In vitro antiviral effects of *Peganum harmala* seed extract and its total alkaloids against Influenza virus

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ABSTRACT

This research was aimed to evaluate the *in vitro* antiviral effect and the mechanism of the effect of *Peganum. harmala* seeds extract against influenza A virus infection using Madin-Darby canine kidney (MDCK) cells. In this research, ethyl alcohol extract of *P. harmala* seeds and its total alkaloids was prepared. The potential antiviral activity of the extract and its total alkaloids against influenza A/Puerto Rico/8/34 (H1N1; PR8) virus was assessed. The mode of action of the extract to inhibit influenza replication was investigated using virucidal activity, hemagglutination inhibition assay, time of addition assays, RNA replication, western blot analysis and RNA polymerase blocking assay. The crud extract of *P. harmala* seed and its total alkaloids showed the best inhibitory effect against influenza A virus replication in MDCK cells using MTT assay, $TCID_{50}$ method and hemagglutination assay. Our results indicated that the extract inhibits viral RNA replication and viral polymerase activity but did not effect on hemagglutination inhibition and virucidal activity. This study showed that, *in vitro* antiviral activity of *P. harmala* seed extract against influenza virus is most probably associated with inhibiting viral RNA transcription. Therefore, this extract and its total alkaloid should be further characterized to be developed as anti-influenza A virus agent.

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1. Introduction

Influenza is an acute respiratory infection caused by influenza viruses, which circulate through all parts of the world. Hospitalization and death occur mainly between high-risk groups. Worldwide, the annual epidemics are estimated to result in about 3—5 million cases of severe illness, and about 250 000 to 500 000 deaths [1]. Pandemic yields different mortality estimates. Segmented ribonucleic acid genome and animal reservoir cause genetic reassortment in the virus. The appearance of new human and non-human source of influenza virus with the ability to cross-species barriers creates with high rate of antigenic drift and shift; also it changes to pathogenic type in their new hosts [2,3].

Annual vaccination is the mainstay strategy for preventing influenza infections and antiviral drugs offer additional preventive and therapeutic benefits [4]. In order to treat anti-influenza A virus two main groups of medicines including matrix protein (M2), ion-channel inhibitors (Amantadine and Rimantadine), and neuraminidase inhibitors (Oseltamivir, Zanamivir, and Peramivir) are confirmed [5]. The neuraminidase inhibitors are widely used in the treatment of both seasonal and pandemic influenza virus infections. However, oseltamivir resistant H1N1 strains were found to be circulated since the 2007—2008 [6,7]. As, the constant evolvement of influenza A virus causes the rapid emergence of resistance to current medicines [8—10], Therefore, it is essential to make the new and efficient anti-influenza medicines in order to treat resistant forms of influenza A virus.

Use of herbal extracts seems to be an alternative. There are some medicinal plants containing active compounds which have been used as remedies and as sources of herbal medicines. Many screening experiments have been carried out to isolate the extracts with antiviral activity from these plants [11—13]. Some of these herbal medicines have been developed into therapeutic agents and have had promising results.

Peganum harmala L. (family Zygophyllaceae) is a perennial,

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glabrous plant which grows spontaneously in the Middle East, Africa, India, South America, southern America, China and most areas of Iran [14]. Seed, fruit, root, and bark of this plant have been used, as folk medicine, for a long time in Iran, Turkey, and China to treat coughs, rheumatism, hypertension, diabetes and asthma [15–17].

Phytochemical compounds from *P. harmala* are alkaloids, flavonoids, amino acids, polysaccharides and anthraquinones [18]. The pharmacologically active compounds of *P. harmala* are several alkaloids, which are found especially in the seeds and the roots. These include β-carbolines such as: harmine, harmaline, harmalol and harman and quinazoline derivatives: vasicine and vasicinone [14].

Literature survey revealed that *P. harmala* and its active alkaloids possess a wide range of pharmacological activities [16,17]. Several studies have revealed anti parasitic [19,20], antifungal, antibacterial [21], insecticidal [22,23] and antiviral [24,25] effects from *P. harmala* and its alkaloids.

This research, most probably as a first work of this kind, was aimed to evaluate *in vitro* anti-viral activity of seed extract of *P. harmala* L. against influenza A virus infection.

2. Methods and materials

2.1. Plant collection, crude extract and total alkaloid extraction

Seeds of *Peganum 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. The seeds were powdered and then extracted using maceration method. The plant material was dissolved in 80% ethyl alcohol and kept at room temperature for 96 h then, the mixture was filtered and concentrated under nearly vacuum pressure and at 40 °C using rotary evaporator.

Total alkaloids were extracted from the ethanol extract as described previously [26]. Briefly, the crude extract was first defatted by using *n*-Hexan to remove all fat soluble (liposoluble) ingredients and fatty oil. The ethanol extract was subsequently dissolved in 5%HCl (to get pH = 5) and filtered. The filtrate was then partitioned three times with ethyl acetate and the aqueous acid layer was combined and dried under reduced pressure to give total alkaloid.

