Antiviral activity of garlic extract on Influenza virus

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Abstract: Influenza virus is the most important cause of annual morbidities and mortalities worldwide with numerous antigenic drifts and shifts. Inaccessibility to effective drugs and vaccines has made world health authorities to be interested in traditional medicine in order to prevent spread of the infectious agent. Garlic is one of the most famous of all plants in human history. It has been shown that garlic extract has various effects on different diseases. The aim of this study was to evaluate garlic extract antiviral activity against influenza virus in cell culture. To study the potential antiviral activity, MDCK (Madin-Darbey Canin Kidney) cells were treated with effective minimal cytotoxic concentration of the extract and 100 $TCID_{50}$ (50% Tissue Culture Infectious Dose) of the virus during infection at different time periods. The viral titers were determined by hemagglutination (HA) and $TCID_{50}$ assays. The antiviral effect of the extract was studied at 1, 8 and 24 hours after treatment on the culture. To measure the amount of the viral genome synthesized at different times after treatment, RNA extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and free band densitometry software were performed. Although the precise mechanism has not been defined yet, it was found that garlic extract with a good selectivity index (SI) has inhibitory effect on the virus penetration and proliferation in cell culture.

Keywords: Influenza virus • Garlic Extract • MDCK • HA • RT-PCR

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Introduction

Influenza virus is capable of causing respiratory diseases worldwide. This virus is constantly evolving and new antigenic variants give rise to epidemics and pandemics. Influenza virus is unique among respiratory tract viruses because of its considerable antigenic variations. These mutations make it extremely difficult to develop effective vaccines and drugs against the virus (2). Therefore it is an essential need to use some of the traditional medicines and combine them with modern medicine to inhibit the viral activity (10).

Garlic may be one of the most famous herbal remedies to be used by in human history- dating back to ancient cultures. Garlic has been an interesting plant for centuries as a medicinal panacea. Broad range of pathogenic organisms, including bacteria, fungi, protozoa and viruses have been shown to be sensitive to fresh crushed garlic (4). Fresh garlic extract with allicin as the main active component of it has been shown to have antiviral activity *in vitro* and *in vivo*. Its beneficial effects may be due in part to sulfur-containing compounds such as allicine, diallyl disulfide, diallyl trisulfide (6) that react with thiol groups of various enzymes which are critical for microorganism surveillance (1). Influenza virus surface glycoproteins are hemagglutinin (HA) and neuraminidase (NA). HA structure consists of HA_1 and HA_2 subunits linked by disulfide bond. In this study various biochemical, virological, and molecular techniques were used to evaluate the antiviral activity of the garlic extract on Influenza virus.

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Material and methods

Virus

Influenza A/New Caledonia/20/99 (H_1N_1) standard virus was obtained from National Institute for Biological Standards and Control (NIBSC). The virus was grown in MDCK cells in presence of 2 µg/ml of Trypsin TPCK (Sigma, St.Louis, MI).

Cell culture

Continuous MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ICN) containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, Gaithersburg, MD), 100 Units/ml Penicillin G and 100µg/ml Streptomycin (Sigma Co.) at 37°C in a humidified 5% CO_2 incubator.

Garlic Extract

Garlic extract was obtained from Shahed University. Briefly, the fresh garlic bulbs were stripped off the outer theca and then chopped, mixed and sterilized by filtration. The transparent extract was preserved at 4°C for further use.

Cytotoxicity assay

MDCK cells were seeded at 5×10^4 cells/well in 96-well microplates, incubated for 24 h at 37ºC.

Different concentrations of the extract were added to the culture and incubated for 48 h at 37ºC in presence of 5% CO_2.

The 50% cytotoxic concentration (CC_{50}), effective minimal cytotoxic concentration (EMCC) and viability of the cells were defined by the MTT method.

MTT assay

Colorimetric MTT assay was performed according to Levi Raphael, et al [8]. Briefly, yellow color of MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide, Sigma) is changed to purple by mitochondrial succinate dehydrogenase of living cells. The medium of the confluent cells was removed, then 100µl of 1x MTT was added to each well. Following incubation at 37°C with 5% CO_2 for 2 h, 100 µl of acidic isopropanol was added and mixed to release the color from the cells. Optical density at 540 nm was measured using ELISA reader (Stat Fax-200).

Inhibitory effect on the virus titer

Equal volume of Influenza virus (100 $TCID_{50}$) and EMCC of the extract were mixed and incubated at 37°C for 1, 8 and 24 h. They were added to the cells with different multiplicity of infection (MOI). Following one hour incubation at 37°C unabsorbed viruses were removed, then the cells were washed with phosphate buffer saline (PBS) and TPCK-containing medium was added (100µl/well). After 48 h incubation at 37°C, viability of the infected and non-infected cells was evaluated by MTT method. The virus titer was determined by HA assay and Karber formula. The viral RNA was extracted and amplified by RT-PCR and evaluated by band densitometry software.

