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ORIGINAL ARTICLE

Antiviral activity of the oseltamivir and *Melissa officinalis L.* essential oil against avian influenza A virus (H9N2)

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Abstract Lemon balm derivatives are going to acquire a novelty as natural and potent remedy for treatment of viral infections since the influenza viruses are developing resistance to the current antivirals widely. Oseltamivir, *Melissa officinalis* essential oil (MOEO) and their synergistic efficacy against avian influenza virus (AIV) subtype H9N2 were evaluated in vitro in MDCK cells at different time exposure by using TCID50, HA, Real Time PCR and HI assay. The results showed that MOEO could inhibit replication of AVI through the different virus replication phase ($P \leq 0.05$). Also the highest antiviral activity of MOEO was seen when AIV incubated with MOEO before cell infection. The TCID50/ml was reduced 1.3–2.1, 2.3–2.8, 3.7–4.5 log 10 than control group (5.6 log 10), HAU/50 µl was decreased 85–94, 71.4–94, 71.4–94 % and viral genome copy number/µl was brought down 68–95, 90–100, 89.6–99.9 % at pre-infection, post-infection and simultaneous stage, respectively. Hemagglutination inhibition result showed the MOEO was not able to inhibit agglutination of the chicken red blood cell (cRBC). Replication of the AVI was suppressed by the different concentration of oseltamivir completely or near 100 %. Also oseltamivir showed a synergistic activity with MOEO especially when oseltamivir concentration reduced under 0.005 mg/ml. The chemical composition was examined by GC–MS analysis and Its main constituents were identified as monoterpenaldehydes citral a, citral b. In conclusion, the findings of the study showed that lemon balm essential oil could inhibit influenza virus replication through different replication cycle steps especially throughout the direct interaction with the virus particles.

Keywords *Melissa officinalis* · Influenza virus · Oseltamivir · Antiviral · Cell culture

Introduction

The H9N2 subtype virus is a conspicuous member of the influenza family because it can infect not only poultry population but also humans. Although H9N2 subtype influenza A virus has been classified as low pathogenic virus, frequent outbreak of the virus with high mortality has been reported in the broiler farms [22, 23]. Moreover, H9N2 influenza viruses are considered to be one of the most important candidates for the next influenza pandemic. So far several cases of human infection with H9N2 have been reported from Hong Kong and some other Asian countries [2]. Furthermore, the viral genome responsible for human pandemic in the last century, either partially or entirely was originated from avian influenza viruses [24].

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Currently only two classes of antiviral drugs have been approved for the treatment and prevention of influenza: inhibitors of the M2 ion channel (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir and oseltamivir) [9, 32]. Recently, all the conventional drugs that target viral proteins and approved by the Food and Drug Administration (FDA) have been associated with emergence of viral resistance. It has been frequently reported that treatment with the M2 blocking antivirals rapidly results in the emergence of resistant variants [8, 10]. Moreover, it showed that resistance to oseltamivir has been increased during these years [9].

Melissa officinalis (lemon balm) is a member of the Lamiaceae (mint) family. The main components of the essential oil are citral (neral and geranial), citronellal, linalool, geraniol and β-caryophyllene-oxide [29]. Other ingredients are tannins unique to the Lamiaceae, such as triterpenylic acid, bitter principles, flavonoids including phenolic acids, terpenes, rosmarinic acid and caffeic acids, also lemon balm have antioxidant, antihistamine, antispasmodic, anti-tumor/anticancer, antibacterial, antifungal and antiviral properties. The extract of *M. officinalis* has been reported to inhibit protein synthesis and exhibit antiviral action against the Herpes simplex virus type 1 (HSV-1) [5].

In the present study, the *M. officinalis* essential oil components were determined by GC/MS and the antiviral activity of the components was analysed in comparison with oseltamivir against AVI (H9N2) replication in the cell culture. Also the synergistic activity of oseltamivir with MOEO was evaluated. The TCID50, HA, Real Time PCR and HI tests were used to evaluate antiviral activity.

