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Assessment of direct immunofluorescence assay in detection of antiviral effect of garlic extract on influenza virus

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Influenza virus belongs to *Orthomyxoviridae* family which is considered amongst the most important causes of morbidity and mortality worldwide. Due to the lack of effective drugs and efficient vaccines, exploring for alternative or complementary traditional medicine seems to be crucial in order to prevent the spread of this important threat. In traditional medicine, garlic is one of the most common herbs used in the treatment of various diseases and ailments especially those associated with the respiratory system. In our previous study, we demonstrated significant antiviral activity of garlic extract (GE) on influenza infection using hemagglutination (HA), methyl tetrazolium (MTT) cytotoxity assay and RT-PCR. Current study was designed to evaluate immunofluorescent assay as a reliable and rapid diagnostic test for this infection. An *in vitro* assay was carried out by infecting MDCK cells with H1N1 influenza A virus (100TCID$_{50}$) in the presence/absence of non-toxic concentration of garlic extract (10 µg/ml). The cells were then subjected to fluorescent staining and the fluorescent intensities were evaluated by randomly counting and averaging the 16 fields of each well. The antiviral activity quantification was provided by calculating the proportion of infected cells in which the production of influenza A virus fluorescent antigens was blocked. It was found that rapid direct fluorescent assay has the ability to detect the effect of garlic extract on the replication of influenza A virus at the first steps of infection.

Key words: Influenza virus, garlic extract (GE), fluorescein isothiocyanate (FITC).

INTRODUCTION

Influenza virus is one of the most important pathogens associated with upper respiratory tract infection in almost all age groups, which cause significant morbidity and mortality world-wide (Fedson, 2008; Wilschut, 2009). The activity of possible and potential antiviral compounds may be assessed *in vitro* by measuring their ability to inhibit a stage or enzymatic activity in viral replication cycle (Fedson, 2008; Wilschut, 2009). The activity of possible and potential antiviral compounds may be assessed *in vitro* by measuring their ability to inhibit a stage or enzymatic activity in viral replication cycle (Takeda et al., 2002; Lei et al., 2011). Immunofluorescent (IF) assay has been recommended for rapid *in vitro* and *in vivo* detection of respiratory viruses including influenza virus(Minnich and Ray, 1980; Ray and Minnich, 1987; Takimoto et al., 1991; Miller et al., 1993; Pianciola et al., 2010). Detection of antigen by fluorescein-labeled antibody was first described by Coons et al. (Coons et al., 1941). Studies on

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cell culture combined with IF techniques have been reported previously to detect cytomegalovirus, herpes simplex virus (Gleaves et al., 1985) and influenza A and B viruses (Espy et al., 1986; Swenson and Kaplan, 1987). A study also described a rapid IF procedure for detection of RSV and influenza A virus in parallel (Todd et al., 1995). FITC-conjugated antibodies specific to the virus, bind to antigens expressed on the surface of the virus, therefore fluorescence intensity can be observed in the infected cells using fluorescent microscopy with proper filters (Oxford and Schild, 1968).

The present study describes the use of specific rapid immunofluorescent cell-counting assay to investigate the quality of being fast for antiviral evaluation of garlic extract (GE) on influenza A virus. The proportion of cells expressing influenza antigens following infection of a given cell population in the presence or absence of GE was used to evaluate the antiviral activity of the test compound. Quantification of the antiviral activity was provided by calculating the proportion of infected cells in which the production of influenza A virus fluorescent antigens was blocked by GE.

MATERIALS AND METHODS

Virus sample

Influenza A/New Caledonia/20/99 (H_1N_1) standard vaccine strain was obtained from National Institute for Biological Standards and Control (NIBSC, London). The virus was cultured in Madin-Darby Canine Kidney (MDCK) cells to create working stock. Progeny virus was harvested three days post-infection and stored at -80°C for further use. During antiviral evaluations, FBS was removed and medium was supplemented with 2 µg/ml of Tosylamide, Phenylethyl Chloromethyl Keton-treated Trypsin (Trypsin_TPCK) (Sigma, Missouri, USA). Trypsin-TPCK is used to help the virus penetration by cleavage of the HA0 to HA1 and HA2. HA test using tissue culture infectious dose ($TCID_{50}$) titration in MDCK cells by Karber formula (Karber, 1931) were conducted to measure virus infectivity.