2.2. Cell culture and influenza virus propagation

Madin Darby Canine Kidney (MDCK) cell line and Influenza virus A/Puerto Rico/8/34 (H1N1; PR8), was 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% Pen/Strep (Gibco, USA) at 37 °C in a 5% CO2 atmosphere and humidified incubator.

2.3. Cytotoxic assay

The effect of crude extract and total Alkaloids of *P. harmala* on the viability of MDCK cells were determined by 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, USA) as described previously [27] with some modifications. Briefly, when the cell monolayer was confluent, the cells were incubated with 200 μL/well of various concentrations of the extract/fractions (in triplicates) in 96 well plates for further 2 days. Then cell monolayers were incubated with 50 μL of 1 mg/mL MTT in PBS at 37 °C for 4 h, then treated with 100 μL of isopropanol with HCl. After shaking the plates for 15 min, the absorbance was read at 570 nm with a reference filter at 640 nm using an enzyme-linked immunosorbent assay (ELISA) reader (StatFax 2100, USA).

2.4. Cytopathic effect (CPE) reduction assay

Confluent MDCK cells monolayer in 96 well plates were infected with 100 μl (100TCID$_{50}$) influenza A (H1N1) virus and incubated at 37 °C for about 1 h to allow virus adsorption. Then, the virus was removed and the cells were treated with serial two-fold dilutions of nontoxic concentration of the extract or total alkaloid (200 μl/well) in serum-free DMEM containing 2 μg/ml TPCK-trypsin and 0.3% BSA. On 48 h post infection cell viability was also determined using previously described MTT assay [27]. A solution of oseltamivir (Sigma, USA) were used as positive controls. The procedure was carried out in triplicate.

The 50% cytotoxic concentration (CC$_{50}$) and 50% inhibitory concentration (IC$_{50}$) were calculated using regression analysis and related models with probit regression model procedure, using the SPSS software (version 16.0). Selectivity index (SI) was calculated as ratio of CC$_{50}$ to IC$_{50}$.

2.5. Hemagglutination (HA) assay

Confluent MDCK cells monolayer in 24-well plates were infected with PR8 virus (100TCID$_{50}$), inoculated with virus at 1 h at 37 °C and cultured in DMEM and TPCK trypsin (0.5 μg/ml; Sigma, USA) either with or without extract/alkaloid treatment. The cell culture supernatants were harvested at 24 and 48 h post infection. Fifty microliters of the two fold serial dilutions of the cell culture supernatants were mixed with the same volume of 0.5% chicken red blood cells (RBCs) in U-bottomed 96-well plate for 45 min at room temperature. The HA assay activity was determined by measuring the dilution factor of the samples required for complete HA assay mediated chicken RBC agglutination [28].

2.6. TCID$_{50}$ virus titration

Confluent MDCK cells monolayer in 24-well plates were infected with PR8 virus (100TCID$_{50}$) in the presence of the extract/alkaloid or control compounds for 24 h at 37 °C. A standard 50% tissue culture infectious doses (TCID$_{50}$) method was used for virus titration in culture supernatants [29]. Briefly, 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 100 μl of a series of 10-fold dilutions was added into the wells and left to incubate for 2 days. After 48 h, 50 μl of culture medium were taken from each well and transferred to a U-bottomed 96-well plate for HA assay [30]. TCID$_{50}$ was calculated based on the Reed and Muench method [31].

2.7. Quantitative reverse transcription-PCR

We used real time PCR to quantify the presence of virus in the media after infection with influenza virus. Confluent MDCK cells monolayer in 12-well plates were infected with PR8 virus (100TCID$_{50}$) in the presence of the extract/alkaloid or control compounds for 24 h at 37 °C. Influenza viral RNA was extracted from the culture supernatant with 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′-AGCAAAAGCAGG-3′). Quantitative PCR was performed using influenza NS1 gene primer (Table 1) and 2x SYBER Green Master Mix (Thermo scientific, Lithuania) with a Rotor-Gene Q (Corbett, Qiagen, Germany). The viral RNA level from the virus-

Table 1
Primers used in real-time RT-PCR.

Gene name	Primer sequence	Melt temperature (°C)	Product length (bp)
Nucleocapsid protein (NP)	F:TGTGTATGGACCTGCCGTAGC	62.1	158
	R:CCATCCACACCAGTTGACTCTTG	61.3	
Hemagglutinin (HA)	F:CCTGCTCGAAGACAGCCACAACG	60.6	98
	R:TTCCCAAGAGCCATCCGGCGA	58.3	
Nonstructural protein (NS1)	F:CATAATGGATCCAAACACTGTGTC	58.1	138
	R:CCTCTTAGGGATTTCTGATCTCGG	60	
Matrix protein (M)	F: GGCAAATGGTACAGGCAATG	57.7	144
	R: AGCAACGAGAGGATCACTTG	57.6	
GAPDH	F: GGAGAAAGCTGCCAAATATGACGA	61.4	140
	R: CGAAGGTGGAAGAGTGGGTGT	61.9	

GAPDH: Canis lupus familiaris glyceraldehyde-3-phosphate dehydrogenase.

infected cells was defined as 100% and the relative viral RNA levels from the test samples were calculated.