Materials and methods

Reagents

MOEO was prepared in pharmacy department of Shiraz University of Medical Science (The *M. officinalis* leaves with voucher number: 25059, were collected from Iran, Fars Province, Shiraz, 2014–2015 in Shiraz University), Antibiotic, trypsin–EDTA, Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were supplied by Gibco BRL (Grand Island, NY). Oseltamivir (F. Hofmann-La Roche Ltd, Switzerland). Tissue culture plates and flasks were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ). Stock solutions (10 mg/ml) of the compounds were made in dimethyl sulfoxide (DMSO) and were subsequently diluted in appropriate culture media. The final DMSO concentration was a maximum of 0.1 %, which had no effect on the MDCK cell cultures, RNX-Plus kit (CinnaGen Co.), AccuPowder® Rocketscript RT PreMix kit (BioNeer), random hexamer, TaqMan probe, BIO-RAD MiniOpticonTM Syste, buffer, MgCl2, DNA Taq polymerase enzyme, dNTP.

Cells and viruses

Avian influenza A virus, A/chicken/Iran/772/1998(H9N2), which was obtained from Avian Diseases Research Centre of Shiraz University was grown in 9 to 11-day-old embryonated chicken eggs free of H9N2 AIV antibody, after incubation at 37 °C for 2 days the allantoic fluid was harvested and subsequently was used for infection. AVI (H9N2) was propagated and passaged in MDCK cells and titer of infectious virus were measured by 50 % Tissue Culture Infective Dose (TCID50), MDCK cells were grown in DMEM with penicillin (100 IU/ml), streptomycin (100 µg/ml) and supplemented with 7 % heat-inactivated Fetal Bovine Serum.

Cell viability assay

The effect of MOEO and Oseltamivir on the viability of MDCK was determined by 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetra zolium bromide (MTT) assay. MDCK cells were grown (1×10^4 cells/well) in 96-well plate for 24 h. The medium was replaced with DMEM containing different concentration of component (MOEO and oseltamivir). Then the cells were further incubated for 48 h. After incubating the cells for specific time at 37 °C, MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for 2 h. After removal of supernatant, 50 µl of DMSO was added and incubated for 30 min. Next, the absorbance was recorded on a micro plate reader at the wave length of 540 nm [14].

Viral inoculation

Two-day-old MDCK cells monolayers were prepared in 96-well plates in DMEM (containing 100 IU/ml penicillin and 100 µg/ml streptomycin and 7 % FBS at a concentration of 1×10^4 cells per well, when 90 % confluent was obtained, the cells were washed twice with phosphate-buffered saline (PBS) to remove residual FBS and infected with the virus at MOI = 0.01 in different stages and the virus infected cells were incubated in the presence of each compound at different concentrations (0.5–0.005 mg/ml) with trypsin (2 µg/ml) at 37 °C in 5 % CO2 for 48–72 h. Briefly, MDCK cells were infected with influenza A virus A/chicken/Iran/772/1998 (H9N2) and incubated with the MOEO and oseltamivir (0.5–0.005 mg/ml) at different times exposure (1 h pre-infection, 1 h post-infection and simultaneous). In the first set phase of experiment, 100 µl medium containing different concentrations of the lemon

balm essential oil were added to the cells culture. Following 1 h incubation at 37 °C, the supernatants were removed and the cells were washed with PBS. Then, 100 µl of the medium containing virus with trypsin was added to each well. (pre-infection exposure), In the post-infection exposure, 100 µl of the medium containing virus with trypsin was added to the cells culture. Following 1 h incubation at 37 °C, the supernatants were removed and the cells were washed with PBS, then 100 µl of the plant essential oil at different concentrations was added to each well. In the third set of experiment, 100 µl of influenza virus was pre-incubated with 100 µl of different concentrations of MOEO for 1 h, and then the whole (200 µl) medium containing virus and MOEO with trypsin was added to the cell culture (simultaneous).

Combination treatment

In the combination treatment, the virus was inoculated in MDCK cells (MOI: 0.01) for 1 h and then the medium was replaced with DMEM containing combination of MOEO (0.5 mg/ml) and oseltamivir (0.001–0.00005 mg/ml). Also different concentration of oseltamivir (0.5–0.00005 mg/ml) were used as control group. The virus replication was detected by a hemagglutination (HA) assay and the virus titration was carried out by Real Time PCR.

Hemagglutination inhibition assay

Hemagglutination inhibition assay was employed to test the effect of compounds on the virus adsorption into target cells. MOEO solutions (50 µl) with twofold serial dilution with PBS were mixed with an equal volume of influenza virus solution. After incubation for 30 min at room temperature, 50 µl of the solution was mixed with an equal volume of 0.5 % cRBC suspension and then it was incubated for 30 min at room temperature.

