

Antiviral activity of water extracts of some medicinal and nutritive plants from the Apiaceae family

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Abstract

During the past two decades, several human infections with avian influenza H5N1 virus have been reported. An increase in the recorded cases of human viral infections led to more public health concern, because of their potential pandemic proportions in the human's society. Moreover, an increase in the cases of drug-resistant influenza A virus has brought the urgent need for alternative anti-influenza drugs. In the present study, water extracts from eight commonly available medicinal and nutritive plants from the Apiaceae family including; Dill, Celery, Caraway, Coriander, Cumin, Fennel, Anise, and Parsley were prepared. The cytotoxicity of each of extract was individually determined in the Madin–Darby canine kidney (MDCK) cells. Thereafter, these extracts were investigated for their *in vitro* antiviral activities against the avian influenza H5N1 virus infection. Current results revealed that water extracts of the eight plants showed antiviral inhibitory activities with percentages ranging from 0- 71 %. Among the tested plants, only anise plant (*Pimpinella anisum*) had significant antiviral activity against the avian influenza H5N1 virus. Thus, the mode of action of this effective anise extract was investigated against the same virus. It was found that water extract of the anise plant induced virucidal effect, as well as direct effect on replication of the avian influenza H5N1 virus. The aim of the present study was to shed light on searching for alternative therapeutic sources for future treatment of the H5N1 influenza virus infection.

Keywords: Avian influenza, Apiaceae family, Antiviral, Medicinal, Cytotoxicity

1. Introduction

Influenza virus belongs to the family Orthomyxoviridae that is classified into four distinct genera mainly; influenza A, B, C and D. Previous studies conducted by [Cao et al., \(2009\)](#); [Hutchinson et](#)

[al., \(2014\)](#); [Kauppila et al., \(2014\)](#); [Li et al., \(2015\)](#) reported that although influenza A, B, and C viruses commonly circulate and cause disease in humans; however, only influenza A viruses (IAVs) pose a

significant threat to zoonotic infections, crossing the host barrier, and causing pandemics. Anti-influenza drugs are needed to overcome the spread of viral infection that could occur even after vaccination, due to antigenic miss matching. Presently, there are two classes of anti-influenza drugs. The first group is virus neuraminidase inhibitor (NAI) such as; Oseltamivir, Zanamivir and Peramivir, whereas the second group is Adamantine M2 ion channel blocker, as Amantadine and Rimantadine. According to [Hurt *et al.*, \(2007\)](#), Oseltamivir is orally administered and is systemically bioavailable. This drug is the main antiviral treatment to the avian influenza H5N1 infections, due to the minimal systemic bioavailability of the inhaled Zanamivir, and the rapid emergence of resistance to the M2 inhibitors.

A study conducted by [Govorkova *et al.*, \(2013\)](#) revealed that specific antiviral drugs can play an important role in the early stages of a pandemic, but the emergence of drug-resistant variants can limit these control options. Previous results of [McKimm-Breschkin *et al.*, \(2007\)](#) showed that resistance to Oseltamivir in clinically derived seasonal IAVs was associated with amino acid substitutions in the neuraminidase (NA).

Egypt is one of the few countries where avian influenza H5N1 virus has become endemic, and has the highest number of human infections. Previous studies of [Earhart *et al.*, \(2009\)](#); [Govorkova *et al.*, \(2013\)](#) demonstrated that some human Egyptian H5N1 isolates had NA mutation, which conferred a 12- 15-fold increase in the IC50 value recorded in the NA inhibition assay. [Hay *et al.*, \(1986\)](#) documented that molecular markers of resistance to Adamantine are amino acid substitutions, at residues of L26, V27, A30, S31, and G34 within the trans-membrane domain of the M2 protein. Recent work of [El-Shesheny *et al.*, \(2016\)](#) confirmed that the Egyptian H5N1 viruses had some molecular resistance markers in the M2 protein that were experimentally confirmed. Limitations of the currently available therapeutics used to control influenza A viruses especially after emerging of resistance strains are the driving forces for the

discovery of novel drugs. Such drugs may act as the first line of defense against the spread of respiratory viral infections, and reduce the time consumed for vaccine development, particularly in a human pandemic setting. The objective of the current study was to investigate the in vitro antiviral efficacy of several water extracts of plants from the Apiaceae family against the avian influenza H5N1 virus.

