effects on viral internalization. Thereafter, the unbound virus and compound were discarded, cells were thoroughly washed with cold phosphate-buffered saline (PBS) and fresh medium containing 3% FBS was added. The culture plates were then switched to 37 °C and cells incubated for 96 h. Finally, supernatants were collected and titrated by CPE-based assay. We observed that treatment with genkwanin reduced the viral titer from 6.6 ± 0.8 log $TCID_{50}$/ml to 4.7 ± 0.4 log $TCID_{50}$/ml (~-98.7%, Fig. 2C).

For the internalization assay, Vero cells were incubated with ASFV

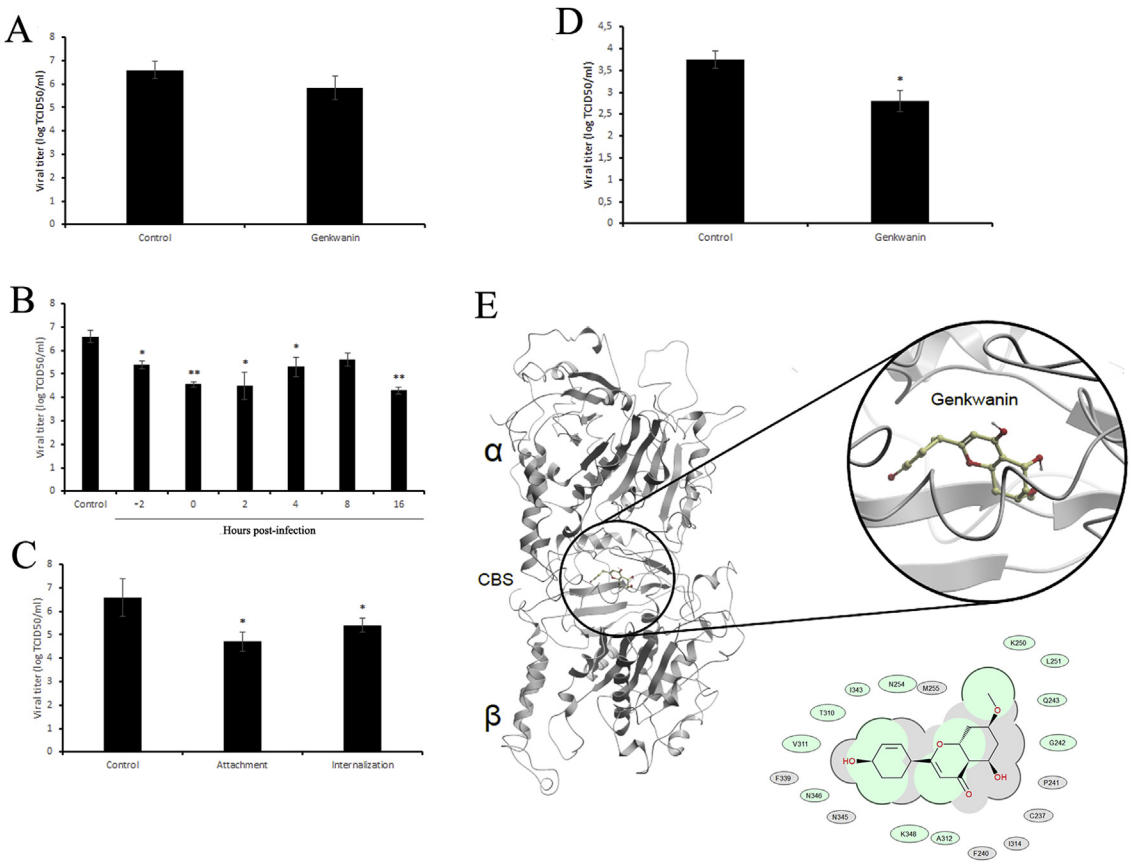

Fig. 2. Evaluation of the antiviral activity of genkwanin against ASFV. (A) Virucidal effect of genistein on extracellular ASFV particles. (B) Effect of genkwanin on ASFV yield when added at different time points: −2; 0; 2; 4; 8; 16 h post-infection. (C) Effect of genkwanin on early stages of ASFV entry. (D) Inhibitory effect of genkwanin on ASFV egress from the host cells. (E) Docking of genkwanin on the colchicine binding site of tubulin. A larger view of the circled area (right side) is presented for clarity. Concentration of genkwanin in all assays was 40 μM. Values represent mean and standard deviation results from three independent experiments. Data were analyzed by Student's *t*-test. Significant differences compared to control are denoted by *($P < 0.05$) and **($P < 0.02$).

