

Original Research Article

***In vitro* and *in vivo* effects of *Peganum harmala* L. seeds extract against influenza A virus**

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Abstract

Objective: Influenza A virus infections are still a major health problem and the choices available for the control and treatment of the disease are limited. This research evaluated *in vitro* and *in vivo* antiviral effects of *Peganum harmala* L. seeds (PHS) extract against influenza A virus.

Materials and Methods: In this research, *in vitro* anti-influenza A virus activity of the extract was assessed in Madin-Darby canine kidney (MDCK) cells. In order to evaluate anti-influenza activity of PHS extract *in vivo*, BALB/c mice were infected with 5LD₅₀ of mouse-adapted influenza virus (H1N1; PR8) and received 200 mg/kg/day of PHS extract or 20 mg/kg/day oseltamivir. Lungs of seven mice per group were removed on day 3 post-infection and lung virus titers were determined by qRT-PCR. Mice survival, body weights and general conditions were observed for up to 14 days post-infection.

Results: The results demonstrated that, the ethanolic extract of PHS possesses high activity against influenza virus with IC₅₀ value of 15.7 (CI95%:11.7-21) µg/ml in MDCK cells. Our results also showed that, oral administration of PHS extract (200 mg/kg/day) or oseltamivir (20 mg/kg/day) to infected mice, increased the survival rate, reduced body weight loss, and decreased lung virus titer.

Conclusion: Based on our findings, *P. harmala* seeds extract can inhibit influenza A virus replication *in vitro* and *in vivo*. Therefore, isolation and characterization of the plant's active compounds and investigation of the underlying mechanisms of its antiviral action are highly suggested.

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Introduction

One of the most common human respiratory tract pathogens with high morbidity and mortality risk, is influenza virus which has been considered as a public health concern (WHO, 2016). The high rates of antigenic drift and shift lead to emergence of novel human and non-human influenza viruses with the ability to cross the species barriers and become pathogenic in their new hosts (Cannell et al., 2008; Dawood et al., 2009).

A group of anti-influenza virus agents including amantadine and rimantadine which are matrix protein (M2) ion-channel inhibitors, interferes with viral uncoating within the host cells. These drugs are only effective against influenza A virus and widespread drug resistance has been observed. The other group, oseltamivir and zanamivir, are neuraminidase (NA) inhibitors (Jackson et al., 2011) and are widely used in the treatment of both seasonal and pandemic influenza virus infections. However, oseltamivir-resistant H1N1 strains with NA H275Y mutation were found to be circulated since 2007–2008 (Dapat et al., 2013; van der Vries et al., 2013). Since no highly immunogenic vaccine with considerable efficiency has been yet developed against this virus and because drug-resistant strains of this virus are emerging (Hayden, 2009; Jefferson et al., 2009; Lackenby et al., 2008), the need for development of new highly effective anti-influenza agents for treatment of both seasonal and pandemic influenza infections, seems to be urgent.

Medicinal plants have been used for many years for the treatment of human diseases (Sewell and Rafieian-Kopaei, 2014). A large number of natural compounds has been examined to be introduced as new herbal medicines and remedies (Akbari et al., 2013; Madihi et al., 2013; Shahrani et al., 2007). The compounds with natural origin, especially those isolated from herbs, have been shown to be reliable sources for development of new drugs (Karimi et al., 2016; Moradi et

al., 2016; Moradi et al., 2016; Moradi et al., 2017). Some of these herbal medicines have become therapeutic agents with promising results (Sarrafchi et al., 2016; Shayganni et al., 2016).

Peganum harmala L. (family *Zygophyllaceae*), a perennial, glabrous plant which grows spontaneously in semi-arid conditions, steppe areas and sandy soils, is native to eastern Mediterranean region. This plant is known as “Espand” in Iran, “Harmel” in North Africa and “African rue”, “Mexican rue” or “Turkish rue” in the United States (Mahmoudian et al., 2002). For a long time, seeds, fruits, roots, and bark of *P. harmala* have been used as folk medicine in Iran, Turkey, and China to treat coughs, rheumatism, hypertension, diabetes and asthma (Mina et al., 2015; Moloudizargari et al., 2013; Zhao et al., 2011).

