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In vitro evaluation of antiviral activity of essential oil from *Zataria multiflora* Boiss. against Newcastle disease virus

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ABSTRACT

Introduction: The study aimed to investigate the antiviral activity of *Zataria multiflora* (ZM) essential oil against Newcastle disease virus (NDV) on Vero cells.
Methods: The cytotoxicity of ZM essential oil was evaluated by MTT assay. Cells were infected with 100 $TCID_{50}$ of a field isolate of virulent NDV (*JF820294.1*). ZM essential oil at concentrations of 1/5000, 1/25 000, 1/125 000 or 1/625 000 was added at different times of infection: 60 minutes pre infection, simultaneously and 60 minutes post infection. Cells were evaluated morphologically. The $TCID_{50}$, neutralizing index (NI) and HA titer were determined.
Results: Cells treated with ZM essential oil in all concentrations 1 hour before or after infection, showed CPE similar to control virus cells. In simultaneous use, cells treated with 1/5000 concentration of the essential oil, remained morphologically normal. $TCID_{50}$ values of all treatments were very close to that of control virus except for simultaneous administration at concentration of 1/5000 which was about 1000 folds lower. Virus titer in different treatments was exactly the same as control virus titer in Haemagglutination (HA) test.
Conclusion: ZM essential oil has some antiviral activity on NDV in vitro, which is possibly by destruction of virus infectivity or inhibition of early phases of viral proliferation cycle.

Implication for health policy/practice/research/medical education:
The essential oil of *Zataria multiflora* (ZM) has antiviral activity on Newcastle disease virus (NDV) in vitro and the outcome of the study can pave the road for further investigation on the potential of ZM essential oil as an antiviral agent.
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Introduction

Despite frequent use and global employment of Newcastle disease (ND) vaccines, the disease is still a constant threat to poultry industry worldwide. It imposes a huge economic burden on the world poultry producers, due to loss of birds, diagnostic laboratory tests, preventive measures, etc in intensive farming units (1) as well as domestic village chickens (2). The disease is caused by Newcastle disease virus (NDV) that belongs to the order Mononegavirals, family Paramyxoviridae, genus Avulavirus and is also known as avian paramyxovirus serotype-1 (APMV-1) (3). Traditional antiviral agents are not routinely used in poultry industry due to high toxicity, considerable increase in production costs and above all legislative limitations with regard to drug residues and possible emergence and spreading of resistant strains.

Finding new natural compounds with antiviral properties has become an interesting and promising research area and different plant-derived preparations may be tested for their antiviral effects. *Zataria multiflora* Boiss. (ZM) is a thyme-like plant belonging to the Lamiaceae family that geographically grows wild only in central and southern areas of Iran as well as in Pakistan and Afghanistan and is a valuable medicinal and condimental plant (4). The essential oil of ZM contain significant amounts of thymol and carvacrol, which are well-known antimicrobial and

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antifungal agents (5,6). There are a few controversial reports on the antiviral activity of ZM essential oil against different viruses (7-9). We previously observed that administration of ZM essential oil shortened the faecal virus shedding period in chickens vaccinated with live ND vaccines (10). This motivated us to evaluate whether the essential oil of ZM has direct antiviral activity against NDV. To this end, we investigated antiviral activity of the ZM essential oil against NDV on Vero cell monolayer by different "time of addition" experiments including 1 hour before infection, simultaneously and 1 hour post infection as well as Haemagglutination (HA) test.

Materials and Methods

Essential oil of ZM

Pure ZM essential oil prepared by steam distillation was provided by Barij Essence Pharmaceutical Co. (Kashan, Iran). It contained 30.6% carvacrol and 29.5% thymol as declared by manufacturer.

Cell line and virus

Green African monkey kidney (Vero) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 7% (growing) or 2% (maintenance) fetal calf serum and 125 µl/100ml gentamicin and 50 µl/100ml amphotericin B. The flasks were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Field isolate of virulent NDV (*JF820294.1*) was propagated in 9-day-old chicken embryo egg. Allantoic fluid was harvested and stored at -70°C until use. The virus was passaged 4 times on Vero cells to gain its highest ability for cytopathic effect (CPE) production and was called NDVP4. The log TCID50/mL calculation was based on the Reed and Muench formula (11).