2. Material and methods

2.1. The influenza virus and the propagating cells

The highly pathogenic avian influenza virus (HPAI) A/chicken/Egypt/M7217B/2013 (H5N1) used in this study, was isolated from infected chickens in Egypt in 2013. This virus was identified and characterized as previously described by [El-Shesheny *et al.*, \(2016\)](#), at the Center of Scientific Excellence for Influenza Viruses, National Research Center, Giza, Egypt. The Madin-Darby Canine Kidney (MDCK) cells were kindly provided by Dr. Richard Webby, St. Jude Children's Research Hospital, Department of Virology and Molecular Biology, USA, as a confluent sheet in 75 cm² tissue culture flasks. These cells were propagated till confluence for several passages, harvested in aliquots and then stored in liquid nitrogen till being used.

2.2. Test plants

Eight common medicinal and nutritive plants belonging to the Apiaceae family were purchased from a herbal store at Giza, Egypt. These plants were characterized and classified according to the United States Department of Agriculture (USDA) natural resources conservation service. All the selected plants were closely related to Angiosperm, and they were purified from the other ambiguous plants.

2.3. Preparation of water extracts from the selected plants

A weight of 3 g of each plant was mixed with 40 ml deionized water, and then boiled for three minutes. Each mixture was covered and then left overnight to

cool at room temperature. The mixture was clarified by centrifugation at 3000 rpm for 3 min., to get each extract in a pure and elegant form. The supernatant was lyophilized in a Petri dish at an oven that was adjusted at 55°C, and then the weight of each extract was determined. Each extract was dissolved in an equal volume (w/v) of deionized water (0.1g/ 0.1ml).

2.4. Detection of cytotoxicity of the prepared water extracts

2.4.1. The MTT assay

Plant extracts were 10-fold serially diluted with Dulbecco's Modified Eagle medium (DMEM). The cytotoxic effect of the extracts were tested in the MDCK cells using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method, according to [Mosmann, \(1983\)](#), with minor modifications.

Briefly, the cells were seeded into 96 well-plates (100 µl/ well at a density of 3×10^5 cells/ ml), and then incubated for 24 h at 37°C in 5 % CO₂. Thereafter, the cells were treated individually with various concentrations of each water extract of all the tested plants, in triplicates. After 24 h of incubating the plates, the media including the extracts were discarded, and the cell monolayers were washed three times with sterile phosphate buffered saline (PBS). Finally, MTT solution (20 µl of 5 mg/ ml stock solution) was added to each well, and then incubated at 37°C for 4 h, followed by medium aspiration. In each well, the formed formazan crystals were dissolved in 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol equivalent to 0.073 ml HCl/ 50 ml isopropanol). Absorbance of the formazan solutions was measured at λ max 540 nm, with 620 nm as a reference wavelength using a multi-well plate reader (FLUOstar, USA).

The percentage of cytotoxicity compared to the untreated control cells was determined using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{(\text{Absorbance of cells without treatment} - \text{Absorbance of cells with treatment}) \times 100}{\text{Absorbance of cells without treatment}}$$

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50 % cytotoxicity (IC₅₀).

