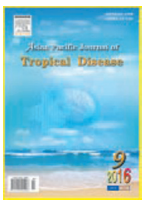
HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

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



Infectious disease research doi: 10.1016/S2222-1808(16)61115-5

Melissa officinalis efficacy against human influenza virus (New H1N1) in comparison with oseltamivir

Parvane Jalali[1], Afagh Moattari[2], Ali Mohammadi[1], Nima Ghazanfari[3], Gholamhosein Pourghanbari[4*]

[1]*Division of Virology, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran*

[2]*Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*

[3]*Department of Pharmacology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*

[4]*Department of Clinical Science, School of Veterinary Medicine, Ardakan University, Ardakan, Yazd, Iran*

ARTICLE INFO

Article history:
Received 23 Jun 2016
Received in revised form 17 Jul, 2nd revised form 8 Aug 2016
Accepted 12 Aug 2016
Available online 15 Aug 2016

Keywords:
Melissa officinalis
New H1N1
Antiviral
Cell culture

ABSTRACT

Objective: To evaluate the antiviral activity of *Melissa officinalis* (MO) extract against the influenza virus H1N1 *in vitro*.
Methods: The cytotoxicity of MO extract was identified on Madin-Darby canine kidney (MDCK) cell culture by MTT assay. The virus was inoculated to the cells (multiplicity of infection = 0.1) in two protocols. In protocol 1, the MO extracts at concentrations of 0.005, 0.050, 0.010, 0.100 and 0.500 mg/mL were incubated with the virus for one hour pre-inoculation. In protocol 2, the mentioned concentrations of MO extracts were added to the cells one-hour post infection. Furthermore, the antiviral effect of oseltamivir with different concentrations was tested as the positive controls. The 50% tissue culture infective dose, neutralizing index and hemagglutination titer were determined.
Results: The medicine oseltamivir and MO extracts were not toxic for MDCK at concentrations less than 1 mg/mL. All utilized concentrations of MO extracts were vigorously efficient to decrease the viral yield in both experiments. The 50% tissue culture infective dose of the groups containing up to 0.100 mg/mL of MO extracts in the first experiment in compare with 0.050 mg/mL in the second experiment reduced to 0. Although hemagglutination tests showed little titers, the viral quantity significantly decreased in both experiments. By the way, the medicine oseltamivir could completely suppress viral replication in MDCK.
Conclusions: The present study suggests that MO extracts have a potent anti-influenza effect in cell culture.

1. Introduction

Influenza A viruses are considered to be one of the most important human pathogens and can cause severe viral respiratory infections. The influenza pandemics such as those occurred in 1918 resulted in high morbidity and mortality rates mainly due to the lack of sufficient protection against the new virus strains[1]. The pandemic new H1N1 virus spread rapidly throughout the world in 2009 and the virus was shown to be more transmissible than the seasonal H1N1[2].

Two groups of antiviral compounds have been approved by the Food and Drug Administration until now, but oseltamivir is regarded as the drug of choice for influenza viruses[3,4].

Lemon balm is one of the most important member of the Lamiaceae family and it is native to Europe, central Asia and Iran.

The main ingredients of the *Melissa officinalis* (MO) are citral (neral and geranial), citronellal, linalool, geraniol and β-caryophyllene-oxide. Tannins such as triterpenylic acid, bitter principles, flavonoids including phenolic acids, terpenes, rosmarinic acid and caffeic acids were belonged to the Lamiaceae[5-8].

Several properties of lemon balm such as antioxidant, antihistamine, antispasmodic, anti-tumor/anticancer, antibacterial, antifungal, antidepressant and antiviral activities were reported[9,10].

It has been shown that the extract of MO is able to prevent protein synthesis in the herpes simplex virus type 1[11]. Some studies have shown that the antiviral activity of lemon balm was due to tannins and polyphenolic compounds[12].

There is no report on the efficacy of lemon balm on the human influenza viruses. In this study, the antiviral activity of MO hydroalcoholics extracts and their synergistic activity with oseltamivir on the replication of the influenza virus subtype H1N1

*Corresponding author: Dr. Gholamhosein Pourghanbari, Department of Clinical Science, School of Veterinary Medicine, Ardakan University, Ardakan, Yazd, Iran.
Tel: 0098-35-32240926
Fax: 0098-35-32240926
E-mail: hpourghanbari@ardakan.ac.ir, hpourghanbari@gmail.com
The journal implements double-blind peer review practiced by specially invited international editorial board members.

were evaluated in Madin-Darby canine kidney (MDCK) cell line and the efficacy were analyzed by the 50% tissue culture infective dose ($TCID_{50}$) and hemagglutination (HA) tests.