Gas chromatography/mass spectrograph (GC/MS) analysis of essential oil

Analyses of the volatile constituents were determined using Agilent 7000 mass spectrometer coupled to Agilent 7890A series gas chromatograph in electron impact mode (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-1MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The GC settings were as follows: initial oven temperature was programmed to increase from 60 to 280 °C at a rate of 3 °C/min and finally held for 4 min. Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The injector temperature was maintained at 250 °C (1). The samples (0.2 µl) were injected neatly with split ratio of 1:30. The transfer line temperature was 280 °C. The mass spectra were recorded over the 46–650 amu range at one scan per second, with an ionizing voltage of 70 eV and an ionization current of 150 mA. The quantitative composition was obtained by peak area normalization. Compounds were identified by comparing their retention times and mass spectra with Adams and Wiley Library [1].

TCID50 method

A standard TCID50 method with a twofold serial dilution was used for virus titration in different experimental stage [27].

Hemagglutination (HA) assay

Standardized chicken red blood cell (cRBC) solution was prepared according to the WHO manual [32]. Virus containing cell culture supernatants was serially diluted twofold and 0.5 % cRBC was added at an equal volume. After 60 min incubation at 4 °C, RBCs in negative wells were settled and formed red buttons, whereas positive wells had an opaque appearance with no sedimentation. HA results are presented as hemagglutination units/50 µl (HAU/50 µl).

RNA isolation

All supernatant samples were immediately frozen following collection and stored at −70 °C until used. RNA of cell culture media samples was extracted using the (CinnaGen Co.) according to the manufacturer's protocol. Briefly, 1 ml of RNX solution was added to 100 µl of cell culture media and mixed well by vortex. After addition of 200 µl chloroform and 15 s shaking, the mixture was centrifuged at 12000 rpm at 4 °C for 15 min. The upper phase was added to equal volume of isopropanol then the mixture incubated on ice for 15 min and centrifuged at 12000 rpm at 4 °C for 15 min to get RNA pellet. The upper phase was removed and 1 ml ethanol (75 %) was added to the tube and centrifuged at 7500 rpm at 4 °C for 8 min for washing step. Then the upper liquid was removed and the precipitate was allowed to dry in room temperature. The pellet was dissolved in a final volume of 50 µl distilled water (DW) and stored at −70 °C until used [21].

Real time PCR

The cDNA was synthesized using AccuPowder® Rocketscript RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's protocol. Reaction was performed with a mixture of 10 µl of total RNA, 20 pmol random hexamer and 20 pmol of forward primer (5′ TCTAACCGAGGTCGAAACGTA 3′) that was specific

to a highly conserved region of matrix protein gene of influenza A virus. The reaction mixture was incubated at 42 °C for 60 min then heated to 95 °C for 5 min, cooled to 4 °C then stored at −70 °C until used.

The quantitative real-time PCR primers (forward: 5′ AAG ACC AAT CCT GTC ACC TCT GA 3′, reverse: 5′ CAA AGC GTC TAC GCT GCA GTC C 3′) and TaqMan probe (FAM 5′ TTT GTG TTC ACG CTC ACC GT 3′ TAMRA) used in this study as described previously [21]. The primers amplified a 104 bp fragment in the M1 gene of influenza A. The probe annealed to the part of the sequence amplified by two primers. The assays were performed on a 48-well microtitration plate of BIO-RAD MiniOpticonTM System. The reaction mixture contained 5 µl of target cDNA, 1 µl of each primer at concentration of 10 pmol/µl, 0.6 µl of the TaqMan probe at concentration of 10 pmol/µl, 0.4 µl of dNTP mix, 2.4 µl of MgCl2 at concentration of 50 mM, 0.2 µl of Taq polymerase enzyme, and 2 µl of ×10 buffer in a final volume of 20 µl. The cDNA was amplified by 40 two-step cycles (15 s at 95 °C for denaturation of DNA, 1 min at 60 °C for primer annealing and extension). Viral cDNA copy numbers (expressed as copies per 1 µg total RNA) were quantified by comparison with a tenfold serially diluted plasmid standard of known concentration. The concentration of the plasmid DNA was calculated with a spectrophotometer. Recombinant plasmids containing 450 bp fragment of matrix protein gene of influenza A, were serially diluted tenfold as a standard in the quantitative real-time PCR, The data and standard curves were obtained during target cDNA and recombinant plasmid amplification [21].