2.8. Time-of-addition experiments

To determine the stage of the viral life cycle that is affected by *P. harmala* seed extract, MDCK cells were seeded into 24-well plates and incubated overnight until 90% confluence. After incubated with virus (10^4TCID$_{50}$) for 1 h at 37 °C, the cells were washed with PBS, and then fresh medium containing TPCK and the extract at its maximum non-toxic concentration (100 μg/ml) was added. The crude extract was added at the following time points: −2 to −1 h (before the adsorption of the virus), −1 to 0 h (adsorption) as well as at three time points post adsorption: 0–2, 2–4, and 4–8 h. The treatment (−2)-8 h post infection was considered as a positive control. In each case after incubation the extract was removed and cells were washed and then fresh medium containing TPCK was added. After 8 h of incubation, supernatants were taken and progeny virus yields were determined using previously described TCID$_{50}$ assay [32,33].

2.9. Virucidal activity

P. harmala seed extract at maximum non-toxic dilution (100 μg/ml) and PBS was incubated with the virus (10^4TCID$_{50}$) for 1 h at room temperature. Then, the infectious titer of the virus in culture medium was determined using previously described TCID$_{50}$ assay [32].

2.10. Inhibition of viral mRNA expression and viral RNA synthesis

Confluent MDCK cells in 12-well plates were infected with the virus, and cultured in the presence of *P. harmala* seed extract (100 μg/ml) for 13 h [34]. Total RNA was extracted from the MDCK cells inoculated with either the treated or untreated viruses using Trizol (Invitrogen, Waltham,MA, USA) according to the manufacturer's instructions. The obtained RNA was transcribed into cDNA by RevertAid First Strand cDNA synthesis kit (Thermo scientific, Lithuania) including an influenza A viral RNA-specific universal primer Uni12 (to detect viral RNA) or oligo-dT (for viral mRNA detection) primers [35], 5X Reaction buffer, RNase inhibitor and dNTP Mix to a final volume of 20 μl. The quantification of viral mRNA (vmRNA) and viral RNA (vRNA) levels was performed using a Rotor-Gene Q (Corbett, Qiagen, Germany) in a total volume of 10 μl containing 1 μl of synthesized cDNA solution, 5 μl of 2x SYBER Green Master Mix (Thermo scientific, Lithuania) and 500 nM of each primer (Table 1). Amplification program included a denaturation at 95 °C for 10 min followed by 45 cycles at 95 °C for 15 s, 54 or 58 °C for 20 s, and 72 °C for 25s. Following the amplification, the specificity of the amplified products was confirmed using melting curve analysis. All the reactions were performed in of 2 independent repeats in duplicate and a mixture containing no cDNA template was used as the negative control. The expression of mRNA was normalized to that of the control housekeeping gene (GAPDH), and the relative changes in gene expression were calculated using the relative quantitative method ($2^{-\Delta\Delta Ct}$). The effects on viral RNA synthesis were shown by log10 copy number decrements in treatments which were calculated through absolute quantification.

2.11. Construction of the plasmid standards for quantification

For quantification of real-time PCR assay, the amplified M2 gene were T/A cloned in pTZ57 R/T vector (Invitrogen, San Diego, CA). Vectors were transformed into *E. coli* TOP10F′ competent cells using calcium chloride solution and under heat shock (42 °C) for 90s. For screening of recombinant vectors, competent cells were cultured in Luria broth (LB) agar media (Merck Co., Germany) containing Ampicillin antibiotic (100 μg/mL), IPTG (0.1 M) and Xgal (20 mg/mL) at 37 °C overnight. The white colonies were selected and cultured again in LB broth media (containing Ampicillin antibiotic) at 37 °C overnight. The recombinant vectors were extracted from bacterial cells by Plasmid Mini Extraction Kit (Bioneer, South Korea) using manufacturer's instructions and PCR analysis was used for confirmation of cloning. The concentration of extracted plasmids were measured spectrophotometrically using a Nano drop system (Implen Nano Photometer™, Germany). The plasmids copy number were calculated taking into account the size of the original plasmid plus the cloned inserts and the concentration of the construct by following formula [36]:

$$\text{Number of copies}/\mu l = [6.02 \times 1023 \text{copies} \\ \times \text{ plasmid concentrations } (g/\mu l)]/ \\ \times [\text{Number of bases pairs} \\ \times 660 \text{ daltons/base}]$$

To plot standard curves, ten-fold serial dilutions of the plasmid (10^7 copies to 100 copy) were prepared and the M2 gene in plasmids were amplified in three replicates for each standard dilution point. Real time RT-PCR quantification was accomplished using the method for measuring the expression of the gene.