at 4 °C for 1 h. Then, the unbound virus was discarded, cells were thoroughly washed with cold PBS and the temperature was shifted to 37 °C to allow virus entry to proceed. Genkwanin was added at 0 h and removed at 1 h following temperature shift. The time point at which cells were shifted to 37 °C was considered as 0 h. After 96 h, supernatant was collected and titrated by CPE-based assay. Significant albeit less prominent inhibition was observed in this assay. The titers were decreased by approximately 1.2 log (Fig. 2C). These findings indicate that genkwanin is able to interfere with the attachment and internalization stages of ASFV infection which are closely associated and essential for viral entry into the host cell. However, the exact mechanism of genkwanin's anti-entry activity remains unexplored, although some studies showed that flavonoids may abolish viral internalization by blocking endosomal acidification in a dose-dependent manner (Mantani et al., 2001; Imanishi et al., 2002; Varghese et al., 2017). Indeed, since acidic pH in endosomes is essential for ASFV entry (Cuesta-Geijo et al., 2012), further studies involving the effect of genkwanin on endosomes will be required to elucidate the mechanism of anti-entry activity.

The time-of-addition experiments also revealed significant antiviral effects when genkwanin was added at 16 h post-infection (Fig. 2B). This time point coincides with the ASFV movement from perinuclear assembly sites (factories) to the cell periphery (Jouvenet et al., 2004). To better understand whether genkwanin may affect the viral egress, we performed a virus egress assay. Briefly, Vero cells grown in 24-well cell culture plates (four wells per experiment) were incubated with ASFV/Ba71V strain (0.2 $TCID_{50}$/cell). At 16 h post-infection, ASFV-infected cells were treated with genkwanin for 1 h. Then, control and treated groups were washed three times with PBS and fresh medium was added. At 24 h post-infection, when the first cycle of ASFV was completed and new viral particles were released (Salas and Andrés, 2013), the medium was harvested and titrated by CPE-based assay. As expected, genkwanin treatment decreased the viral progeny release in about 1 log (Fig. 2D).

Since ASFV transport to the cell surface is dependent on microtubules, tubulin polymerization inhibitors like colchicine and nocodazole are able to suppress ASFV release from cells (Carvalho et al., 1988; Jouvenet et al., 2004). Apigenin has been reported to be a highly potent inhibitor of tubulin polymerization by targeting the colchicine-binding site (Choudhury et al., 2013). Thus, we suppose that genkwanin may also act as an inhibitor of tubulin assembly, thereby disrupting the viral egress. To test this hypothesis, we studied the interaction of genkwanin with tubulin using *in silico* approaches. We conducted docking of genkwanin with tubulin to identify a binding site. According to the docking highest score, genkwanin bound to the intermediate domain of β-tubulin located between α-helixes H7 (221–236), H8 (247–258) and β-sheets S8(309–316), S9(344–352), which form the colchicine binding site (Ravelli et al., 2004). Further, we performed molecular dynamics simulations to get a more accurate binding conformation (Fig. 2E; Video 1S). The binding free energy calculated by MM-GBSA method was −31.6 kcal/mol (Table 1S). More specifically, Phe-240, Asn-254, Met-255 Thr-310, Ala-312 amino acids had highest contribution in the tubulin-genkwanin binding process (Table 1S). Thus, it is highly probable that this interaction can inhibit tubulin polymerization. Taken together, our results suggest that genkwanin inhibits the exit of ASFV from the host cell likely through disrupting the virus movement along microtubules.