Statistical analysis

Results are depicted as means ± standard deviations for three independent experiments, one-way ANOVA analysis of variance was used to evaluate the difference between the treatments and untreated control. Significant differences among treatments ($P \leq 0.05$) were identified with the Duncan test.

Result

Composition of the essential oil

The chemical composition of lemon balm oil was characterized by GC–MS analysis. The chemical composition of the essential oil extracted from *M. Officinalis* were shown in supplementary material in the Table I. The Geranial (citral A; 26 %) and Neral (citral B; 19 %) were identified as the main components of the *M. Officinalis* essential oil.

Cytotoxicity assay

In order to obtain the safe concentration of MOEO and oseltamivir for MDCK in cell culture, different concentrations of the component were added to MDCK cells using the MTT test. According to the MTT results, MOEO and oseltamivir had no serious effect on viabilities of MDCK cells as the concentration reduced under to 1 and 2 mg/ml respectively.

Antiviral activity in TCID50 results

According to the results, influenza virus replication was suppressed by the different concentration of MOEO in the three experimental stage (1 h pre-infection, 1 h post-infection and simultaneous) and there was significant difference between the three experimental stage.

Overall, the virus titer decreased from 5.6 (TCID50/ml) in the control group of the Pre-infection, Post-infection and simultaneous stage to 0.98–2, 3.1–4.3 and 2.3–3.3 (log 10) respectively. In the post-infection stage, the groups that were received 0.5, 0.1 and 0.05 mg/ml MOEO had higher reduction in TCID50 index than other groups. Also in the simultaneous stage, the groups that receiving 0.5 and 0.1 mg/ml of the MOEO showed to be more effective against virus replication.

The antiviral activity of MOEO in the Pre-infection stage (1 h before inoculation of the virus to cell culture) was obtained to be more effective than other time addition of MOEO to the cells significantly. Furthermore, TCID50 in the groups containing 0.5 and 0.005 (mg/ml) of MOEO were reduced to 0.98 and 1.9 (log 10) respectively ($P \leq 0.05$; Fig. 1).

Antiviral activity in hemagglutination test

The MOEO showed a significant effect against influenza virus replication according to the HA test findings in all experimental stage when compromised to the control group. Moreover, the antiviral activity was not affected by MOEO time addition to the cell culture and HAU unit was decreased by different concentration of MOEO from 42/50 µl (control) under to the 6.7/50 µl in the different groups (Fig. 2; $P \leq 0.05$).

Antiviral activity in real time PCR assay

As presented in the Fig. 3, the results of the Real Time PCR indicated that Influenza virus replication was suppressed by MOEO significantly. The antiviral activity was not affected by MOEO time addition to the cell culture when the experimental stages compared with the control group ($P \leq 0.05$). The virus genome copy number reduced

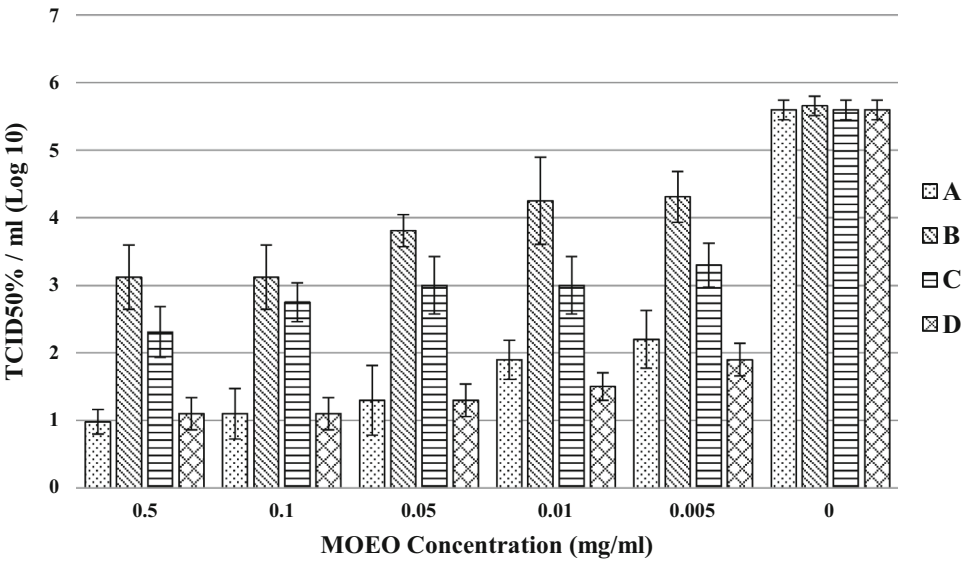

Fig. 1 The AIV (H9N2) titer at TCID50 test (log 10). TCID50/ml was increased as the concentration of *M. Officinalis* essential oil decreased. *a* Adding MOEO at pre infection stage, *b* adding MOEO at post infection stage, *c* adding MOEO and virus at same time, *d* Oseltamivir. Each value is the result of mean ± SD of three independent experiments ($P \leq 0.05$)