P. harmala contains alkaloids, flavonoids and anthraquinones (Bukhari et al., 2008). The pharmacologically active compounds of *P. harmala* are several alkaloids which are found especially in the seeds and roots. These include β -carboline such as harmine, harmaline (identical with harmidine), harmalol and harman and quinazoline derivatives like vasicine and vasicinone (Mahmoudian et al., 2002).

A literature review revealed that *P. harmala* and its active alkaloids possess a wide range of pharmacological activities like cardiovascular (Aarons et al., 1977; Berrougui et al., 2006), neurologic (Berrougui et al., 2006; Fortunato et al., 2009; Nasehi et al., 2010; Spletstoeser et al., 2005), anticancer (Hamsa and Kuttan, 2010; 2011; Zaker et al., 2007), antidiabetic (Mina et al., 2015; Moloudizargari et al., 2013), antispasmodic, anticholinergic, antihistaminic and antiadrenergic effects (Aqel and Hadidi, 1991) as well as angiogenic inhibitory properties (Moloudizargari et al., 2013). Several studies have reported anti-parasidal (Astulla et al., 2008; Sathiyamoorthy et al., 1999), antifungal, antibacterial (Nenaah, 2010), insecticidal (Jbilou et al., 2008; Rharrabe et

al., 2007), and antiviral (Asgarpanah and Ramezanloo, 2012; Kiani et al., 2008) effects for *P. harmala* alkaloids.

This research evaluated *in vitro* and *in vivo* antiviral activity of seeds extract of *P. harmala* L. against influenza A virus infection.

Materials and Methods

Plant collection and extraction

Seeds of *P. harmala* were purchased from a reliable drugstore. Then, in the Herbarium of Medical Plants Research Center of the Shahrekord University of Medical Sciences (Iran), genus and species of the plant were identified and confirmed (herbarium number MPSKUMS-188). The seeds were powdered and then extracted using maceration method. The plant material was dissolved in 96% ethyl alcohol and kept at room temperature for 96 hr. Then, the mixture was filtered and concentrated under nearly vacuum pressure at 40°C using rotary evaporator. The extracts were dissolved at 37°C in 10% dimethylsulphoxide (DMSO) to give a stock solution of 10 mg/mL, filtered and stored at 4°C until analysis. The little percentage of DMSO present in the wells (maximum 0.1%) has no effect on the results of the experiments (Jadhav et al., 2012).

Cell culture and influenza virus propagation

Madin Darby Canine Kidney (MDCK) cell line, and mouse-adapted influenza virus (A/Puerto Rico/8/34 (H1N1; PR8) and swine influenza virus (A/Iran/12/2014 (H1N1)) were obtained from Influenza Unit, Pasteur Institute of Iran. MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA), at 37°C in humidified incubator with 5% CO₂.

Virus titration

A standard 50% tissue culture infectious doses (TCID₅₀) method was applied for virus titration (Kim et al., 2010). When, 90% confluent MDCK cells were prepared in 96-well plates, the cell culture medium was aspirated and washed twice with phosphate-buffered saline (PBS). Then, 200µl of 10-fold dilutions of virus in DMEM with 0.5µg/mL trypsin TPCK, was added into the wells and incubated for 2 days. Consequently, 50µl of culture medium was taken from each well and transferred to a U-bottomed 96-well plate for hemagglutination assessment (WHO, 2011). TCID₅₀ was calculated based on the method of Reed and Muench (Reed and Muench, 1938).

Cytotoxicity assay

The effects of PHS extract on the viability of MDCK were determined using 3-(4, 5-dimethylthiazol-2-yl) 2, 5 diphenyl tetrazolium bromide (MTT; Sigma, USA) assay. When the cell monolayer was confluent, the cells were incubated with 100 µL/well of various concentrations of PHS extract (in triplicates) in 96-well microtiter plates for another 2 days. After the incubation period, the supernatants were removed from the wells and 50 µL of an MTT solution (1 mg/mL in PBS) was added to each well. The plates were incubated for 4 hr at 37°C, and 100 µL of DMSO (Samchun, Korea) was added to the wells to dissolve MTT crystals. The absorbance was read using an enzyme-linked immunosorbent assay (ELISA) reader (StataFax 2100, USA) at 570 nm. The percentage of toxicity was calculated using the following formula:

Toxicity (%) = [100– (ODT/ODC) ×100], where ODT and ODC refer to the absorbance of the test substance and the control, respectively (Mosmann, 1983). The 50% cytotoxic concentration (CC₅₀) was defined as the cytotoxic concentration of the extract by regression analysis.