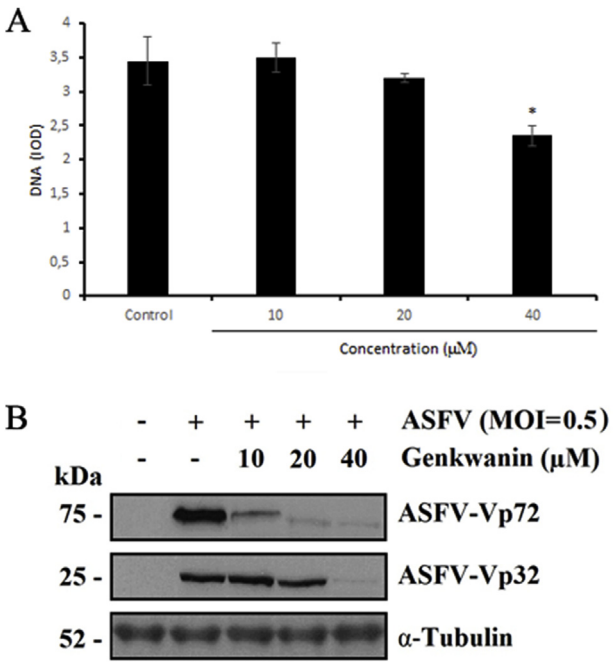

Fig. 3. Inhibition of viral DNA and protein synthesis by genkwanin. (A) The DNA content of ASFV factories in genkwanin-treated cells. The measurement was carried out for 100 viral factories per sample. (C) Viral protein synthesis analyzed by Western blotting. α-tubulin was used as a loading control. Molecular weights (kDa) of evaluated proteins are indicated on the left of immunoblot image. Values represent mean and standard deviation results from three independent experiments. In both assays cells were treated with genwkanin at 10, 20 and 40 μM concentrations. Data were analyzed by Student's *t*-test. Significant differences compared to control are denoted by *($P < 0.05$).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.antiviral.2019.04.008.

Video 1S - Molecular dynamics of genkwanin-tubulin interaction. Docking coordinates was used as starting position for MD simulations. Genkwanin was stabilized after ~100 ns of the GaMD. Further, it had stable position in CBS during cMD.

To further confirm the antiviral effect, we also measured the quantity of viral DNA in ASFV factories upon treatment with genkwanin. Briefly, Vero cells grown on coverslips and infected with ASFV/Ba71V strain (2 $TCID_{50}$/cell) were exposed to genkwanin at 10, 20 and 40 μM concentrations from 1 hpi. At 16 h post-infection, cells were fixed in a 96% ethanol solution for 30 min and stained in fresh Schiff's reagent by the method of Feulgen (Arabyan et al., 2018; Karalyan et al., 2018). The DNA content of viral factories was measured by computer-equipped microscope-cytometer at 575 nm and expressed as Integrated Optical Density (IOA). As shown in Fig. 3A, in Vero-treated cells the ASFV factories contained significantly less DNA than in mock-treated cells, indicating that genkwanin at 40 μM concentration may also hamper viral DNA replication. Similar results were obtained when ASFV-infected cells were exposed to apigenin and genistein (Hakobyan et al., 2016; Arabyan et al., 2018).

We also evaluated the expression of an early (p32) and a late (p72) viral protein in the presence of genkwanin by immunoblotting analysis, as previously described (Simões et al., 2015). Briefly, Vero cells grown in 30 mm dishes were infected with ASFV-Ba71V isolate (MOI of 0.5) and when indicated, exposed to genkwanin at 10, 20, 40 μM concentrations, after the adsorption period (1 h). Before protein extraction, cells were lysed in ice-cold modified RIPA buffer supplemented with a protease-inhibitor cocktail and a phosphatase-inhibitor cocktail. Clarified whole-cell lysates were subjected to SDS-PAGE gel electrophoresis using 8–16% (w/v) polyacrylamide separating gels and transferred to a 0.2 μm pore diameter nitrocellulose membrane by electroblotting. Blot membranes were then blocked with phosphate-buffered saline plus 0.05% (v/v) Tween-20 (PBST), containing 5% (w/v) of BSA, during 1 h at RT, and thereafter incubated with specific primary antibodies (1:200 of an in-house produced swine anti-ASFV serum; 1:100 of an anti-VP73, #1BC11 from Ingenasa; 1:220 of an anti-α-tubulin # 2125 from Cell Signalling Technology), for 1 h at RT. Then, membranes were incubated with appropriate secondary antibodies conjugated with HRP (1:100,000 of an anti-swine IgG #114-035-003; 1:50,000 of an anti-rabbit IgG #111-035-003; and 1:75,000 of an anti-mouse IgG #315-035-003, all from Jackson ImmunoResearch Lab), for 1 h at RT. A final wash step in PBST (3 × 10 min) was performed before protein detection with a chemiluminescence detection kit. The results showed that the synthesis of both proteins was significantly reduced following treatment with genkwanin at 40 μM concentration (Fig. 3B). Interestingly, the presence of genkwanin at lower concentrations also had effect on the synthesis of late but not early protein, suggesting that this compound may mainly interfere with the expression of late ASFV genes by yet unknown mechanism.