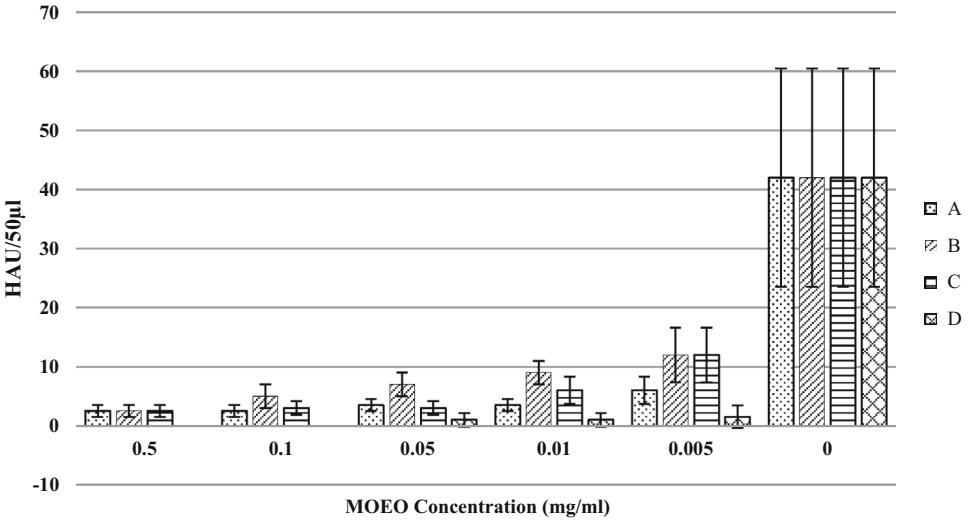

Fig. 2 The AIV (H9N2) titer at HA test (HAU/50 µl). HAU/50 µl was increased as the concentration of *M. Officinalis* essential oil decreased. *a* Adding MOEO at pre infection stage, *b* adding MOEO at post infection stage, *c* adding MOEO and virus at same time, *d* oseltamivir. Each value is the result of mean ± SD of three independent experiments ($P \leq 0.05$)

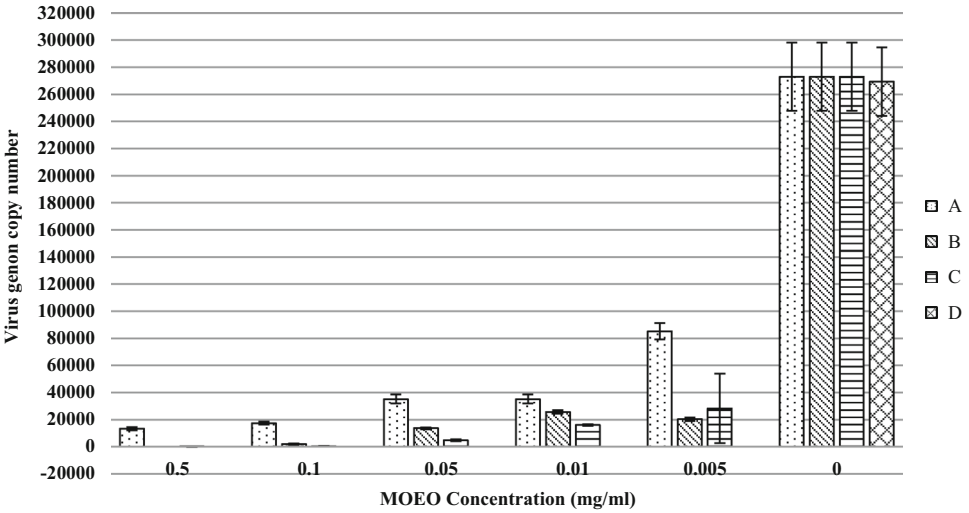

Fig. 3 As indicated, different concentration of *M. Officinalis* essential oil reduced replication AVI (H9N2) as the virus genome copy number/µl decreased using Real Time PCR test. *a* Adding MOEO at pre infection stage, *b* adding MOEO at post infection stage, *c* adding MOEO and virus at same time, *d* oseltamivir. Each value is the result of mean ± SD of three independent experiments ($P \leq 0.05$)