Anti-influenza virus activity of PHS extract

Confluent monolayers of MDCK cells were prepared in 96 or 12-well plates. swine influenza A virus was inoculated into the cells at 100 TCID₅₀, and the virus-infected cells were incubated in the presence of each compound at various concentrations. Oseltamivir served as the positive control. Virus replication and virus titration were assessed using different methods including cytopathic effect reduction assay, hemagglutination assay, and real-time quantitative RT-PCR.

Cytopathic effect (CPE) reduction assay

When the cell monolayer was 90% confluent, the cell culture medium of cells was aspirated. Next, cells were washed with PBS and infected with 100 TCID₅₀ of influenza A (H1N1) virus for 1 hr. Then, the virus was removed and the cells were treated with 2-fold diluted nontoxic concentrations of PHS extract (6.25, 12.5, 25, and 50 µg/ml) and incubated for 48 hr. DMSO 0.1% and oseltamivir (Sigma, USA) were used as negative and positive controls, respectively. Cell viability was also determined using a previously described MTT assay (Kodama *et al.*, 1996). The procedure was carried out in triplicate. The 50% inhibitory concentration (IC₅₀) was determined based on a curve of inhibition versus the concentration of the extract. Selectivity index, as a marker of antiviral activity, was determined as the ratio of CC₅₀/IC₅₀.

Hemagglutinin (HA) assay

Confluent MDCK cells in 12-well plates were infected with 100 TCID₅₀ of influenza A (H1N1) virus, and incubated for 1 hr at 37°C. Then, the virus was removed and the cells were treated with 2-fold diluted nontoxic concentration of PHS extract (5, 10, 20, 40 and 80 µg/ml). To evaluate the presence of the virus in cell culture, the cell culture supernatants were harvested at 24 and 48 hr post-infection. Serial dilutions of the cell culture

supernatants were mixed with the same volume of 0.5% chicken red blood cells (RBCs) in a U-bottomed 96-well plate for 45 min at room temperature. The hemagglutination activity was determined by measuring the dilution factor of the samples required for complete chicken RBC agglutination (Jang *et al.*, 2014).

Quantitative reverse transcription-PCR

We used real-time PCR to quantify the presence of virus in the media of MDCK cells after infection with influenza virus. Next, in 12-well plates, 90%-confluent MDCK cells were infected with the virus and treated with 2-fold dilutions of nontoxic concentrations of PHS extract (5, 10, 20, 40 and 80 µg/ml) or control for 48 hr. Viral RNA was extracted from the culture supernatant, using a viral nucleic acid extraction kit (Yekta tajhiz azma Co., Iran), and reverse-transcribed to cDNA using RevertAid First Strand cDNA synthesis kit (Thermo scientific, Lithuania) and an influenza A viral RNA-specific universal Uni12 primer (5'-AGCAAAGCAGG-3'). Quantitative PCR was performed using influenza M gene primers (5'-GGCAAATGGTACAGGCAATG-3' and 5'-AGCAACGAGAGGATCACTTG-3') (Mehrbood *et al.*, 2012) and 2X SYBER Green Master Mix (Thermo scientific, Lithuania) with a Rotor-Gene Q (Corbett, Qiagen, Germany). The viral RNA level in the virus-infected cells was considered 100%, and the relative viral RNA levels in test samples were calculated.

***In vivo* study**

Female BALB/c mice (weighing 18–23 g) were obtained from Pasteur institute (Tehran, Iran). Mice were housed at 25°C with controlled 12 hr-12 hr light-dark cycles. Food and water were freely available. All experiments were executed in accordance with the Guide for the Care and Use at Laboratory Animals and approved by research and ethics committee of Shahrekord University of Medical

Antiviral effects of *Peganum harmala* L. seeds extract

Sciences (approval No. IR. SKUMS.REC.1395.160).