Cytotoxicity assay

The cytotoxicity of ZM essential oil was evaluated by MTT assay kit (Bio-Idea Co., Tehran, Iran). Vero cells were seeded in 96-well plates at an initial density of 5×10^3 cells per 0.1 ml. The cells were incubated with increasing concentrations of essential oil for 72 hours at 37°C and 5% CO_2. One hundred microliters Roswell Park Memorial Institute medium (RPMI) and then 10 µL of MTT solution were added to the cells, which were further incubated for 4 hours. MTT was removed, and 50 µL of DMSO was added for 10 minutes. The optical density was measured at 550 nm. Each experiment was performed in heptoplicate. Tenfold (10^{-1} to 10^{-10}), fivefold (1/5000 to 1/9765625000) and twofold (1/5000 to 1/256000) concentrations of the essential oil were tested. Percentage of cell viability was calculated as [mean absorption of treated cells/mean absorption of control cells] × 100. Concentrations that showed viability percentage above 50% were used for further experiments.

Time of addition assays

Vero cell monolayers cultured in 96-well microtiter plates and were infected with 100 $TCID_{50}$ of NDVP4. 0.1 ml of ZM essential oil at concentrations of 1/5000, 1/25000, 1/125000 or 1/625000 was added at different times of infection: 60 minutes pre infection, simultaneously and 60 minutes post infection. Virus control (positive control) wells (column 9) and negative control wells (columns 10, 11 and 12 as well as row H) which contained only medium without virus or essential oil, were considered in each microtiter plate. Each concentration of ZM essential oil was used in heptoplicate. For each treatment, cells were incubated with ZM essential oil for 1 hour and then washed two times with PBS, and maintenance medium was added. Mono layers were incubated for 72 hours at 37°C and 5% CO_2. Cells were then evaluated morphologically under inverted microscope. The content of each well was removed and used for $TCID_{50}$ and neutralizing index (NI) determination. NI was described as $\log_{10}$ of virus control titer_$\log_{10}$ virus titer in ZM treated wells. NI ≥ 2 was considered as an appreciable virus inhibition (12,13).

Haemagglutination test

The HA method was used as described by Grimes (14). Using a 96-well V-shaped microtiter plate, 50 µL normal saline was added to wells. Consequently 50 µL of medium from virus control or ZM treated wells was added to each well and was serially diluted. Fifty µL of 0.5% chicken red blood cell (RBC) was added to all wells. After 30 minutes, dilution of the last well showing agglutination gave the titer of the virus sample. Haemagglutination units were expressed as HAU/mL.

Results

Morphological features of cells under inverted microscope

As previously stated, after 72 hours of incubation, Vero cells were evaluated under inverted microscope. Control cells had normal morphology while CPE was observed in virus control cells. CPE was characterized by granularity in cytoplasm, dark and small shrunk nuclei and clustering of infected cells (Figure 1).

Cells that were treated with ZM essential oil in all concentrations 1 hour before or 1 hour after infection with VDVP4, showed obvious CPE similar to control virus cells. When ZM essential oil was used simultaneously with virus infection, only cells that were treated with 1/5000 concentration of the essential oil, remained morphologically normal without CPE observation.

$TCID_{50}$ and neutralizing index values

Results of $TCID_{50}$ values as wells as NI related to different treatments are summarized in Table 1. The only treatment that showed NI ≥ 2 was simultaneous administration of ZM essential oil at the concentration of 1/5000. $TCID_{50}$ values of all treatments were very close to that of control virus except for simultaneous administration at concentration of 1/5000 which was about 1000 folds less

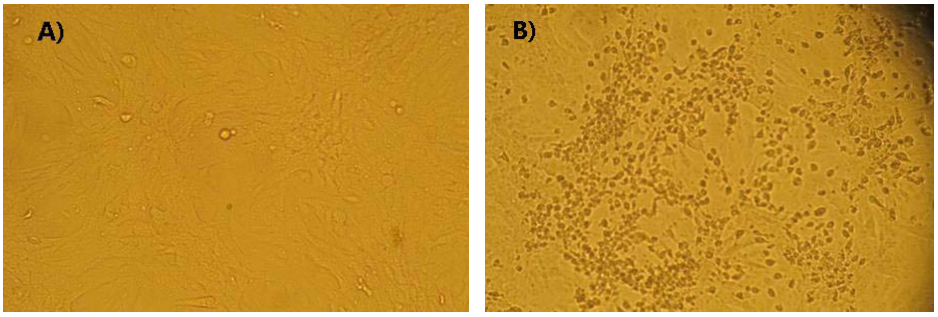

Figure 1. Photomicrographs of A) normal Vero cells (cell control) and B) Vero cells infected with NDVP4 virus (virus control cells) showing CPE (Mag. 100).