from 2.7×10^5 in the control group under to 4×10^4 in different treatments (Fig. 3). The groups containing 0.5 and 0.1 mg/ml of MOEO at the simultaneous stage (inoculation of the MOEO and virus at the same time to cells) showed the most inhibition on the virus replication. Also this pattern repeated by the groups receiving 0.5 and 0.1 mg/ml of MOEO at the post-infection stage (inoculation of the MOEO 1 h after cell infection with the virus) and the virus genome copy mean number reduced under to 1×10^3/µl at these groups ($P \leq 0.05$). Also antiviral activity followed a MOEO dose dependent pattern.

Oseltamivir efficacy

The cells were infected with influenza A virus (H9N2) and incubated for 1 h, then supernatants were removed and Oseltamivir was added at different concentration (0.5–0.00005 mg/ml) to each well. Virus replication was suppressed by oseltamivir when different concentration of oseltamivir added into the infected MDCK cells. The Evaluation of viral replication inhibition using Real Time PCR indicated that the viral replication was suppressed by different concentrations of oseltamivir. Genome copy number interestingly decreased from 2.7×10^5/µl in control group to 0/µl at the concentrations that including higher than 0.005 mg/ml of oseltamivir.

Also genome copy number reduced under to 1.2×10^4/µl when oseltamivir concentration decreased under to 0.005 mg/ml and it had a significant difference with the control group (Figs. 1, 2, 3).

Antiviral activity in combination treatment

The combination of oseltamivir and MOEO interestingly increased the oseltamivir efficacy on the AIV and the release of virus from infected MDCK cells was reduced by the combination treatment. As presented in the Table 1, virus genome copy number was reduced significantly when different oseltamivir concentrations (0.001–0.00005 mg/ml which was combined with the 0.05 mg/ml of MOEO. For instance, virus genome copy number was reduced under to 12000/µl when infected cells treated by 0.00005 mg/ml of osletamivir. On the other hand, combination of 0.00005 mg/ml with 0.5 mg/ml of MOEO suppressed virus replication completely.

Effects of MOEO on hemagglutination activity

To study the effectiveness of MOEO in preventing the ability of the virus particle to bind to cell surface receptors, the researchers employed hemagglutination inhibition (HI) assays. This study was an attempt to see if MOEO could interfere with the viral adsorption to RBC resulting in hemagglutination inhibition. The result of the study showed that MOEO had no effect on the hemagglutinin protein and the RBC did not show to get cross linked by the virus to form a type of lattice in this case.

Discussion

Antiviral activity of some medicinal plants against influenza viruses such as green tea, pomegranate, licorice root, milk thistle, Echinacea purpurea, have been reported previously due to the safety and development of resistance by influenza viruses [9, 25, 30, 32]. In the present study, for the first time the researchers demonstrated that lemon balm essential oil ingredients inhibit AVI (H9N2) proliferation and mechanism action of MOEO against AIV was evaluated at different steps of influenza virus replication cycle. Also oseltamivir showed synergistic activity against AIV with MOEO.

Interestingly, the data suggested that MOEO acts as an antiviral in different steps of AIV replication cycle. Based on the real time PCR finding, the virus genome copy number was reduced significantly when the MDCK cells were treated by MOEO before inoculation of the AIV to the cells (pre-infection stage). It appears to interfere with the cell surface proteins such as masking the cell surface. Therefore, it can prevent the binding of the virus particle to cellular receptors. Ehrhardt et al. [9] reported the extract of special variant of the Mediterranean plant *Cistus incanus*, CYSTUS052 interferes with the virus surface proteins and inhibits binding of the virus particle to cellular receptors.

Table 1 Combination efficacy of the oseltamivir and MOEO on the copy genome number of the AVI (H9N2)

Component	Concentration (mg/ml)									
Oseltamivir	0.001	0.0005	0.0001	0.00005	–	0.001	0.0005	0.0001	0.00005	0
MOEO	0.5	0.5	0.5	0.5	0.5	0	0	0	0	0
	Real time PCR results									
Genome number/µl	0	0	0	0	180	983 ± 812	2866 ± 162	5512 ± 546	11927 ± 2767	273300 ± 25166

Also Imanishi et al. [17] suggested that catechins interfere with the virus-cell membrane fusion by inhibition of acidification of endosome.