2.12. Hemagglutination inhibition (HI) assay

The hemagglutination inhibition (HI) assay was employed to test the effect of the extract on influenza virus hemagglutinin [37]. Briefly, 50 μL of two fold serial dilutions of the extract diluted in PBS containing 0.1% BSA was mixed with 50 μL of the virus in PBS containing 0.1% BSA. After 1 h of incubation, the hemagglutination assay was carried out to measure virus titration. Titers are given as
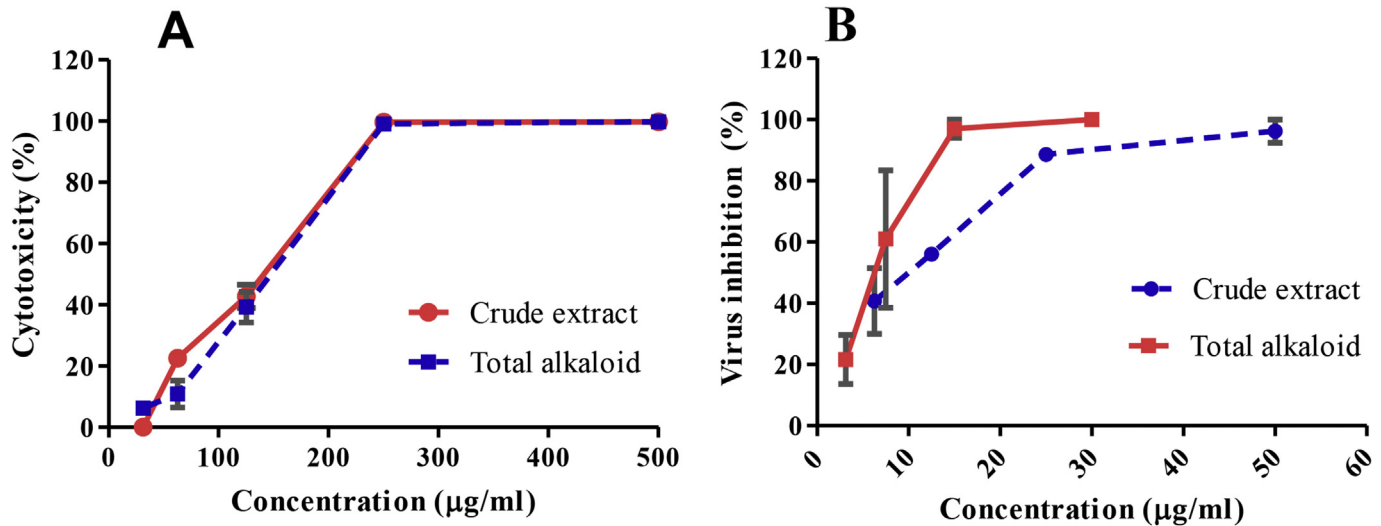
Fig. 1. Cytotoxicity and anti-influenza virus activity of crude extract and total alkaloids of *Peganum harmala* L. Confluent MDCK cells were exposed to different concentrations of crud extract or total alkaloids for 48 h without virus (A) or after influenza viruses (PR8) infection (B). Cell viability was measured in MTT test. Results are means ± SD from three experiments.

Table 2
Hemagglutination titers of PR8-infected MDCK cell supernatants in the presence of the *Peganum harmala* L. seed crud extract and total alkaloid.

Extract/fractions	Concentration (µg/ml)	Log2 HA titer/50 µl supernatant	
		24 h[a]	48 h[a]
Crude extract	100	0.5 ± 1	1.33 ± 1.5
	31.6	2.25 ± 1.5	3.66 ± 1.15
	10	3.5 ± 1.29	5.66 ± 1.15
	3.1	4.75 ± 1.7	6 ± 2
	virus control	5.25 ± 1.7	7 ± 1
Total alkaloid	31.6	0.25 ± 0.5	2.33 ± 0.58
	10	2.25 ± 1.7	5 ± 2
	3.6	3.5 ± 1.3	5 ± 1
	1	4.5 ± 2.1	6.33 ± 1.53
	virus control	5.25 ± 1.7	7 ± 1
Oseltamivir (µmol)[b]	5	0	0
	2.5	0	0.5 ± 0.71
	1.25	0	4
	virus control	5 ± 1.4	8

[a] Hours post-infection.
[b] Oseltamivir was used as positive controls.

HA units/50 µL (HAU/50 µL) in comparison with the control treatment (virus without extract).

2.13. Western blot analysis

MDCK cells in 6-well plates were inoculated with PR8 at ($100TCID_{50}$) in the presence of crude extract (100 and 50 µg/ml) for 24 h at 37 °C. The culture medium was removed and cells were washed twice with cold PBS and lysed in ice-cold radio immune precipitation assay buffer (6 ×) containing protease inhibitor cocktail. Protein concentration was measured by Bradford assay (BioRad), and the equal amounts of protein from each sample were subjected to blotting.