For *in vivo* toxicity determinations, the dose of PHS extract which was lethal to mice was determined. Mice were randomly divided into 7 groups of 5 mice and treated with 2-fold diluted doses of PHS extract (80, 161, 312.5, 625, 1250, and 2500 mg/kg/day) or control by oral gavage once a day for 5 days. The mice were monitored daily for signs of toxicity and death for 21 days. Lethal dose (LD₅₀) was calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). We also calculated the median infectious dose (LD₅₀) of PR8 in mice using Reed and Muench method (Reed and Muench, 1938).

Protective efficacy in mice

In order to evaluate anti-influenza activity of PHS extract *in vivo*, mice were randomly divided into 4 groups of 14. Mice were anesthetized by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Parke-Davis, Pontypool, UK) and 4 mg/kg xylazine (Bayer, Bury St, Edmunds, UK) and infected with 5LD₅₀ of mouse-adapted Influenza virus (A/Puerto Rico/8/34 (H1N1; PR8) in 50 µL sterile PBS via the intranasal route. Group 1 was infected with virus and did not receive treatment. Groups 2 and 3 were infected with virus and received 200 mg/kg/day of PHS extract or 20 mg/kg/day of oseltamivir (sigma, USA) orally via gavage once a day during days 1–5 post-infection (PI) starting 1 hr post-infection. Group 4 received PHS extract 200 mg/kg/day orally for 5 days without virus inoculation.

For the evaluation of body weight and survival rate, seven mice from each group were observed daily for morbidity and weighed once a day for 14 days. Based on our data, percentage of mortality (M), index of protection (IP) = $(M_p - M_e)/M_p \times 100$, where M_p and M_e are mortality in virus control group and drug-treated groups,

correspondingly) and mean day to death (MDD) were calculated.

In order to determine infectious titer of the virus in lung tissue, lungs of the other seven mice per group, were removed on day 3, and PI (peak of virus titer (Perrone et al., 2008) and Lung virus titers were determined by real-time qRT-PCR using primers for NS1 gene (Zhang et al., 2013).

Lung viral titers analysis

Total RNA was extracted from the lung homogenates using Trizol Reagent (1 ml of Trizol for 30 mg tissue), according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (RT-PCR) reaction was performed using a Rotor-Gene 3000 (Corbett, Qiagen, Germany) at a total volume of 10 µl containing 1µl of synthesized cDNA solution, 5µl of 2X SYBER Green Master Mix (Thermo scientific, Lithuania) and 500 nM of each primer. Amplification program included a denaturation at 95°C for 10 min followed by 45 cycles at 95°C for 15 sec, 54 or 58°C for 20 sec, and 72°C for 25 sec. Following the amplification, the specificity of the amplified products was confirmed using melting curve analysis. Viral RNA was normalized against RNA loading for each sample using the β-actin RNA as an internal standard. The primers for PR8 NS1 gene were: forward 5'-CATAATGGATCCAAACACTGTGTC -3' and reverse 5'-CCTCTTAGGGATTCTGATCTCGG -3'. The primers for β-actin were: forward 5'-CGGTCAGGTCATCACTATCGG -3' and reverse 5'-TCTTTACGGATGTCAACGTCACAC -3'.

The levels of viral RNA were expressed as the ratio of viral RNA to β-actin mRNA (viral RNA/β-actin mRNA). Each assay was performed in duplicate for each sample. Relative quantification of viral RNA to β-actin was determined using the $2^{-\Delta C_t} = 2^{-(C_t, NS1 - C_t, \beta\text{-actin})}$ equation (Schmittgen and Livak, 2008).

Statistical analyses

The IC₅₀ and CC₅₀ values were calculated using GraphPad Prism Demo (GraphPad software, San Diego, California, USA). The data were analyzed by Kruskal-Wallis test, and expressed as mean±SD. Survival times were compared using the log-rank test. Differences in values among different groups at p≤0.05 were considered statistically significant.