Percent protection

Percent protection of the extract was calculated by Anova test (Tukey) using viability of mock-infected and infected cells after 48h exposure (8).

Hemagglutination assay

To evaluate presence of the virus in cell culture, serial dilutions of the culture media were added to 96-well U-shape microplates. Chicken red blood cells (cRBCs) (0.5%) were added to each well. Following incubation at least for one hour at room temperature, precipitation of the RBCs demonstrated absence of the virus while hemagglutination indicated presence of the virus (5).

RNA Extraction

Viral genomic RNA was extracted from the infected cells using Viral RNA Extraction Kit (Qiagen,Hilden,Germany). Briefly, 70µl cell culture media was used and RNA was bound to glass fibers fixed in a column and finally was isolated and eluted in 30µl of elution buffer. Since influenza A virus was assessed in this study, influenza B virus was added to the samples as internal control (70µl).

RT-PCR

Complementary DNA of the viral RNA was synthesized by cDNA synthesis Kit (Fermentas,Vilnius, Lithuania). Ten microliter RNA sample along with random hexamer primers and dNTPs mix were incubated at 56°C for 5 min, added to a mixture of RNase free buffer using RT-kit. The cDNA products were stored at -20°C.

PCR

PCR reaction was performed as described by Shahidi, et al (7). Primers used in this study amplified NP gene of influenza B and M gene of influenza A.

PCR products were visualized on 1.5% agarose gel by ethidium bromide staining after electrophoresis.

Semi-Quantitative analysis

The end of exponential phase of the virus amplification cycles by Real-time PCR was determined at 24 cycles. PCR products were measured semi-quantitavely by determining band densitometry ratio using "Image Tool" software.

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Results

Cytotoxicity

The viabilities were evaluated by MTT method. The results showed that Garlic extract had no serious effect on viabilities of MDCK cells at concentration up to 100µg/ml. EMCC of the extract was determined 10µg/ml through MTT standard curve by Anova test (Tukey) (Table1). It did not show any serious cytopathic effects on the uninfected cells as control while reduced CPE of the virus infected cells.

Percent protection

The extract was exposed to the infected cells and after 48h viability of mock-infected and infected cells measured by MTT. Percent protection was obtained by analysis of MTT results using SPSS software version 11.0 (Table 2).

Table 1: Cytotoxicity of Garlic extract on MDCK cells (MTT test)

VIRUS	CC_{50}	EMCC (EC_{50})	SI
A/H_1N_1	100 µg/ml	10 µg/ml	10

CC50: concentration which causes 50% cytotoxic effect
EC50: concentration of the drug required to inhibit 50% of virus-induced CPE
SI: Selectivity Index: CC50/EC50

Table 2: Percent protection results (MTT test). Values are averages of four independent experiments

TIME	MEAN ± SD	
	Virus	Extract treated Virus
1h	0.156±0.018	0.253±0.013*
8h	0.175±0.023	0.275±0.051*
24h	0.168±0.029	0.274±0.063*

*: Significantly different values obtained for extract-treated compared to untreated samples ($p<0.0001$).

It was found that extract-treated virus compared to untreated samples had significantly different MTT results using Anova test (Tukey) analysis ($p<0.0001$).

Hemagglutination assay

Antiviral effect of the extract was determined against influenza A (H1N1) in different sets of experiments. Inhibitory effect of the extract was shown by reducing HA titer (Figure 1 and Table 3).

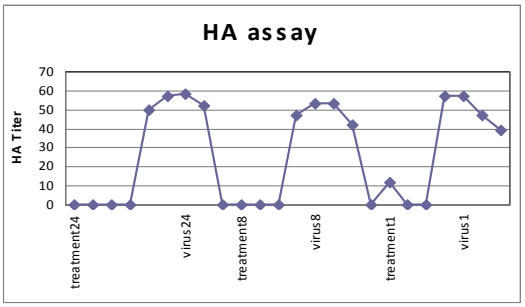


Fig. 1: HA of Garlic extract treated influenza virus A at different time periods.
Effect of the Garlic extract on HA titer of influenza virus at three time points: 1) 24h, 2) 8h, 3) 1h, treatments.

Table 3: Antiviral activity of Garlic extract against influenza virus A (H1N1) by HA test. Values are averages of four independent examinations for HA assay.

TIME	MEAN ± SD	
	Virus	Extract treated Virus
1h	0.156±0.018	0.253±0.013*
8h	0.175±0.023	0.275±0.051*
24h	0.168±0.029	0.274±0.063*

*: Significantly different from values obtained for extract-treated compared to untreated samples ($p<0.05$).

Table 4: Quantitative analysis of RT-PCR products.