In pigs, monocytes and macrophages are the main targets for ASFV infection. Therefore, we conducted additional experiments to define the effect of genkwanin on the virulent ASFV isolate (Armenia/07) in porcine macrophages. Preparation of porcine alveolar macrophages was done as previously described (Carrascosa et al., 2011). Although the cytotoxicity of genkwanin against porcine alveolar macrophages was lower than against Vero cells (data not shown), we treated ASFV-infected macrophages with the same concentration of genkwanin (40 μM) as used in experiments with Vero cells. All antiviral assays were carried out as described above with some modifications. Particularly, macrophages were seeded in 96-well plate and titration was performed by hemadsorption (HAD) assay (Carrascosa et al., 2011). The titer was expressed as $HADU_{50}$/ml. Time-of-addition assay showed that the ASFV yields were dramatically reduced when genkwanin was added at early or late stages of infection (Fig. 4A). That these two stages were affected by genkwanin indicates that it may block the viral entry to and exit from porcine macrophages as shown in Vero cells. Treatment of porcine macrophages with genkwanin at virus attachment step resulted in decrease of ASFV yield from 5.0 ± 0.3 log $HADU_{50}$/ml to 3.6 ± 0.2 log $HADU_{50}$/ml (~-96.0%, Fig. 4B). A significant antiviral effect was also observed when genkwanin was added during the internalization stage of ASFV infection. Finally, the virus egress assay showed a decrease of 1.2 log in the released viral progeny after the first cycle of ASFV infection in porcine macrophages (Fig. 4C). Taken together, these results indicate that genkwanin is able to inhibit not only ASFV Ba71V but also highly virulent ASFV isolate now circulating in Europe and China.

In conclusion, we have identified genkwanin as potent anti-ASFV compound that targets viral entry and exit stages of infection. The observation that genkwanin can inhibit the replication of highly virulent ASFV isolate in porcine macrophages emphasizes its value as candidate for antiviral drug development. Furthermore, to the best of our knowledge, this is the first report on antiviral activity of genkwanin *in vitro*, suggesting that future studies may expand the scope of its application as an antiviral agent against other viruses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.04.008.

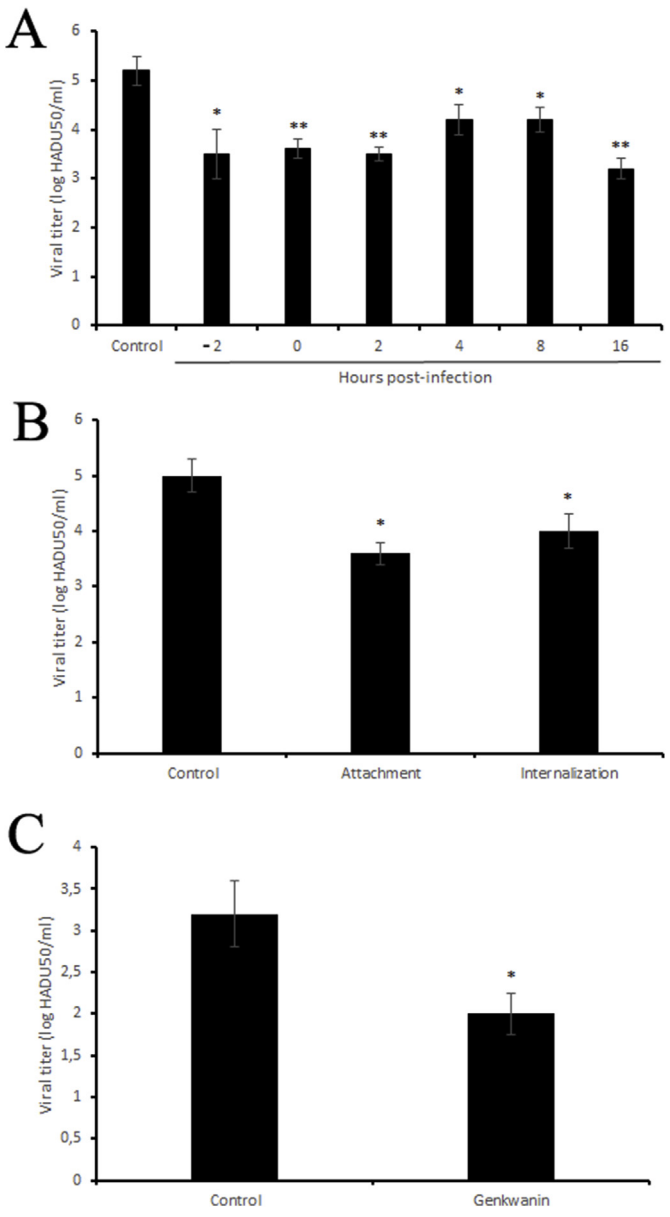

Fig. 4. Antiviral activity of genkwanin against ASFV Armenia/07 isolate. (A) Antiviral effect of genkwanin depending on the time of addition: −2; 0; 2; 4; 8; 16 h post-infection. (B) Inhibition of ASFV entry into porcine macrophages. (C) Inhibitory effect of genkwanin on ASFV egress from porcine macrophages. Concentration of genkwanin in all assays was 40 μM. Values represent mean and standard deviation results from three independent experiments. Data were analyzed by Student's *t*-test. Significant differences compared to control are denoted by *($P < 0.05$) and **($P < 0.02$).

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