The protein lysates were mixed with SDS loading buffer (0.125 M Tris-HCl 4%, SDS 20%, glycine 10%, and 2-mercapto-ethanol), followed by boiling for 5 min and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to polyvinylidene difluoride (PVDF; Bio-Rad, USA) membrane using semi-dry transfer system (Bio-Rad,

USA). Viral nucleoprotein (NP) and M1 protein, and cellular β-actin protein (loading control) were detected with anti-NP (Thermo Fisher Scientific, USA), anti-M1 (Thermo Fisher Scientific, USA) and β-actin antibodies (Abcam; USA), respectively. The horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-rabbit/IgG) were used and the bound antibodies were detected using C-DiGit® Blot Scanner (LI-COR, USA).

2.14. Statistical analysis

The data were statistically evaluated using a kruskal-wallis to compare differences between the groups. A p-value of <0.05 was considered to be significant. The IC_{50} and CC_{50} values were calculated by regression analysis and related models with probit regression model procedure, using the SPSS software (version 16.0).

3. Results

3.1. Cytotoxicity and antiviral activity of the extracts and total alkaloid

The results showed that the CC_{50} value of crude extract and total alkaloide on MDCK cells were 122.9 (CI95%: 107.8−140) and 133.9 (CI95%: 121.9−147) µg/ml respectively. The analysis showed a significant relationship between the concentration of the extract/alkaloid and cell death (the more the increase in the extract concentration was, the more the cell death was ($P < 0.05$; Fig. 1).

MDCK cells were inoculated with PR8 virus for 1 h. Then, the virus was removed and the cells were treated with serial two fold dilutions of nontoxic concentration of the extract or total alkaloid. Our results showed that more the extract concentration, more the CPE inhibition (Fig. 1, $p < 0.05$). Based on regression analysis, IC_{50} of crud extract and total alkaloid influenza virus were 9.87 (CI95%: 7.3−11.3) and 5.8 (CI95%: 3.7−8.9) µg/ml with SI value of 12.45 and 23.1 respectively. In comparison, The IC_{50} and CC_{50} of oseltamivir were 539.4 (CI95%: 378.9−768.5) and 0.873 (CI95%: 0.55−1.37) µmol with SI value of 617.8.

Antiviral activity of the extract/alkaloid against influenza virus was assessed by HA endpoint test. According to the results, the titer of virus was reduced upon treatment with crude extract and total alkaloid in a dose-dependent manner (Table 2).
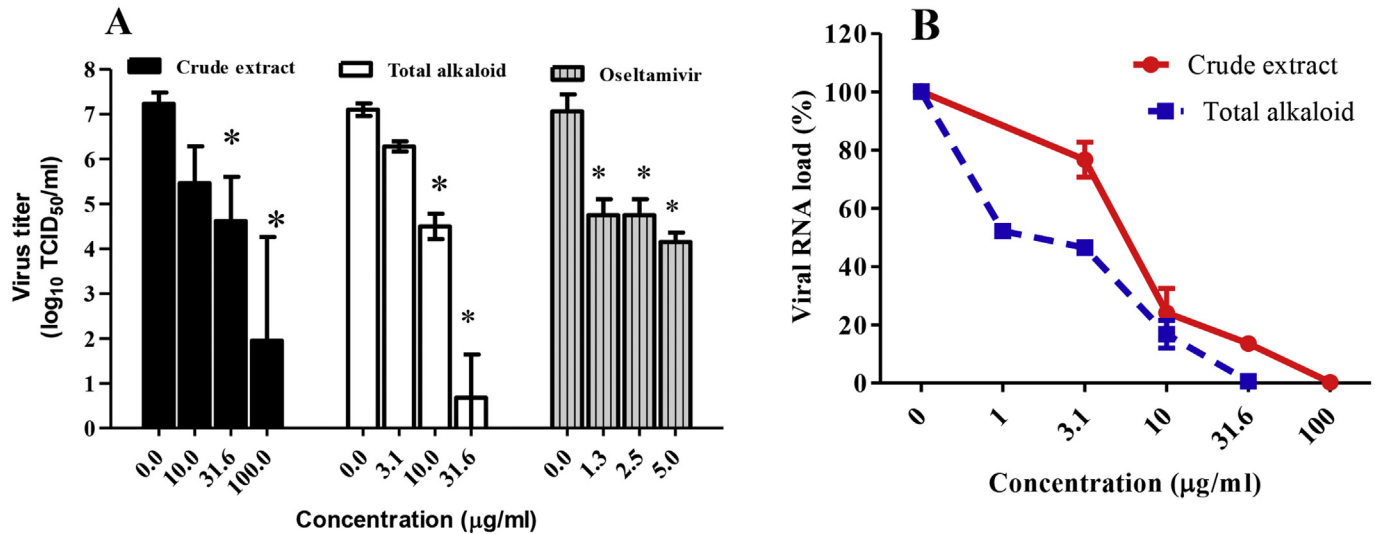
Fig. 2. Decrease of influenza viral titers in the culture supernatants by the *Peganum harmala* L. seed crud extract and its total alkaloids. PR8-infected MDCK cells were incubated with different concentrations of the extract/alkaloid for 24 h and the supernatants were used for $TCID_{50}$ titration (A) or for viral RNA quantification by quantitative RT-PCR (B). Oseltamivir was used as positive controls (μmol). The data are the mean values of three independent experiments (mean $\pm$ SD). P values were calculated against virus control (untreated sample) using Kruskal Wallis test,*$P < 0.05$.
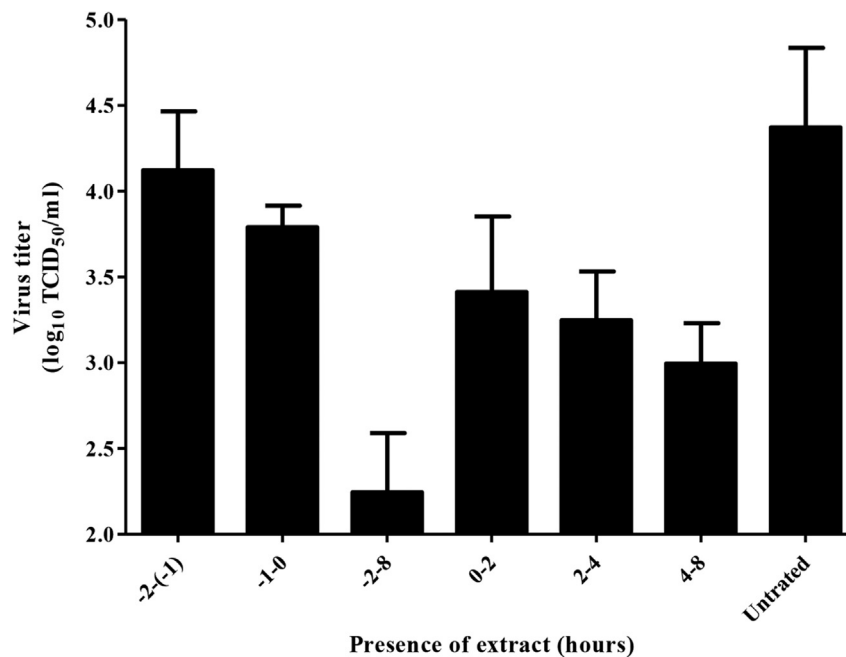