Table 1. $TCID_{50}$ and NI values related to different time of addition assays with different concentrations of Zataria multiflora essential oil (ZMEO)

Concentration	Time of administration					
	1 hour pre infection		Simultaneous		1 hour post infection	
	$TCID_{50}$	NI	$TCID_{50}$	NI	$TCID_{50}$	NI
0 (control virus)	$10^{5.85}$	-	$10^{6.17}$	-	$10^{6.31}$	-
1/5000	$10^{5.94}$	-0.09	$10^{2.89}$	3.28	$10^{6.28}$	0.03
1/25000	$10^{5.5}$	0.35	$10^{5.56}$	0.61	$10^{6.35}$	-0.04
1/125000	$10^{5.79}$	0.06	$10^{5.86}$	0.31	$10^{6.13}$	0.18
1/625000	$10^{5.67}$	0.18	$10^{5.74}$	0.43	$10^{6.35}$	-0.04

Abbreviation: NI, neutralizing index.

than virus control.

Virus titer in haemagglutination test

Virus titer in different treatments was exactly the same as control virus titer (HAU/50µl = 8).

Discussion

Like many other viral diseases of the poultry, currently there is no treatment available for ND. Preventive measures especially vaccination against the disease are widely used, however; velogenic strains are still a major threat to poultry production in many countries. Finding safe and efficient antiviral agents with relatively low concerns about potentially hazardous residues in edible tissues of the poultry is a promising aspect of research especially on compounds with natural origins. Aerial parts of ZM is used as a popular condiment in Persian cuisine and also as a traditional folk remedy for its antiseptic, analgesic, carminative, anthelmintic and anti-diarrheal properties. There are few reports about the effect of ZM essential oil on viruses with mostly inconsistent results. Mardani et al (7) reported that essential oil of ZM had a significant inhibitory effect on HSV-1 on Vero cells. In a study by Azizkhani et al (8), ZM essential oil emulsions of 3%, 5% were used by spray on baby-leaf salad which showed no effect on feline calicivirus. Elizaquível et al (9) mixed different concentrations of ZM essential oil with feline calicivirus and murine norovirus and the infectivity of the recovered viruses was evaluated by cell-culture assays. They reported that 2% concentration of ZM essential oil could decrease feline calicivirus titer.

In the present study, we investigated antiviral effects of different concentrations of essential oil of this plant on NDV in Vero cell line by time of addition assay and HA test. We observed that concentrations of 1/5000 (0.02%) and lower of the essential oil had cytotoxicity of less than 50% on Vero cells in MTT assay. This is in compliance with the results of a previous study by Mardani et al that reported a 50% cytotoxic concentration (CC50) of 0.067% for ZM essential oil on Vero cells.

In our study, among the 4 different concentrations of ZM essential oil which was assayed for their antiviral effects, only the highest dose showed about 3 logs (1000 fold) decrease in $TCID_{50}$. In pretreatment assay in which the cells were incubated with essential oil for 1 hour before virus infection, no activity was observed. This may suggest that the compound cannot bind to virus receptors on the cell surface involved in the initial attachment of the virus. However, when essential oil was added to the monolayer during virus adsorption (simultaneously with virus), a marked inhibitory effect due to the highest concentration of ZM essential oil was observed. From these, it may follow that the effect of ZM essential oil on NDV is mostly on early stages of viral life, including entry of the virus to the cells or direct destruction of the virus or its infectivity outside the cell. Moreover, addition of this concentration of ZM essential oil 1 hour post infecting cells had no considerable effect on $TCID_{50}$ values that rules out possible effect of the essential oil on later viral replication phases. It should be recognized that further experiments on separate phases of the viral life cycle as well as a direct

virucidal assay are needed for confirmation.

As previously stated, in our study, the lower $TCID_{50}$ value related to simultaneous administration of 1/5000 concentration was not reflected in HA test and virus titer in different treatments was exactly the same as control virus titer. The HA test does not discriminate between viral particles that are infectious and particles that are degraded and no longer able to infect cells and both can cause the agglutination of RBCs (14). Therefore, the HA titer of virus treated with simultaneous administration of 1/5000 concentration may be due to degraded viral particles. This observation potentiates the suggestion that the lower $TCID_{50}$ value which was observed in this treatment is related to direct virucidal activity of the essential oil or destruction of viral infectivity.

Conclusion

In conclusion, ZM essential oil has some antiviral activity on NDV in vitro which is possibly by destruction of virus infectivity or inhibition of early phases of viral proliferation cycle.

Authors' contributions

The first two authors (AM and NM) had influential roles in conducting the study and data acquisition as well as data analysis. TS prepared the manuscript and proposed the study design. The last two authors (MA and SS) contributed in experments.

Conflict of interests

Authors have no conflict of interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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