Results

Cytotoxicity and antiviral activity of PHS extracts in MDCK cells

The results showed that the CC₅₀ value of PHS extract in MDCK cells was 139.2 (CI95%:118.2-163.9) µg/ml. The analysis showed a significant relation between the concentration of the extract and cell death (p<0.01). To evaluate the effect of PHS extract on influenza virus replication, virus-infected MDCK cells were treated with non-cytotoxic concentrations of PHS extract for 48 hr to investigate the cytopathic effect and the viability of uninfected and infected cells measured by MTT. Based on nonlinear regression analysis, IC₅₀ of PHS extract on influenza A (H1N1) was 15.7 µg/ml (CI95%:11.7-21) with SI value of 8.87 (Figure 1).

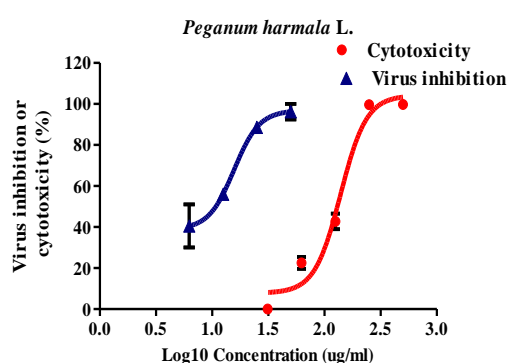


Figure 1. Anti-influenza virus activity and cytotoxicity of *Peganum harmala* L. extracts in MDCK cell lines. Confluent MDCK cells without virus or after influenza A virus infection, were exposed to different concentrations of the extract for 48 hr. Cell viability was measured using MTT assay. Results are expressed as means ± SD from three experiments.

Antiviral activity of PHS extract against influenza virus was assessed by hemagglutination endpoint test. PHS inhibitory effect resulted in a significant decrease in log₁₀ virus titer as tested by hemagglutination assay (Figure 2A). In addition, PHS extract effects on viral genome load levels were reflected as decrements in log₁₀ copy number in treatments which were calculated through absolute quantification. Quantitative analysis of the M gene of influenza A virus PCR products exposed to PHS extract, showed statistically significant decrements in viral load compared to the virus sample (Figure 2B).

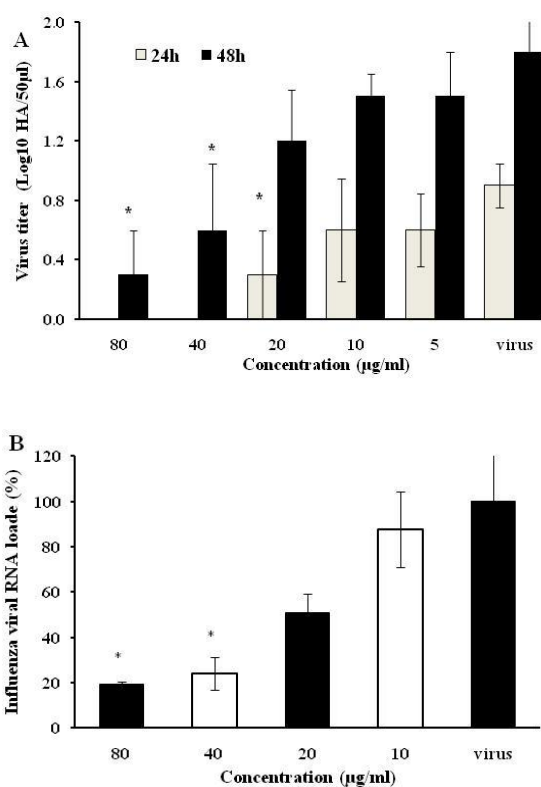


Figure 2. Reduction in influenza viral titers in the culture supernatants treated with the *Peganum harmala* seeds extract. The influenza A H1N1-infected MDCK cells were incubated with different concentrations of the extract for 24 and 48 hr. The supernatants were used for hemagglutination assay (A) or for viral RNA quantification by quantitative RT-PCR using influenza M gene (B). * p<0.05 from values obtained for treated samples compared to virus control (untreated sample). The data were analyzed by SPSS using Kruskal-Wallis test. The data are expressed as mean±SD of three independent experiments.

