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Original Article

Evaluation of Antiviral and Antioxidant Activity of Selected Herbal Extracts

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Abstract

Background: Herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. The major reasons for using medicinal herbs as a good alternative to chemical drugs, is that they have fewer side effects than synthetic drugs.
Objective(s): The main objectives of this study were to investigate the antiviral activity of some popular herbal extracts in Egypt, and to determine their antioxidant activity.
Methods: Experimental studies were carried out in different labs of the National Research Center in Cairo, Egypt. Ethanol extracts of three herbal plants with a history of use in traditional medicine namely; Zingiber officinale (Ginger), Nigella sativa (Black seeds) and Foeniculum vulgare (fennel) were tested for their antioxidant potency and antiviral activity against influenza virus. *In vitro* cytotoxicity assay was made for the extracts by 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay for the determination of the concentration that causes inhibition to half of the viable cells (50% growth inhibition TC50) for each extract and selection of the safe concentration for the antiviral experiment. Antiviral activities were determined by Plaque reduction assay using Madin-Darby canine kidney (MDCK) cell line. Percentage of radical scavenging was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method to evaluate the antioxidant activity of the extracted plants.
Results: Each herb had a certain cytotoxic effect to MDCK cells at the different tested concentrations, TC50 calculated ranged from 50 to 308 µg/µl. The extracts inhibited the growth and development of H5N1 virus in a dose-dependent manner with varying antiviral activity. The most potent viral inhibitor was reported by ethanol extracts of Foeniculum vulgare. All the tested extracts showed high antioxidant activities with some variations ranged from 77.8 to 87.2 percentage scavenging activity at 30 minutes of incubation as determined by the DPPH assay.
Conclusion: Some traditionally used medicinal plants are promising sources for potential antiviral and antioxidant compounds.

Keywords: Zingiber officinale, Nigella sativa, Foeniculum vulgare, cytotoxic, antiviral, antioxidant, Plaque reduction, DPPH, Free radical scavenging.

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INTRODUCTION

Despite the successes in the treatment of some viral diseases during the past three decades, the search for new antiviral drugs remains an area of active investigation. Effective treatment is not available for many viral infections. Moreover, the selection of resistant and cross-resistant mutants caused partially by the narrow spectrum of the mechanism of action, as well as potential toxic side-effects demand the discovery of new drugs.(1)

Influenza A viruses are negative strand RNA with a segmented genome that belongs to the family Orthomyxoviridae. Both influenza A and B viruses can infect humans and cause annual influenza epidemics which result in significant morbidity and mortality worldwide. There are 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of the influenza A virus that infect a wide variety of species.(2)

The introduction of avian virus genes into the human population can happen at any time and may give rise to a new pandemic. There is even the possibility of a direct infection of humans by avian viruses, as evidenced by the emergence of the highly pathogenic avian influenza viruses of the H5N1 subtype that were capable of infecting humans.(3)

There has been increasing interest in the possibility of using herbal extracts as antioxidants. The food and cosmetic industries have attempted to select natural extracts to replace synthetic phenolic antioxidants, such as butylated hydroxyl anisole (BHA) and butylated hydroxyl

toluene (BHT).[4-6] Furthermore, the possible use of natural antioxidants for the prevention of some human diseases has stimulated increasing interest in this field. In fact, it is well known that arteriosclerosis, ischaemia events, human cancer and inflammatory diseases are related to significant exposure of cells to oxidative stress.[7-10]

One of the possible methods which can be used for the discovery of active substances is the screening of plant extracts for their bioactivities followed by bioassay guided fractionation of active extracts to identify the active substance. The selection of the plant species for the present study was mainly based on the common use of these species for the treatment of various diseases, as indicated by traditional health care systems.[11-14]

The main objective of the present study was to investigate the *in vitro* antiviral activity of the following medicinal herbs; Foeniculum vulgare, Nigella sativa and Zingiber officcinale against Influenza virus H5N1 and their antioxidant effect spectrophotometrically.

METHODS

Three replicate samples of each selected plant extract were tested for their virostatic efficacy using serial concentrations of each extract against Influenza virus H5N1. Before evaluating the antiviral activity, the cytotoxic effect of the selected extracts were carried out against MDCK cell line at different concentrations to determine the TC50; the concentration that causes inhibition to half of the viable cells (50% growth inhibition) by MTT assay. Then the nontoxic concentrations calculated were used in the plaque reduction experiment for the determination of the antiviral activity.

- Preparation of the crude herbal extracts

The tested plant materials were obtained from the local herbarium stores. Plant materials consisting of mature seeds of Foeniculum vulgare (Sweet Fennel), Nigella sativa (Black seeds), and rhizomes of Zingiber officcinale (Ginger).