2.4.2. The plaque titration assay

The Plaque titration assay was carried out for counting the plaque forming units (PFUs), as previously described by [Tobita, \(1975\)](#). Briefly, six-well tissue culture plates were seeded with MDCK cells (10^5 cells/ well). At 90-100 % confluence (one day post-seeding), the cells were washed twice with PBS. The viruses were 10-fold diluted in DMEM. About 100 µl of the undiluted virus and each dilution were mixed with 400 µl DMEM, and then inoculated into the MDCK cells. Plates were incubated at 37°C for 1 h. After incubation, the wells were aspirated to remove the residual inoculum. Each well was then immediately covered with 2 ml of DMEM overlaying medium containing; 1 % agarose type 1 (Lonza, Basel, Switzerland), 1% antibiotic-anti-mycotic mixture, and 1 µg/ ml of Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin. Plates were then incubated at 37°C with 5 % CO₂ for 2 d. The formation of the plaques was microscopically observed daily. Once clear plaques could be visualized, 1 ml of 10 % formaldehyde was added to each well for 1 h at room temperature, for cell fixation and virus inactivation. The formaldehyde was then discarded; the plates were rinsed with water and then dried. For visualization of the plaques, 1 ml of the staining solution, consisting of 1 % crystal violet and 20 % methanol in dist. water, was added to each well and then incubated at room temperature for 5 min. The dye was discarded and the wells were rinsed with water, and then dried. Viral plaques were then counted, and the virus titer was calculated through the following equation according to [Tobita, \(1975\)](#):

$$\text{Plaque forming unit (PFU)/ ml} = \text{Number of plaques} \times \text{Reciprocal virus dilution} \times \text{multiple and number to complete the inoculum volume to 1 ml.}$$

2.5. Plaque reduction assay to screen the water extracts for their anti-H5N1 activity

The plaque reduction assay was carried out according to the method described by [Hayden *et al.*, \(1980\)](#) in a six well plate, where MDCK cells (10^5 cells/ ml) were cultivated in these plates for 24 h at 37°C. The H5N1 virus was diluted to give countable plaques at the dilution of 10^5 , mixed with the safe concentration of the tested extracts based on the cytotoxicity assay, as shown in the Table (1), and then incubated for 1 h at 37°C before being added to the MDCK cells. The growth medium was removed from the cell culture plates, and then mixtures of the virus-plant extract were inoculated (100 µl/ well). After 1 h of contact time for virus adsorption, 3 ml of DMEM supplemented with 2 % agarose was added onto the cell monolayer; plates were left to solidify, and then incubated at 37°C for 3-4 d till formation of the viral plaques. Formalin (10 %) was added for 2 h, and then the plates were stained with 0.1% crystal violet in dist. H₂O. Control wells were included, where the untreated virus was incubated with MDCK cells. Finally, the plaques were counted, and then the percentage reduction in plaques formation in comparison to the control wells was recorded as the following equation, as previously described by [Tobita, \(1975\)](#):

$$\% \text{ Inhibition} = \frac{\text{viral count (untreated)} - \text{viral count (treated)}}{\text{viral count (untreated)}} \times 100$$

2.6. Mechanism of virus inhibition

The possible mechanism of A/chicken/Egypt/M7217B/2013 (H5N1) virus inhibition by the water extract of anise plant was studied, in reference to three different assumed mechanisms. The anise water extract was tested at four different concentrations mainly; 4, 8, 16, and 32 µg/ µl.

2.6.1. Viral replication

The assay was carried out in a 6 well plate, where the MDCK cells were cultivated (10^5 cell / ml) for 24 h at 37°C. The tested virus was diluted to give 10^5

PFU/ well, applied directly to the cells, and then incubated for 1 h at 37°C. Unabsorbed viral particles were removed by washing the cells three successive times, using supplements of free-medium. Water extract from anise was applied individually at different concentrations ranging from 4 to 32 µg. After 1 h of contact time; about 3 ml of DMEM medium supplemented with 2 % agarose was added to the cell monolayer. Plates were left to solidify, and then incubated at 37°C till appearance of the viral plaques.

Cell monolayers were fixed in 10 % formalin solution for 2 h and then stained with crystal violet. Control wells were included, where the MDCK cells were incubated with the virus. Finally, the plaques were counted and the percentage of reduction in plaques formation was recorded as above mentioned, compared to the control wells, in reference to [Kuo *et al.*, \(2002\)](#).

2.6.2. Viral Adsorption

According to the previous method conducted by [Zhang *et al.*, \(1995\)](#), the MDCK cells were cultivated in a 6 well plate (10^5 cell/ ml) for 24 h at 37°C. Water extract of anise plant was applied individually at different concentrations ranging from 4 to 32 µg in 200 µl of infection medium without supplements, and then co-incubated with the cells for 2 h at 4°C. The unabsorbed extract was removed by washing the cells three successive times with supplements free-medium.