2. Material and methods

2.1. Reagents

Lemon balm extracts were prepared in the Pharmacy Department of Shiraz University of Medical Science. Antibiotic, trypsin–ethylene diamine tetraacetic acid, fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were supplied by Gibco BRL (Grand Island, NY, USA). Oseltamivir (F. Hofmann La Roche Ltd, Switzerland) was purchased from a pharmacy in Iran. Tissue culture plates and flasks were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, USA). Stock solutions (10 mg/mL) of the materials were solved in dimethyl sulfoxide (DMSO) and were subsequently diluted in appropriate culture media. The maximum DMSO concentration reached to 0.1%.

2.2. Cells and viruses

Human influenza A virus, 2009 pandemic new H1N1, which was taken from Influenza Virus Research Centre of Shiraz University of Medical Science, the Influenza virus (new H1N1) was replicated and passaged in the cells and virus titers were evaluated by using $TCID_{50}$. MDCK cells were grown in DMEM with penicillin (100 IU/mL), streptomycin (100 μg/mL) and supplemented with 7% heat-inactivated FBS.

2.3. Cell viability assay

The extract and oseltamivir efficacy against the MDCK was measured by MTT test. MDCK cells were propagated (1×10^4 cells/well) in a 96-well plate for 26 h. The medium was taken place with DMEM including different concentrations of the extracts. After incubating the cells at 37 ˚C for 48 h, 100 μL of the Roswell Park Memorial Institute medium (without phenol red) with 10 μL MTT (5 mg/mL in phosphate-buffered saline) was added to each well and the cells were incubated for 4 h. Then, the supernatant was removed and 50 μL of DMSO was inoculated to the each well and incubated for 30 min. A micro plate reader at the wave length of 540 nm was used for recording the absorbance[13].

2.4. Virus inoculation

MDCK cells were grown in the plates (including 96 wells) by using DMEM (1×10^4/well), when the cells confluence was up to 90%. The residual FBS was removed by washing the cells with phosphate-buffered saline twice.

The inoculation of the cells with the virus at multiplicity of infection = 0.1 was carried out in two protocols. In protocol one (pre-infection), the virus was added with the different concentrations of MO extracts (0.500, 0.100, 0.050, 0.010 and 0.005 mg/mL) for 1 h then the inoculation was occurred. In protocol two (post-infection), the cells were incubated with the virus for 1 h, and then the medium containing those concentrations of MO extracts were added to the wells and incubated for 72 h. The medium applied in both protocols contained trypsin at the concentration of 2 μg/mL. The incubations were performed at 37 ˚C in 5% CO_2 and 80% humidity.

The oseltamivir was also used as the positive control. The medium containing the various concentrations (0.500, 0.100, 0.050, 0.010, 0.005 mg/mL) of oseltamivir were inoculated to the MDCK cells for 1 h after the virus inoculation (multiplicity of infection = 0.1) and incubated for 72 h.

The cell culture supernatants were collected and the viral HA titres and $TCID_{50}$ were calculated.

2.5. $TCID_{50}$ test and HA assay

A standard protocol by using a 2-fold dilution of each sample was used for $TCID_{50}$ test[14].

Cell culture supernatants containing virus was diluted 2-fold serially and 0.5% chicken red blood cell was inoculated at an equal amount. Then, the plate was incubated for 60 min at 4°C, the red buttons were composed in negative wells, whereas positive wells did not show any red buttons and the opaque appearance was observed. HA results are given as hemagglutination units/50 μL (HAU/50 μL).

2.6. Neutralizing index (NI)

The NI test was used to obtain the antiviral activity of the extracts or drugs. The NI of virus inactivation was calculated by subtracting the log10 titer of collected virus from the infected MDCK cells with extract/drug treated virus from the collected viral titer of the infected negative control cells[1]. Inactivation of the virus was evaluated to be effective when NI $\geqslant$ 2.8 and the NI in positive control group was 4.0[15].

3. Results

3.1. Cell viability

The safe concentration of MO extract and oseltamivir in MDCK cells were calculated by using different concentrations of the components and adding them to the cells and the cytotoxicity was evaluated with MTT assay. The cytotoxicity of the MO extract and oseltamivir on the MDCK cells was reduced under to 50% as the concentration was decreased to 1 mg/mL.

3.2. MO extract efficacy in protocol 1

3.2.1. HA and $TCID_{50}$ test

The mean virus titer in the negative control reached to 53 HAU/50 μL, while it decreased to 20 HAU/50 μL at the least concentration 0.005 mg/mL and to 15 HAU/50 μL at 0.010 mg/mL. The mean virus titer was 5.5 HAU/50 μL at the rest concentrations.

The viral $TCID_{50}$ based on log10 reached to 4 in the negative control while it was 2 at the concentration of 0.005 mg/mL. It decreased to 0 at the concentrations of 0.100 and 0.500 mg/mL of MO.

3.2.2. NI

According to the findings, NI was shown to be equal or more than