In vivo therapeutic efficacy of PHS extract against PR8 (H1N1) influenza virus

Oral treatment with PHS extract (once a day for 5 days) indicated an approximate LD₅₀ of 519 mg/kg/day for the extract.

The effect of oral treatment with PHS extract on the survival rate of influenza-infected mice was evaluated. We observed that treatment of mice with 200mg/kg PHS extract and 20 mg/kg oseltamivir decreased body weight loss (Figure 3A) and increased survival rates (Figure 3B) of

infected mice. While the MDD of virus control mice was 8.57±2.07 days, the mice administered with 200 mg/kg PHS extract and 20 mg/kg oseltamivir survived for 11.85±3.97 days and 12.7±3.9 days, respectively (p<0.05; Table 1).

The effect of the extract on viral loads in lungs of seven mice per group was examined on day 3 PI. We observed that treatment of mice with oseltamivir reduced viral titers. Similarly, viral load in virus-infected lungs was reduced in PHS extract (200mg/kg/day)-treated group, compared to the infected mice without therapy (Table 1).

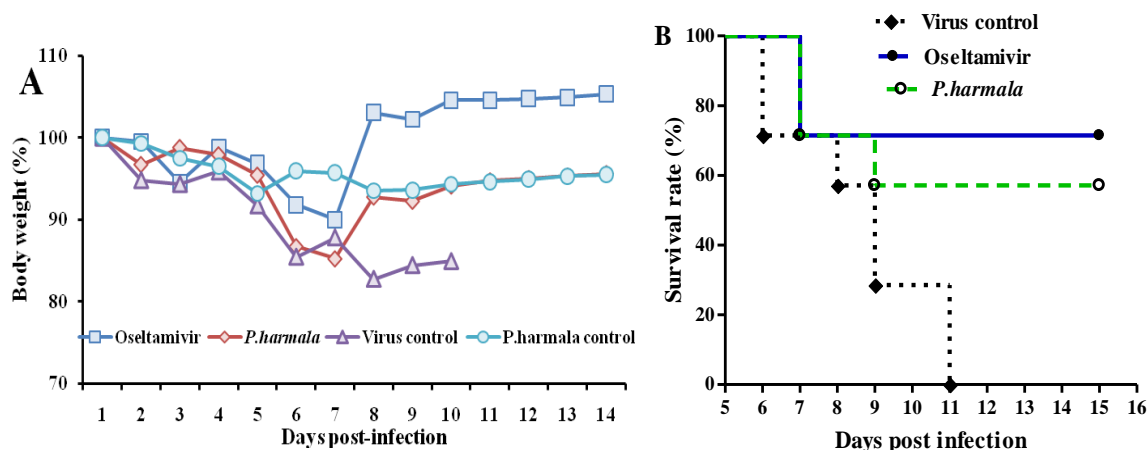


Figure 3. *In vivo* antiviral activity of *Peganum harmala* seeds extract against PR8 (H1N1) influenza virus in mice. Female BALB/c mice were intranasally infected with a mouse-adapted PR8 (H1N1) influenza virus (5LD₅₀). One hour later, seven mice per group received placebo, *P. harmala* seeds extract (200 mg/kg/day) or oseltamivir (20 mg/kg/day) once daily for 5 days, orally. (A) Body weights and (B) survival were monitored daily.

Table 1. Effect of oral treatment with *Peganum harmala* seeds extract on lung virus yield and mice survival in PR8 influenza virus-infected mice

Group	Dead/total (M, %)	IP, %	MDD ^{a,b}	Viral RNA titer / β-actin gene expression ^{b,c}
PHS +virus	3/7 (42.85)	57.15	11.85±3.97	1.07×10 ⁵ ± 7.87×10 ⁶ *
oseltamivir + virus	2/7 (28.57)	71.43	12.7±3.9*	6.08×10 ⁷ ± 5.03×10 ⁷ *
Virus control	7/7 (100)	N/A	8.57±2.07	8.43×10 ⁵ ± 2.96×10 ⁵
PHS control	0/7 (0)	-	>14	N/A