The efficacy of the MOEO ingredient on the cell surface proteins such as, oligosaccharides or sialic acids (SA) that are receptors for influenza viruses, was not described until now. Moreover, the SA is the most important surface cellular protein for adsorption of AIV. Thus, binding MOEO ingredient to the cell surface proteins can cause prevention of the virus adsorption directly or indirectly.

According to the post-infection stage findings (inoculation of the MOEO 1 h after cell infection by AVI), the virus titration was reduced in the TCID50 and HA test. Also the results of Real Time PCR exhibited a significant reduction in virus genome copy number at different concentrations of MOEO comparing to control group and it appears to interfere with intracellular step of the virus replication. The inhibitory effect did not relate to the virus attachment to MDCK cells because MOEO was inoculated to the cells after virus absorption phase (1 h post-infection). Song et al. [31] showed that high concentration of catechins from green tea such as epigallocatechin gallate suppress viral RNA synthesis and neuraminidase activity of Influenza viruses. Influenza virus proliferation was also inhibited by pomegranate components such as punicalagin [14]. Furthermore, Song and Choi [30] indicated that silymarin suppress influenza virus proliferation by viral mRNA synthesis inhibition. In this regard, extracts of *M. officinalis* have been reported to inhibit protein biosynthesis in a cell-free system from rat liver cells, and it was suggested that this effect may be due to its caffeic acid-like components [7].

Moreover, according to the findings of the study at simultaneous stage when AIV was incubated with MOEO for 1 h and then the mixture was inoculated to the cells (inoculation of MOEO and AIV to MDCK cells at same time), MOEO ingredients showed an antiviral activity against AIV. It appeared that AIV was affected by MOEO before adsorption. Also based on the HI results, HA activity was not affected by the essential oil. Therefore, inhibition of viral propagation was not performed throughout the HA inhibition and it suggests other mechanisms such as structural damage to the virion can be responsible for the direct antiviral activity of the MOEO. The inactivation of different influenza virus subtypes like as H9N2 by direct exposure to hydroxytyrosol, a phenolic derivative of the leaves and fruits of olive were associated with damage to virion integrity, but HA and NA functions were unaffected [33]. Furthermore, it has been shown that quercetin 3-rhamnoside inhibits virus replication in the initial stage of virus infection by indirect interaction with virus particles [6]. However, Haidari et al. [14] evaluated the direct effect of a pomegranate polyphenols on influenza virus and observed inactivation was associated with loss of HA activity.

An overall view, as presented in Table 2, showed that there was no significant difference between the experimental stages (pre-infection stage, post-infection stage and simultaneous stage) when their virus genome copy number was compromised to each other. But the lowest virus replication inhibition belonged to the simultaneous stage as the viruses were treated by MOEO before cell infection.

Based on the results of this research, it can be concluding that different ingredients of MOEO may exhibit distinct antiviral mechanisms of action against influenza viruses. It has been reported the extract of lemon balm cannot prevent the entry of Herpes simplex virus type 2 virus to the cells but it acts after penetration of the virus into the host cell [20]. However, typical reduction in viral infectivity was observed when HSV-1 and HSV-2 viruses were treated with lemon balm oil prior to infection, thus a high antiviral activity probably due to direct drug–virus interaction was detected [28]. Moreover, aqueous extract of lemon balm as well as phenolic compounds drastically affected the infectivity of HSV-1 only at the early stage of virus replication [5].

In a GC/MS analysis, the main component of the essential oil was identified as Citral that is a natural mixture of two isomeric acyclic monoterpene aldehydes, geranial (26.8 %), neral (19.1 %) and sesquiterpene such as Caryophyllene-E (11.3 %) and Caryophyllene oxide (11.8 %). Antiviral activity of citral against HSV-1 and yellow fever virus has been demonstrated [4, 13]. Astani et al. [3] observed the high antiviral activity for β-caryophyllene when herpesvirus was incubated with these drugs prior to host cell infection. The exposure of the enveloped viruses such as HSV-1, HSV-2 and dengue viruses to the antiviral components prior to cell infection resulted in prevention of cell infection [3]. On the other hand, in a few studies, the plant antiviral ingredients showed to be effective against the intracellular steps of enveloped viruses such as HSV-1, bovine herpesvirus type 2, human immunodeficiency virus type 1, influenza A virus, influenza B virus, and human respiratory syncytial virus [11, 12, 16, 19].