Garlic extract and toxicity effects

The GE used in this study was obtained from Shahed University. Briefly, the fresh garlic bulbs were stripped off the outer theca on crushed ice, chopped, smashed and filter sterilized through sterile 0.22 µm syringe filter and stored at 4°C for further use. The effective minimal cytotoxic concentration (EMCC) of the extract was determined 10 µg/ml as previously reported (Mehrbod et al., 2009). Briefly, confluent MDCK cells in 96-well microplate (Nunc, Denmark) were exposed to different dilutions of GE in triplicates and incubated for 48 h. Colorimetric MTT assay was performed as described elsewhere. The absorbance of the color in the solution was analyzed at 540 nm with plate reader machine (Stat Fax-200, FL, USA) to calculate the 50% cytotoxic concentration (CC_{50}), effective minimal cytotoxic concentration (EMCC) and viability of the cells by two way ANOVA, SPSS.

Antibody

Anti-influenza A/H_1N_1 specific FITC-conjugated antibody was purchased from US Biological Co. (Genentech, USA). This antibody specification was Goat polyclonal anti- influenza A H1N1 (#I7650-83G) with ~4.5 mg/ml concentration. The optimized dilution for this experiment was determined at 1:50 in blocking buffer after different concentrations trials on the infected cell culture.

Direct immunofluorescent assay

A direct immunofluorescent assay was performed according to the guidelines reported by Oxford and Schild (Oxford and Schild, 1968). Briefly, MDCK cells were cultured in 8-well chamber slides (Lab-Tek II Chamber-Slides, Nunc, USA) at 6×10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech Cellgro, USA) containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY, USA), 100 Units/ml Penicillin G and 100 µg/ml Streptomycin (Sigma, St. Louis, Missouri, USA) in 5% humidified incubator at 37°C.

Then, semi-confluent monolayer of MDCK cells were infected with tenfold dilutions of H1N1 at 0.01-0.5 multiplicity of infection (MOI) in the presence and absence of EMCC of GE obtained by MTT assay which had no significant cytotoxic effect on the cell viability shown by inverted microscope. Untreated cells were considered as negative control. Following 1 h incubation at 37°C, the cells were incubated with DMEM containing 2 µg/ml of TPCK following washing with phosphate-buffered saline (PBS) and incubated for 1 h. After removing the chamber compartments, the cells were washed with PBS, fixed in ice-cold acetone and blocked using 3% non-fat dry milk in PBS for 1 h at 4°C and finally labeled with FITC-conjugated antibody (1:50 dilution equal to ~0.1 mg/ml concentration). 1 h incubation at room temperature in dark made the slide ready to examine by fluorescent microscope (Nikon E200) equipped with fluorescein filter (DM 505, EX 450-490, BA 520).

Immunofluorescent cell-counting assay

The Garlic extract effect on the development of influenza A virus propagation in a short time of 1 h incubation time was evaluated using FITC-conjugated anti-influenza antibody. The number of fluorescent intensity was counted in at least 16 randomly selected fields for each well of treatments using the 40X objective. A field of a confluent cell sheet in each well contained approximately 100,000 cells (Oxford and Schild, 1968). Statistical analysis was performed using SPSS 11.0. Data were represented as means ± SD. One-way analysis of variance (ANOVA) post-hoc LSD test was used for data analysis.

RESULTS

MDCK cell monolayer infected with serial ten-fold dilutions of the influenza A virus in the presence and absence of GE in a short time of 1 h was assessed by direct immunofluorescent assay using specific anti-influenza A/H_1N_1 FITC-conjugated antibody and the number of fluorescent cells was counted randomly. Assessing the virus titer from each treatment showed the linear relationship between inoculated virus concentration and the proportion of the percentage of fluorescing cells (Figure 1). Following the combined treatments of the virus in the presence and/or absence of GE, the cells were fixed and processed for direct immunofluorescent staining. The expression of the influenza A virus surface glycoprotein knobs on the infected cells as shown in Figure 2 was obvious after 1 h inoculation time, however,