Fig. 3. Time-of-addition activity of *Peganum harmala* L. seed extract on influenza virus. PR8 virus was absorbed to MDCK cells (10^4 $TCID_{50}$) for 1 h at 4 °C. After washing of non-absorbed virus, the crude extract was added at its maximum nontoxic concentration (100 μg/ml) to the cell-virus system at the following time points: -2 to -1 h (before the adsorption of the virus), -1 to 0 h (adsorption) as well as at several time points post adsorption: 0—2, 2—4, and 4—8 h. Virus titer was determined after 8 h of incubation in MDCK cells using $TCID_{50}$ assay.

To address whether *P. harmala* and total alkaloid could have inhibitory effect on infectious virus yield, the virus was titrated by HA endpoint test, $TCID_{50}$ method and quantitative RT-PCR analyses. The results showed that, crude extract and total alkaloid significantly reduced the levels of infectious viral titers and viral RNA levels (Table 2 and Fig. 2).

3.2. P. harmala L. seed extract inhibits late stages of influenza virus infection

To determine the stage of viral cycle when the extract demonstrates its activity, time-of-addition experiments were performed. Evaluation of virus titer indicated that crude extract did not interfere with virus absorption (-1 to 0 h) or receptor binding (-2 to -1) step. In addition, decrease in the virus titer was observed at time points 4 to 8 and 2—4 h. Maximum virus inhibition by the extract was seen at the time point -2 to 8 h post infection (Fig. 3).