The extraction was done at room temperature. About 500 g of the dried, ground plant materials were soaked in 1 L ethanol (98%) separately for 5-7 days. The soaked material was stirred every 18 h using a sterilized glass rod. The final extracts were passed through Whatman filter paper No.1 (Whatman Ltd., England). The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40 ^{o}C and stored at 4^{o}C for further use. The stock solution of crude extracts was prepared by dissolving a known amount of dry extract in dimethyl sulphoxide (DMSO).[15]

-MTT cytotoxicity assay (TC50)

The tested crude extracts were 10-fold serially diluted with Dulbecco's Modified Eagle's Medium (DMEM) for obtaining the working solutions (100,200,400 and 800 µg/ml) of the extracts.

The cytotoxic activity of each extract was tested in MDCK cells by using the MTT method according to Mossman with minor modifications.[16]

The assay detects the reduction of MTT by mitochondrial dehydrogenase to orange formazan product, which reflects the normal functioning of mitochondrial and cell viability. The amount of the formazan produced is proportional to the number of viable cells.

Briefly, the cells were seeded in 96 well-plates (100 µl / well at a density of 3×105 cells/ml) and incubated for 24 hrs at 37^{o}C in 5 % CO_2. After 24 hrs, cells were treated with various concentrations of the tested compounds in triplicates. After 24 hrs, the supernatant was discarded and cell monolayers were washed with sterile phosphate buffer saline (PBS) 3 times, then MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 hrs followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCL in 50 ml isopropanol). Absorbance of formazan solutions was measured at λmax 540 nm using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

% cytotoxicity = (Absorbance of cell without treatment - Absorbance of cell with treatment) / Absorbance of cell without treatment *100

TC50 for cell growth was calculated from the plot of percentage cytotoxicity versus sample concentration.

-Plaque reduction assay

The assay was carried out according to the method described by Hayden et al., 1980,[17] which is a six well plate where MDCK cells (105 cells/ml) were cultivated for 24 hrs at 37^{o}C. The virus A/CHICKEN/7217B/1/2013 (H5N1) was diluted to give 10^{5}PFU/ well and mixed with the safe concentration of the tested compounds, then incubated for 30 minutes at 37^{o}C before being added to the cells. The growth medium was removed from the cell culture plates, then the virus-extract and Virus-oseltamivir mixtures were inoculated (100 µl / well).

After 1 hour contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose were added onto the cell monolayer, plates were left to solidify and incubated at 37^{o}C till formation of viral plaques (3 to 4 days). After incubation, formaldehyde (10%) was added for two hours then plates were stained with 0.1% crystal violet in distilled water. Control wells were included where untreated virus was incubated with MDCK cells. Finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as following

% inhibition = viral count (untreated) - viral count (treated)/ viral count (untreated) x 100

-Assessment of antioxidant activity

The free radical scavenging activity (RSA) was assessed

by the decolorization of an ethanolic solution of DPPH radical that have been evaluated spectrophotometrically at 517 nm according to Brand-Williams *et al.*[18]
Samples were dissolved in ethanol and the ethanolic DPPH served as a control. An amount of 2 ml DPPH solution and 100 µl (10 mg) of samples were mixed together. The mixture was shaken vigorously and left to stand for 10, 20, 30 and 40 min in the dark, and the absorbance was measured at 517 nm against blank using UV/Visible spectrophotometer 2401 PC (Shimadzu, Kyoto, Japan). The experiment was carried out in triplicate and averaged. The scavenging activity was calculated as follows:
Scavenging ability (%) = ($A_{517\ of\ control}$ - $A_{517of\ sample}$ / $A_{517\ of\ control}$) ×100.

RESULTS

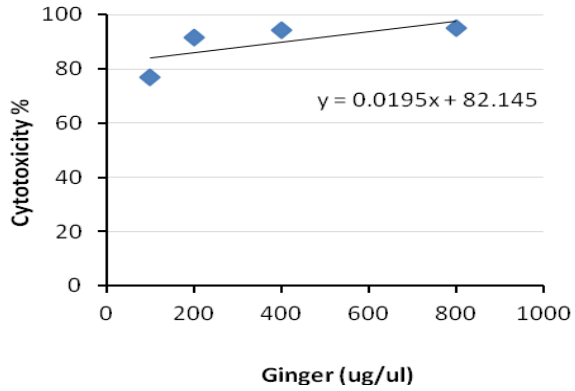
1-Zingiber officcinale

Table 1 shows that Zingiber officinale had a high cytotoxic percent at the lowest dilution used; 100 µg/ml and the percentage of cytotoxicity increased gradually till the highest concentration tested; 800 µg/ml giving 94 percent of cytotoxicity.