PHS: *Peganum harmala* seeds extract (200 mg/kg/day), oseltamivir (20 mg/kg/day), M: Mortality, IP: Index of protection, ^aMDD: Mean day to death of mice dying prior to day 14; ^bData is represented as mean ± SD; ^cthe levels of viral RNA were expressed as the ratio of viral RNA to β-actin mRNA (viral RNA/β-actin mRNA). Relative quantification of viral RNA to β-actin was determined using the 2^{-ΔCt} = 2^{-(Ct, NS1- Ct, β-actin)} equation. * p<0.05 versus virus control.

Discussion

Infection with influenza A virus is still a major health issue, and the available choices for the control and treatment of the disease are limited. Natural products and their derivatives have, historically, been considered as invaluable therapeutic agents (Mirhosseini *et al.*, 2014; Rouhi-Boroujeni *et al.*, 2016). Recent technological advances have allowed the researches for the development of anti-viral and also anti-influenza drugs from natural products (Ge *et al.*, 2010).

In this study, we used MTT cytotoxicity method, hemagglutination assay and qPCR method to evaluate anti-influenza A virus activity of PHS extract *in vitro* using MDCK cell line. Based on our results, the CC₅₀ (on MDCK cell) and the IC₅₀ value (on influenza A virus) of PHS extract were 139.2 (CI95%:118.2-163.9) µg/ml and 15.7 (CI95%:11.7-21) µg/ml, respectively. The recommended IC₅₀ value, characteristic of herbal extract against infectious diseases is less than 100 µg/mL (Cos *et al.*, 2006). The PHS extract used in this study had an IC₅₀ value of 15.7 µg/mL which is far below the recommended cut-off and this may indicate that this extract is a potent anti-influenza A virus *in vitro*. Since PHS extract showed antiviral effects *in vitro*, BALB/c mice were used to determine *in vivo* effects of this extract against mouse-adapted influenza A virus. Orally administered PHS 200 mg/kg/day (after infection with influenza virus) for 5 days significantly increased the survival rate, prolonged the mean survival time and reduced the viral titers in the lung. Many plant extracts and compounds have been shown to possess anti-influenza A virus activity, while only a few of which exhibited a protective effect against influenza infection, *in vivo* (Kim *et al.*, 2010; Yu *et al.*, 2016; Zarubaev *et al.*, 2015).

PHS contains some alkaloids compounds and has been considered from long time ago as a herbal medicine. The pharmacological studies of the PHS

extract has shown that this herb contains flavonoids, saponins, tannins, compounds reducers, volatile oils, anthraquinones, triterpenes, sterols, and alkaloids. These studies also showed that pharmacologically active compounds of *P. harmala* include several alkaloids, β-carbolines (such as harmine, harmaline, harman and harmalol) and the quinazoline derivatives (vasicine and vasicinone) (Mina *et al.*, 2015).

It has been reported that some of the β-carboline alkaloids also have antimicrobial (Shahverdi *et al.*, 2008), antiviral (Hudson *et al.*, 1986; Ishida *et al.*, 2001) and antiplasmodial (Astulla *et al.*, 2008) activities. It has been also shown that two alkaloid compounds of the β-carbolines (Zhang *et al.*, 2013) and the quinazoline (Dang *et al.*, 2014; Pan *et al.*, 2015) have anti-influenza A virus activity (Chiou *et al.*, 2011; He *et al.*, 2013; Zeng *et al.*, 2006). Therefore, the anti-influenza A virus activity of PHS extract used in this study could be attributed to its alkaloid components. The spectrum of anti-influenza activities and the underlying mechanisms as well as more preclinical evaluations of PHS extract will be done in our future works.

In conclusion, the results presented here suggest that PHS extract can inhibit influenza A virus replication *in vitro* and *in vivo*. Therefore, further characterization of PHS active compounds and investigations of the underlying mechanisms of its antiviral activity need to be carried out to enable us to introduce PHS as an anti-influenza A virus agent.

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Conflict of interest

The authors of this research declare no conflict of interest.

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