3.3. P. harmala seed extract inhibit viral protein synthesis

The levels of influenza viral mRNA were compared between the crude extract treated and untreated infected cells. RNA extraction
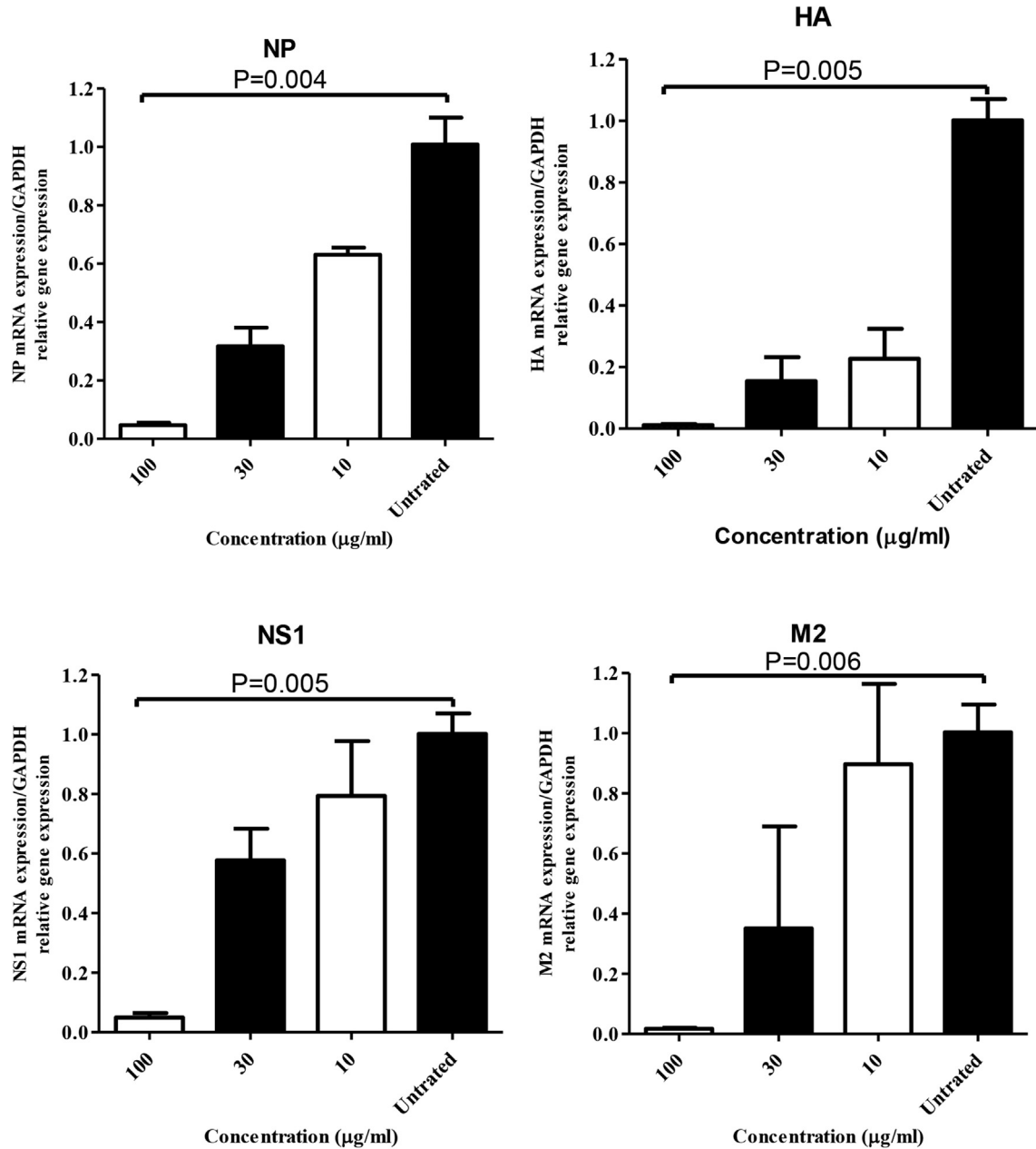
Fig. 4. Effects of *Peganum harmala* L. seed extract on the expression of viral mRNA in MDCK cells. Cells were infected with PR8 virus and treated with different concentrations (100, 31.6 and 10 μg/mL) of the extract. Total RNA was extracted at 13 h after influenza virus infection and the levels of intracellular mRNA influenza genes (NS1, NP, M1, and HA) were measured. Influenza viral mRNA levels normalized to GAPDH. The data are the mean values of 2 independent repeats in duplicate (mean ± SD). P values were calculated against virus control (untreated sample) using Kruskal Wallis test.

was performed at 13 h after influenza virus infection and the levels of intracellular influenza virus mRNA (NS1, NP, HA and M2 genes) were measured. Quantitative real-time PCR showed a significant decrease in influenza mRNA expression level from the crude extracts treated cells dose-dependently compared with untreated virus infected cells (P < 0.05; Fig. 4).

In order to detect viral protein synthesis in the cells following the inoculation of the virus, viral NP and M1 were studied using western blot analysis. We found that the extract inhibits the viral protein synthesis in a dose-dependent manner (Fig. 5). These results indicate that the *P. harmala* extract have inhibitory effects on the viral protein synthesis.

3.4. *P. harmala* extract inhibit viral RNA replication

The synthesis of viral RNA in MDCK cells were compared between various concentration of crud extract treated or untreated was analyzed at 13 h after the inoculation of the virus.

The extract effects on viral RNA load levels were shown by log10 copy number decrements in treatments which were calculated through absolute quantification. Quantitative analysis on the M2 gene of influenza virus showed statistically significant decrements in viral load compared to the virus sample in a dose-dependent manner (Table 3).
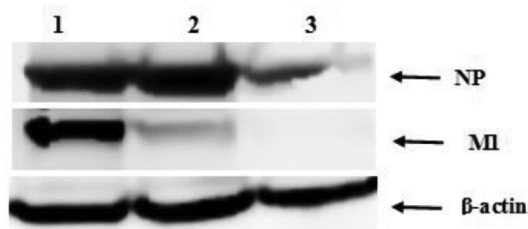
Fig. 5. Effects of *Peganum harmala* L. seed extract on the protein synthesis in MDCK cells. MDCK cells were infected with PR8 virus and then incubated with different concentrations (100 and 50 μg/ml) of the extract for 24 h at 37 °C. The viral proteins, NP and M1, were detected using their specific primary antibodies and HRP-conjugated secondary antibodies. Cellular β-actin was used as a loading control. Well 1: virus control, well 2: virus +50 mg/mL crude extract, well 3: virus +100 mg/mL crude extract.