Table 1: Cytotoxic activity of Zingiber officinale extracts against MDCK Cells at different concentrations by MTT Assay

Concentration (µg/µl)	Cytotoxicity %	Mean	Control
100	78.8445	0.24767	1.0695
200	91.4920	0.091	1.0695
400	94.4830	0.059	1.0695
800	94.0448	0.053	1.0695

Figure 1 shows the plot between the calculated percentage of cytotoxicity and the concentrations tested of Ginger extract giving the Growth inhibition curve. Regression equation from Excel sheet was used for determining TC50.



TC_{50} = 50 µg/µl

Figure1: Cytotoxic activity of Zingiber officinale in the form of growth inhibition curve

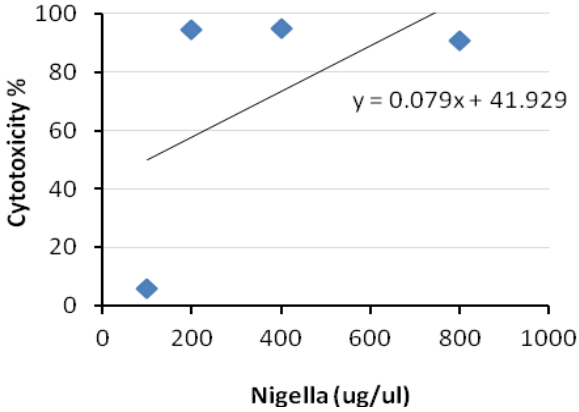
2- Nigella sativa

Table 2 reveals that Nigella sativa had a low cytotoxic effect at the lowest dilution used; 100 µg/ml and the highest effect was determined at the concentration 400 µg/ml giving 95 percent of cytotoxicity.

Table 2: Cytotoxic activity of Nigella sativa extracts against MDCK cells at different concentrations by MTT assay

Concentration (µg/µl)	Cytotoxicity %	Mean	Control
100	5.9755	1.00567	1.0695
200	94.5150	0.05867	1.0695
400	95.0759	0.05267	1.0695
800	90.6194	0.10033	1.0695

Figure 2 shows the growth inhibition curve for Nigella sativa extract; cytotoxic concentration that caused inhibition of 50 percent of the viable cells was calculated from the graph to be 102 µg/ml.



TC_{50} = 102 µg/µl

Figure 2: Cytotoxic activity of Nigella sativa in the form of growth inhibition curve

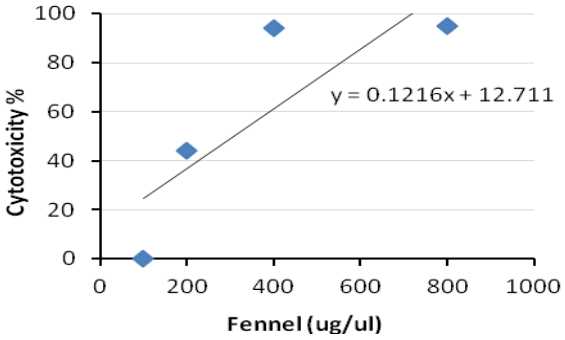
3-Fennel

In Table 3, Foeniculum vulgare had no cytotoxic activity at 100 µg/ml, the percentage of cytotoxicity increased gradually till the highest tested concentration (800 µg/ml) giving 94 percent of cytotoxicity.

Table 3: Cytotoxic activity of Foeniculum vulgare extracts against MDCK cells at different concentrations by MTT Assay

Concentration (µg/µl)	Cytotoxicity %	Mean	Control
100	0	1.09967	1.0695
200	44.1837	0.597	1.0695
400	94.1098	0.063	1.0695
800	94.8890	0.058	1.0695

Figure 3 shows the plot between the calculated cytotoxic percentage and the concentrations tested of fennel extract giving the growth inhibition curve, 308 µg/ml caused cytotoxic effect to 50 percent of the cells.



TC_{50}= 308 µg/µl

Figure 3: Cytotoxic activity of Foeniculum vulgare in the form of Growth inhibition curve

4-Antiviral activity

Table 4 demonstrates the antiviral activity of the selected herbal extracts determined by plaque reduction method. Most of these extracts had partial activity at the low concentration used and the activity was increased by increasing the concentration of the extract. At the non - cytotoxic concentrations used; the ethanolic extract of Zingiber officcinale, had the lowest activity while it was moderate in Nigella sativa with 48 percent activity.

Foeniculum vulgare showed a high percentage of inhibition value at the maximum non-cytotoxic concentration with 83 percentage of inhibition. All these results were compared to Oseltamivir; the current antiviral used.