After that, the A/chicken/Egypt/M7217B/2013 (H5N1) virus was diluted to give 10^5 PFU/ well, co-incubated with the pretreated cells for 1 h, followed by adding 3 ml DMEM supplemented with 2 % agarose. Plates were left to solidify, then incubated at 37°C to allow formation of the viral plaques, fixed and then stained. The percentage of reduction in plaques formation was calculated as above mentioned, in comparison to the control wells; where the untreated MDCK cells were directly infected with the A/chicken/Egypt/M7217B/2013 (H5N1) virus.

2.6.3. The virucidal effect

To detect the possible virucidal mode of action of the water extracts, an assay previously reported by [Schuhmacher *et al.*, \(2003\)](#) was followed. This assay was carried out in a 6 well plate, where the MDCK cells were cultivated (10^5 cell / ml) for 24 h at 37°C. A volume of 200 µl of serum free DMEM containing 10^5 PFU of A/chicken/Egypt/M7217B/2013 (H5N1) virus was added to the concentration (ranging from 4 to 32 µg) of the tested extract. After 1 h of incubation, the mixture was diluted three times using serum free medium so that each 10 fold dilution still allows the existence of viral particles to grow on MDCK cells, but leaves nearly no extract. Then, about 100 µl of each dilution was added to the MDCK cell monolayer. After 1 h of contact time, 3 ml of DMEM overlaying medium containing; 1 % agarose type 1 (Lonza, Basel, Switzerland), 1 % antibiotic-anti-mycotic mixture, and 1 µg/ ml of Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, was added to the cell monolayer. Plates were left to solidify, incubated at 37°C to allow formation of viral plaques, and then fixed.

Finally, these plates were stained as above mentioned, to calculate the percentage of reduction in plaques formation compared to the control wells; where cells were infected with the virus that was not pretreated with the tested extract.

3. Results

3.1. The cytotoxicity assay

The MDCK cells were treated with different concentrations of the eight tested plants extract's for 24 h, and then the MTT compound was added on the next day. After addition of the MTT on the cells for 4 h, formazan was formed which is an insoluble crystal precipitate of purple color, which results from the metabolism of the viable cells to the MTT. Therefore, the formazan quantity is directly proportional to the number of viable cells.

The quantity of formazan is measured by recording changes in absorbance at 570 nm, using a plate reading spectrophotometer. There were eight

plant extracts tested during the current study. At least four different concentrations of each tested plant extract were prepared in triplicates. The IC₅₀ was calculated by plotting the concentrations of the plant extract against the percentage of the cytotoxicity. The calculated IC₅₀ for each water extract is illustrated in Table (1). The extracts have a broad range of 50 % toxicity to the MDCK cells that ranged from 621 µg/ µl to >800 µg/ µl.

3.2. Inhibitory potential of the plant extracts against the HPAI H5N1 virus

The plaque infectivity titer of A/chicken/Egypt/M7217B/2013 (H5N1) virus was initially calculated and shown to be 2×10^7 PFUs/ ml.

Water extracts from eight plants from the Apiaceae family were investigated for their in vitro antiviral activities against the avian influenza H5N1 virus infection, using the plaque reduction assay at four different concentrations that illustrated in Table (2). Results revealed that water extracts of the eight plants showed antiviral activities, thus caused viral inhibition by percentage ranged from 0-71 %, as shown in Table (2). Among the tested plants, anise plant extract had significant antiviral activity against avian influenza H5N1 virus recording (71%), followed by fennel (50 %), cumin (44 %), parsley (40 %), caraway (33 %), coriander (33 %), and dill (23 %), at the highest safe concentration of; 32, 32, 6, 32, 16, 8, and 8 µg of each plant extract, respectively. However, the water extract from celery had no antiviral effect against the H5N1 virus, recording 0% viral inhibition. The water extract of anise was tested at four different concentrations mainly; 4, 8, 16, and 32 µg/ µl. The concentrations of 4 and 8 µg/ µl showed 47 % inhibition of the H5N1 virus, while the extract at 16 and 32 µg/ µl showed 71 % viral inhibition.