Table 3
Effects of *Peganum harmala* L. seed extract on the viral RNA copy number.

Concentration (μg/ml)	Ct (Mean ± SD)	Log10 copy numbers (Mean ± SD)
100	28.76 ± 0.89	1.88 ± 0.225*
31.6	25.18 ± 1.16	2.78 ± 0.29
10	23.9 ± 1.25	3.10 ± 0.315
Virus control (untreated)	17.59 ± 0.41	4.69 ± 0.10

Cells were infected with PR8 virus and treated with different concentrations (100, 31.6 and 10 μg/ml) of crude extract. Total RNA was extracted at 13 h after influenza virus infection and the Log10 copy numbers relating to Ct values of intracellular influenza M gene were measured. Data were averages of 2 independent repeats in duplicate. *($p < 0.05$) comparison with the virus untreated sample using Kruskal Wallis test.

3.5. Virucidal activity and hemagglutination inhibition activity of P. harmala seed extract

To elucidate the mechanism of antiviral activity of the extract, another series of experiments was conducted Study of virucidal activity showed that virus yield in the extract and control was 5.5 ± 0.56 and 5.08 ± 0.59 (log 10-TCID50) respectively ($p > 0.05$). This confirms that the extract has no virucidal activity and this mechanism should not be considered for the extract.

To determine whether the crud extract can prevent the ability of virus particles to bind to cell surface receptors, we used HI assays. The HA assay results indicated that pretreatment with the crude extract could not prevent the binding of virus to RBCs (data not shown).

4. Discussion

P. harmala seed has been considered from long time ago as a herbal medicine. It has been reported that *P. harmala* have antimicrobial [38], antiviral [39,40], and antiplasmodial [19] activities. To the best of our knowledge, to date, there has been no report on the anti-influenza virus activity of *P. harmala* seed extract. Therefore, in this study anti-influenza virus activity of *P. harmala* seed extract was evaluated through inhibiting the viral RNA transcription and replication in MDCK cell line.

In the present study, the crude extract inhibited influenza A PR8 virus replication in this cell line, with IC_{50} value of about 9.87 (CI95%: 7.3–11.3)μg/ml and SI value of 12.45. Using additional antiviral assays, which measured the titers of HA or infectious viral particles in the culture supernatants, as well as the viral RNA and viral protein levels in the cell lysates, it was ensured that the crude extract of *P. harmala* seed could suppress production of infectious influenza viruses. As an IC_{50} value of a characteristic herbal extract against infectious diseases is less than 100 μg/mL [41], this extract with IC_{50} of 9.87 should have strong activity against influenza virus.

Our results showed that the total alkaloids of this extract had antiviral activity, with IC_{50} value of about 5.8 (CI95%: 3.7–8.9)μg/ml and SI value of 23.1, better than that of the crude extract. Other studies showed that pharmacologically active compounds of *P. harmala* include several alkaloids, β-carbolines (such as harmine, harmaline, harman and harmalol) and the quinazoline derivatives of vasicine and vasicinone [16]. It has been reported that some of the β-carboline alkaloids also have antiviral [39,40], activities. It has been also shown that alkaloid compounds of the β-carbolines [42] and the quinazoline [43,44] have anti-influenza A virus activity. Therefore, the anti-influenza A virus activity of the *P. harmala* seed extract used in this study could be attributed to its β-carboline alkaloids.

In this study, using quantitative real-time PCR, western blot analysis, and time course study, the effect of *P. harmal* extract on influenza virus replication in MDCK cell line was investigated. Based on our study, the effect of extract on reduction of the RNA level and the viral polymerase activity was evident. In influenza virus particle, the genomic RNAs (vRNAs) are associated with multiple copies of nucleoproteins (NPs) and a small number of RNA-dependent RNA polymerase complexes, that consist PA, PB1, and PB2, which together form the ribonucleoprotein complex (RNP) which is essential for the transcription and replication of the viral RNAs [45]. Based our results, *P. harmala* seed extract may decrease the level of NP and viral polymerase activity, and thus affects the RNP complexes' activity, leading to inhibition of both viral RNA transcription and replication.

Time course study of one cycle of influenza virus replication showed that the extract inhibited the influenza replication but did not interfere with virus absorption or receptor binding step. The recent claim was supported by HI assay that the attendance of the extract was not show any inhibitory effect on virus-induced HA. The maximal virus inhibition was achieved at time points 4–8 and 2–4 h post infection during late stages of the viral replication cycle. This stage include the synthesis of influenza virus late proteins and their subsequent assembly into functionally active complex. Further studies are needed to identify the precise target of this extract on the influenza virus life cycle.

This study showed that, *in vitro* antiviral activity of *P. harmala* seed extract against influenza virus is most probably associated with inhibiting viral RNA transcription. Therefore, this extract and its total alkaloid should be further characterized to be developed as anti-influenza A virus agent.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Competing interests

The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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