5-Antioxidant activity

Table 5 shows the antioxidant activity calculated as percentage of radical scavenging activity of the tested extracts at four different interval time (10, 20, 30 and 40 min). The antioxidant activity was increased by gradual increasing the contact time of DPPH.

Table 4: Percentage of inhibition of influenza virus by the tested herbal extracts measured using Plaque reduction assay

Extract name	Conc. µg/µl	Initial viral count	Viral count (PFU/ml)	Percentage of inhibition
Zingiber officcinale	20	29 X 10^5	29 X 10^5	0
	40		27 X 10^5	6.9
Nigella sativa	50	29 X 10^5	24X 10^5	17.2
	100		15 X 10^5	48.3
Foeniculum vulgare	150	29 X 10^5	10 X 10^5	65.5
	300		5 X 10^5	82.8
Oseltamivir	1 µM	29 X 10^5	0	100

Table 5: Antioxidant capacity measured in percentage of scavenging activity of the tested herbal extracts using DPPH method

Extract name	10 min	20 min	30 min	40 min
Nigella sativa	74.9	78.9	83.1	84.4
Foeniculum vulgare	62.4	69.3	77.8	78.5
Zingiber officcinale	79.3	83.1	87.2	88.9

DISCUSSION

The results of this preliminary investigation provide evidence to the importance of ethnopharmacology as a guide for the screening of biologically active plant materials. This study has investigated anti-Influenza (H5N1) activity (*in vitro*) of the hydroethanolic extracts, prepared from some plant species of different botanical families.

To the best of our knowledge, no published results (national or international) were found exactly similar to this antiviral study, concerning the method used for extraction, the antiviral activity screening program, type of cell line, concentrations of the tested extracts and the cytotoxic assay

Percentage of plaque reduction or antiviral potential exhibited variations at the selected herbs compared to Oseltamivir, the potent antiviral for influenza virus. From the tested plant extracts; Zingiber officcinale showed very low activity with 6.9 % of plaque reduction. Nigella sativa had partial activity at higher concentrations with percentage of plaque reduction equal to 48.3 percent. The highest activity was determined by the foeniculum vulgare extracts with 82.8 % of plaque reduction at 300 µg/ml.

Higher results were reported by Chanqet et al.,[19] who investigated the antiviral activity of Zingiber officinale against human respiratory syncytial virus (HRSV) by plaque reduction assay in both human upper (HEp-2) and lower (A549) respiratory tract cell lines. It was found that the hot water extracts of fresh ginger dose-dependently inhibited HRSV-induced plaque formation in both HEp-2 and A549 cell lines, 300 µg/ml fresh ginger could decrease the plaque formation to 12.9% when given before viral inoculation.

Salem et al.,[20] examined the antiviral effect of black seed oil (BSO) from Nigella sativa using murine cytomegalovirus (MCMV) as a model. The results showed that BSO exhibited a striking antiviral effect against MCMV infection, which may be mediated by increasing the innate immunity. The Plaque forming unit (PFU)

decreased from 45x104 to 7 x104 after treatment with BSO.

Another study examined the antiviral activity of the essential oil of fruit sample of Foeniculum vulgare against the DNA virus Herpes simplex type-1. The essential oil displayed strong antiviral effects against HSV, the extracted oil inhibited the virus significantly by a maximum cytopathic inhibitory concentration ranging between 0.8 and 0.025 µg/mL.[21]

Free radicals have been implicated in many disease conditions, especially superoxide radicals, hydroxy radicals, peroxyl radicals and singlet oxygen. Herbal drugs containing free radical scavengers are gaining importance in preventing the deleterious consequences of oxidative stress, and so they may be used as prophylaxis for many diseases. Plant extracts exhibit efficient antioxidant properties due to their phytoconstituents, including phenolics.[22-24]

In this study, the tested plant extracts were evaluated for their free radical scavenging activity using the DPPH radical assay. Reduction of DPPH radicals can be observed by the decrease in absorbance at 517 nm. All the plant extracts exhibited good antioxidant activity. At 30 min of incubation; extracts of Zingiber officcinale and Nigella sativa exhibited 87.2 and 83.1 percent of free radical scavenging respectively. Less activity was determined by Foeniculum vulgare extract with percentage activity of 77.8 percent. These differences in their activities may be due to their different antioxidant mechanisms. Similar studies on plant extracts exhibited nearly similar results.[25, 26]

Thus, it is concluded that these herbal extracts have an important antiviral and antioxidant activity, It is recommended to study their fractionation properties to identify their active agents. Future research in our laboratory is focused on the fractionation of the active extracts and isolation of the active principles (s) from these extracts.

Conflict of Interest: None to declare.

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