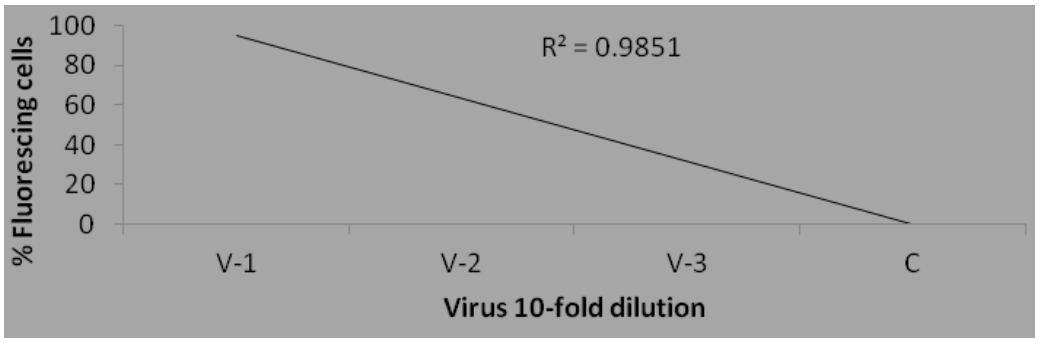

Figure 1. Linear relationship between virus dilution and proportion of fluorescence in 1 h incubation time. The figure clarifies the linear relationship between the virus concentrations and visible fluorescence in different dilutions of the virus inoculated to MDCK cells. V-1, virus dilution 10^{-1}; V-2, virus dilution 10^{-2}; V-3, virus dilution 10^{-3}; C, negative control.

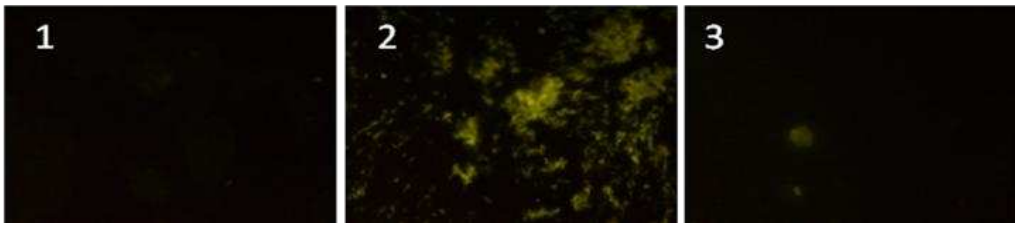

Figure 2. Decrements in viral antigen expression and detection in treated samples as compared to untreated ones by immunofluorescent staining. 1, negative control; 2, virus dilution 10^{-1} without GE; 3, virus dilution 10^{-1} with GE.

this expression was decreased obviously in the presence of GE. (Lower virus titer data not shown).

Statistical analysis from randomly counted data of each treatment using post-hoc LSD test showed significant decrements in combined treatments ($p \leq 0.05$), as compared to the virus inoculated sample (Figure 3). It shows strong antiviral potential of GE against influenza A virus on its HA glycoprotein knobs. Additionally, no viral antigen was visualized in the immunofluorescent staining in negative control sample.

DISCUSSION

Influenza infection is still a major cause of severe respiratory disease with greatly variations in pathogenicity (Lamb and Takeda, 2001; Fedson, 2008). Due to its unpredictability and not having promising antiviral drugs for continuous usage in emergency situations (Pathumwadee et al., 2008), this type of virus requires appropriate, reliable, and safe diagnostic methods that are also accessible to clinical laboratories. Hence, herbal medication is widely used because of the effectiveness, less side effects and low cost. Investigation on traditional medicinal plants has become more important in present time studies on medical sciences. Garlic (*Allium sativum*), a member of the Liliaceae family is one of the most popular herbs since antiquity plays a promising role to reduce various risk factors associated with several diseases with broad-spectrum therapeutic ingredients (Rivlin, 2006; Thomson et al., 2007; Mahesar et al., 2010). Garlic directly kills different types of viruses such as herpes, vaccinia and influenza (Ankri and Mirelman, 1999).

In our previous study, we showed significant antiviral activity of GE on influenza infection by conventional methods especially in the first steps of infection through inhibition of penetration. In that study, we focused on three time points of treatments (1, 8 and 24 h) in cell-free combination of GE (10 µg/ml) and influenza virus ($100TCID_{50}$) (0.5 MOI) in 37°C and finally 1 h incubation with the cell and incubating with TPCK for 48 h. Evaluating the results by HA, MTT and RT-PCR clarified significant preventive effect of GE on the adsorption of the virus particle to the cell by decreasing HA titer, increasing cell viability and decreasing the viral genome level even in short time of 1 h incubation time (Mehrbod et al., 2009). In this regard, we designed the current study to evaluate immunofluorescent assay as a reliable and rapid diagnostic test for this infection for this short time of 1 h incubation.