TIME	BAND DENSITY (MEAN ± SD)	
	Virus	Extract treated Virus
1h	0.123±0.003	0.077±0.002*
8h	0.184±0.001	0.097±0.001*
24h	0.079±0.001	0.042±0.002*

Gel electrophoresis of RT-PCR products of the viral RNA after exposure to the extract (Mann-Whitney U test) at time points.
Values are averages of four independent examinations for RT-PCR.
* Significantly different values obtained for extract-treated compared to untreated samples ($p<0.0001$).

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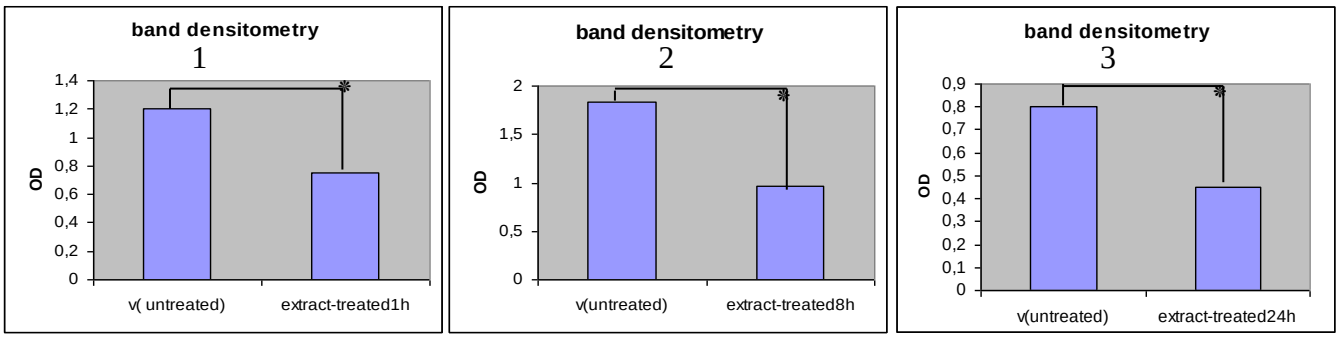


Fig. 2: Semi-quantitative analysis of the virus genome contents before and after treatment with the extract at 3 time points: 1) 1h, 2) 8h, 3) 24h.
*: The results showed meaningful difference ($p<0.05$) between exposed and non-exposed samples.

Semi-Quantitative analysis

The PCR was ceased at this cycle to be able to have semiquantitative results. The effect of the extract on the viral genome was shown by reduction in the content of the PCR products on gel electrophoresis (data not shown). Semi-Quantitative analysis using band densitometry on PCR products showed statically meaningful decrease in genome content after 1, 8 and 24 h direct exposure of the extract to the virus by Mann-Whitney U test. (Fig. 2 and Table 4).

Discussion

As long as influenza virus remains a serious cause of disease, there will be a need to identify and develop some anti-influenza compounds with distinct mechanisms of action (11). Current therapeutic antiviral drugs have limited clinical efficiency and toxic side effects (3). Antiviral agents of plant origin are easily accessible, mostly nontoxic, and inexpensive (10). Garlic, with a long history of traditional medications, is shown to be effective against different microorganisms including viruses. Sulfur containing compounds in garlic extract such as allicine, diallyl disulfide, diallyl trisulfide and others (6) react with thiol groups of various enzymes, e.g. alcohol dehydrogenase, thioredoxin reductase and even disulfide bonds which are critical for microorganism surveillance (1).

We studied garlic extract's inhibitory effect against influenza virus A/H_1N_1. The virus was cultured on MDCK cells. CC_{50} value of this extract was obtained nearly100µg/ml by MTT method. The concentration of 10µg/ml (EMCC) was found to inhibit virus growth to a large extent as was measured by reduction in HA titer and CPE induction.

Inhibition of influenza virus adsorption to MDCK cells and chicken red blood cells by the Garlic extract was estimated by MTT and HA methods. Its effect on the viral genome replication was determined by RT-PCR method. Our results showed concordance among methods used in this study; high percent protection following exposure of Garlic extract to the virus cell culture using MTT method is in concordance with reducing HA titer of the virus cell culture after incubation with this extract. The inhibitory effect of the extract was seen in RT-PCR products, too. By semi-quantitative analysis of PCR products with free band densitometry software, decrease in the content of the treatment bands was obvious in compared with untreated virus band.

For all three time periods of treatments, 1, 8 and 24h exposures, viral infectivity was reduced. Although it was effective and meaningful even after one hour contact compared with 8 and 24h exposures. This may involve interference of viral membrane fusion by inhibition of penetration phase (9).

In conclusion, the biochemical and molecular methods used to evaluate of the antiviral activity of Garlic extract demonstrated that this compound could be suggested as a suitable potent antiseptic agent.

The molecular basis of antiviral effect of Garlic extract on influenza virus would be an interesting field for future studies.

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