The IC₅₀ was calculated by plotting the concentration of the anise water extract against the percentage of viral inhibition. From the obtained straight line, the recorded IC₅₀ of the anise extract is 5.5 µg/ µl, as demonstrated in Fig. (1).

Table 1: Cytotoxicity assay of water extracts from the eight tested plants on the MDCK cells, using the MTT assay

Name of the plant extract	IC50 (µg/ µl)
Caraway	> 800
Cumin	621
Parsley	> 800
Fennel	> 800
Anise	> 800
Dill	713
Celery	> 800
Coriander	742

*Where; IC50: The concentration of plant extract which exhibited 50 % cytotoxicity

Table 2: Inhibitory activity of the eight different plant extracts from the Apiaceae family against the HPAI H5N1 virus

No.	Plant extract	Conc. (µg/ µl)	Virus control (PFU/ ml)	Viral count (PFU/ ml)	Viral inhibition (%)
1	Caraway	16	30×10^5	20×10^5	33 %
		8		20×10^5	33 %
		4		30×10^5	0 %
		2		30×10^5	0 %
2	Cumin	6	27×10^5	15×10^5	44 %
		3		27×10^5	0 %
		1.5		25×10^5	7 %
		0.75		29×10^5	0 %
3	Parsley	32	25×10^5	15×10^5	40 %
		16		13×10^5	48 %
		8		15×10^5	40 %
		4		14×10^5	44 %
4	Fennel	32	20×10^5	10×10^5	50 %
		16		9×10^5	55 %
		8		13×10^5	35 %
		4		11×10^5	45 %
5	Anise	32	25×10^5	5×10^5	71 %
		16		5×10^5	71 %
		8		9×10^5	47 %
		4		9×10^5	47 %

6	Dill	8	13×10^5	10×10^5	23 %
		4		12×10^5	8 %
		2		15×10^5	0 %
		1		13×10^5	0 %
7	Celery	8	25×10^5	33×10^5	0 %
		4		28×10^5	0 %
		2		33×10^5	0 %
		1		23×10^5	8 %
8	Coriander	8	15×10^5	10×10^5	33 %
		4		12×10^5	20 %
		2		11×10^5	27 %
		1		14×10^5	7 %

Where; * PFU: Plaque forming unit

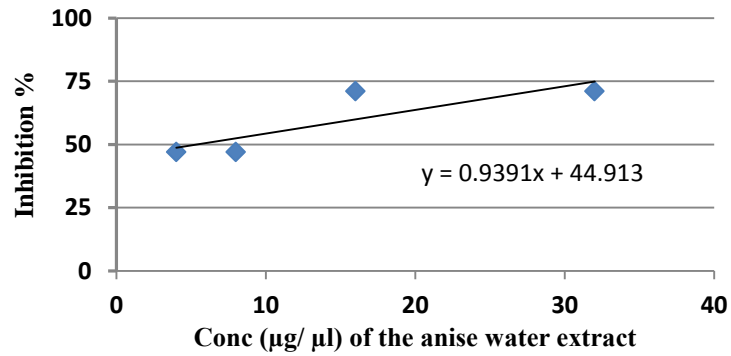


Fig. 1: The effect of different concentrations ($\mu\text{g}/\text{ml}$) of the anise water extract on causing inhibition (%) of the (H5N1) virus. A linear trend line was added to calculate the IC50.

3.3. The mechanism of action of the promising anise water extract against the H5N1 virus

The effective extract of anise plant was tested to determine its antiviral mechanism of action, based on three main possible mechanisms: (i) Inhibition of viral replication and budding. (ii) The ability of the extract to inhibit of attachment of virus to infected cells-membrane fusion known as blocking the viral entry (viral adsorption); and (iii) The direct effect of extract to inactivate the virus viability (virucidal activity). Additionally, the above-mentioned mechanisms could account for the recorded antiviral activities either independently, or in combinations. In this regards, the interaction between the anise plant extract and the H5N1 virus could be explained by means of the following three different mechanisms:

3.3.1. Effect on virus replication

In order to determine the effect of tested extract of anise on viral replication, inoculation of such extract was applied after one hour of the H5N1 virus inoculation. Results demonstrated that the anise water extract had a potent effect on virus replication, giving 94 % inhibition at the highest tested concentration (32 µg/ µl).