The present study strongly shows that GE inhibits the formation of influenza A virus antigens which is detectable

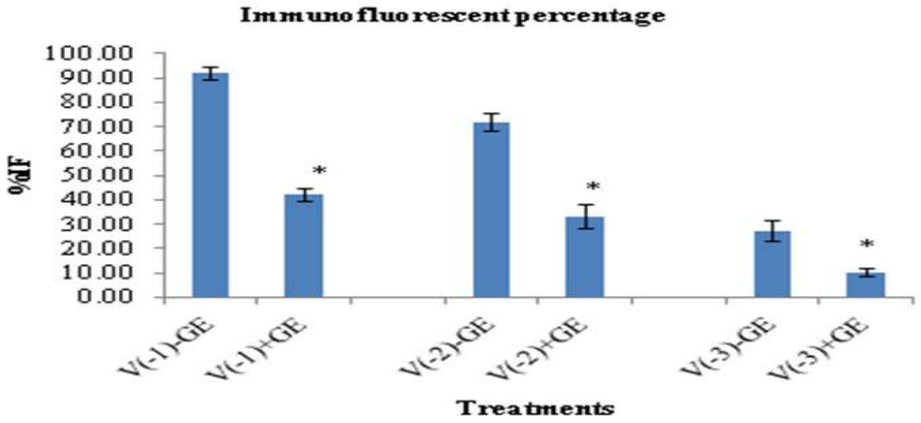

Figure 3. Quantitative results of immunofluorescent assay for the viral antigen detection on the cell surface in presence and absence of GE at 3 different dilutions of influenza A virus: V-1, virus dilution 10^{-1}; V-2, virus dilution 10^{-2}; V-3, virus dilution 10^{-3}; *the results (mean ± SD) showed significant decrements in samples exposed to GE (+GE) in comparison with non-exposed samples (-GE) in each group ($p<0.05$).

by immunofluorescence at very early steps following infection of MDCK cells. It also describes the use of specific immunofluorescent technique to investigate the antiviral activity of the GE on influenza A virus. The advantage of this method is allowing the study of the local effects of this compound in individual cells in a rapid way. Furthermore, using the immunofluorescent cell-counting assay, antiviral activity of the compound could be quantified.

The use of MDCK cells with an immunofluorescent cell-counting technique has been a relatively sensitive and rapid method to investigate the properties and mode of action of certain antiviral compounds since 1960's (Shigeta et al., 1992). It has also been proved for rapid preliminary screening of compounds for virustatic activity (Oxford and Schild, 1967).

Qualitative immunofluorescence techniques were used successfully to investigate the inhibition of Sendai virus (paramyxovirus type 3) by n-isobutylbiguanide hydrochloride (Kashiwazaki and Ishida, 1965) and to study the effect of various inhibitors of DNA viruses on the formation of Adenovirus or SV_{40} virus antigens (Rapp et al., 1965). In addition, the results of a study illustrated that the immunofluorescent techniques are useful for investigation the antiviral activity even in experimental animals (Denk and Kovec, 1967).The present study has shown that FITC-conjugated antibodies directly against type-specific antigens of influenza A virus are useful diagnostic reagents to detect influenza virus by immunofluorescence testing. It was also concluded that chamber slide assay is a rapid, sensitive, and specific technique for diagnosis of influenza virus as well. The proportion of the inoculated cells developing influenza antigens in the presence or absence of GE can be estimated by this assay. The results of this study were in accordance with our previous study. There is good coincidence between the results of the virus load reduction in presence of GE in these two studies.

In conclusion, the method of direct IF staining in combination with immunofluorescent cell-counting appears to produce fast outcome comparable to those obtained by standard and conventional methods. This accelerated method results in a substantial decrement in turn-around times, making it comparable to the most rapid tests and resulting in greater sensitivity based on specific antibodies. Since GE exhibits antiviral effects, it can be introduced as preventive or therapeutic compound in alternative or complementary remediation and it is speculated that the mechanisms of antiviral activity of GE are not limited and most important pathways involved in the pathogenesis of influenza A virus should be considered in future.

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