In accordance with these studies, and in our view, MOEO ingredients can inhibit AIV replication by different mechanisms of action such as masking the host cellular surface protein, intracellular steps and direct virucidal effect by structural damage but not through the HA.

In our knowledge, there is a few study about the oseltamivir efficacy on the AVI subtype H9N2, our experiment has shown that different concentrations of oseltamivir that were used in this experiment inhibit propagation of AVI strongly than the control group. Comparison of MOEO efficacy with to the oseltamivir showed that components of

Table 2 Overall view of the means comparison of different time exposure of MDCK cells to compounds

Experiments	TCID50 (log 10)	HA	Real time PCR
Pre-infection[1]	2.91 ± 0.40^{b}	3.6 ± 1.43^{ab}	36000 ± 28000^{b}
Post-infection[2]	3.72 ± 0.58^{c}	6.7 ± 3.49^{b}	12000 ± 11000^{a}
Synchronize[3]	1.53 ± 0.53^{a}	5.3 ± 3.99^{b}	8990 ± 10000^{a}
Oseltamivir[4]	1.4 ± 0.3^{a}	0.7 ± 0.6^{a}	0^{a}
Positive control[5]	5.66^{d}	42^{c}	273300 ± 25166^{c}
Negative control[6]	0	0	0

The data represent the mean $TCID_{50}$/ml (log 10), HAU/50 µl and virus genome copy number/µl values ± standard deviations overall in every stage

[1] MOEO was added to MDCK cells and after 1 h, the cells washed with PBS then virus was added. [2] Virus was added to MDCK cells and after 1 h, the cells washed with PBS then MOEO were added. [3] MOEO and Virus were added at the same time. [4] Virus was added to MDCK cells and after 1 h, the cells washed with PBS then oseltamivir was added. [5] Virus was added to MDCK cells and after 1 h, the cells washed with PBS then DMEM was added. [6] DMEM was added and after 1 h, the cells washed with PBS then DMEM was added

[a,b,c] Different superscripts denote significant differences among means in column for main effects ($P \leq 0.05$)

lemon balm oil can act as an antiviral substance for treatment and prophylaxis of the influenza viruses. In our pervious study [26], oseltamivir strongly blocked AIV (H9N2) proliferation in the MDCK cells when different concentrations of oseltamivir (0.5–0.005 mg/ml) added to infected MDCK cells [26].

Therefore, in the present study, the synergistic activity of oseltamivir in combination with MOEO was evaluated as the oseltamivir concentration decreased under to 0.005 (mg/ml). The researchers of this study demonstrated that combination of MOEO with oseltamivir increased the inhibitory effect of oseltamivir on AVI especially when the oseltamivir concentration was reduced under to 0.005 mg/ml. Also the viral genome copy number was not detected when 0.5 mg/ml of MOEO added to the groups containing different concentration of oseltamivir. Therefore, it can be concluded that oseltamivir shows synergistic activities with MOEO. It was previously detected that natural products such as polyphenols show synergistic activities with antimicrobial agents [15]. Haidari et al., [14] obtained that combination of pomegranate polyphenol extract and oseltamivir increased the anti-influenza efficacy of oseltamivir synergistically. Several advantages of employing synergistic activities as a kind of therapy and treatment can be mentioned such as higher potential, less toxicity and fewer side effects and the lower cost. Researchers of this study concluded that, MOEO ingredients can act as an antiviral substance with the different mechanisms of action and they believe that the ingredients provide a much higher level of antiviral activity than single drug therapy. Furthermore, the combined use of two or more drugs with different mechanisms of action can potentially reduce the incidence of drug resistance [16, 18].

In conclusion, lemon balm essential oil ingredient seems to act as natural and novel antiviral substance through the different stages of influenza virus (H9N2) replication. Also AIV was suppressed with MOEO before cell infection with the direct drug–virus interaction but not through the HA inhibition. Moreover, in accordance to the results, different mechanisms of action seem to be present. Also a synergistic activity was observed between oseltamivir and MOEO. However, the mechanisms underlying the antiviral efficacy of MOEO against influenza viruses have not been determined completely and it supports the need for further studies to explore the therapeutic and prophylactic potential of lemon balm products in treating influenza A viruses.

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