3.3.2. Effect on virus adsorption

To examine the possible role of the tested anise extract on the adsorption process, the MDCK cells were subjected to different concentration of water extract for 2 h before inoculation of the H5N1 virus. Results showed that the highest used concentration of the anise extract (32 µg/ µl) has a limited effect, causing 31 % viral inhibition. Accordingly, these results demonstrated that the tested anise extract has no direct effects on the adsorption process of the H5N1 virus.

3.3.3. Virucidal effect

Results of detecting the virucidal mechanism of action of the anise extract against an the AI

A/chicken/Egypt/B13825A/2017 (H5N1) virus, showed that the highest tested concentration of anise extract caused 84% viral inhibition. Thus, it seems that the effect of the anise extract is on the virus itself. In this case, this extract has been described as a virus inactivating agent (Virucidal effect).

4. Discussion

Influenza virus, especially H5N1, remains a major global human health issue, due to the risk of a major pandemic. Therefore, several studies on the inhibitors or vaccines of H5N1 have attracted worldwide attention. Previous study of [Hartley, \(1998\)](#) reported that plants are considered as rich sources of simple phenols, tannins, alkaloids, saponins, lignans, anthocyanins, carotenoids, carbohydrates, flavonoids, isoflavones, phenolic acids and many more compounds. These phytochemical compounds have been proven to be responsible for multiple antimicrobial and antiviral activities.

The Apiaceae family is one of the aromatic flowering plants, and members of this family are widely distributed. The antiviral inhibitory activity of water extracts of eight plants of the Apiaceae family, ranged from 0-71 %. In line with previous studies conducted by [Ohira *et al.*, \(2009\)](#); [Shahrajabian *et al.*, \(2019\)](#), an extract from anise had significant antiviral activation compared with the other tested plants.

In the pharmaceutical industry, shikimic acid was extracted from the Chinese star anise plant by hot water extraction method, as revealed by [Ohira *et al.*, \(2009\)](#). Moreover, shikimic acid is a leader compound in the manufacture of the drug Oseltamivir phosphate, commercially known as Tamiflu, which is an oral antiviral drug used to treat influenza viruses.

Current results demonstrated that fennel plant extract showed 50 % inhibition against the avian influenza H5N1 virus. This is in accordance with the previous results of [Choi, \(2018\)](#), which showed that essential oils extracted from seeds of fennel had

antiviral activity (<50 %) against the human influenza A/WS/33 (H1N1) virus. In addition, previous studies of [Astani *et al.*, \(2011\)](#); [Badgujar *et al.*, \(2014\)](#), showed the antiviral activity of the essential oil extracted from fruit of fennel plant, against Herpes simplex type-1 and para-influenza type-3.

During this study, cumin showed only 44 % of viral inhibition against H5N1 virus. Results of [Mohamadein *et al.*, \(2015\)](#) demonstrated the antiviral activity of 1-(2-Ethyl, 6-Heptyl) phenol, extracted from cumin seeds against the HAV, Cox B4, and HSV-1 viruses. Limited data are associated with the antiviral activities of parsley, coriander, caraway, and dill plants, may be due to their restricted antiviral effects.

Current results showed that water extracts from the natural plants could be used as potential natural antiviral agents. However, future *in vivo* studies are needed to assess the true antiviral activities of these plants, and to determine their possible mode of action in infected animal models.

Conclusion

Water extracts of eight plants of the Apiaceae family showed variable inhibitory activities against the avian influenza virus subtype H5N1. Only the anise plant extract had significant antiviral activity against this virus. Water extract of the anise plant induced virucidal effect, as well as direct effect on replication of the avian influenza H5N1 virus.

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Conflict of interest

The authors declare that there is no any conflict of interests.

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Ethical approval

Ethical approval was obtained from the Local Ethics Committee at the National Research Center, (NRC), Giza, Egypt.

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