Microbial Pathogenesis 110 (2017) 42–49

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

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

Mohammad-Taghi Moradi [a], Ali Karimi [b, *], Mahmoud Rafieian-Kopaei [b], Fatemeh Fotouhi [c]

[a] Students Research Committee, Shahrekord University of Medical Sciences, Shahrekord, Iran
[b] Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran
[c] Department of Influenza and other Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran

ARTICLE INFO

Article history:
Received 20 May 2017
Received in revised form
10 June 2017
Accepted 15 June 2017
Available online 16 June 2017

Keywords:
Influenza A virus
Antiviral activity
Total alkaloid
Peganum harmala L.

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.

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,

* Corresponding author.
E-mail address: kakarimi42@gmail.com (A. Karimi).

http://dx.doi.org/10.1016/j.micpath.2017.06.014
0882-4010/

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-