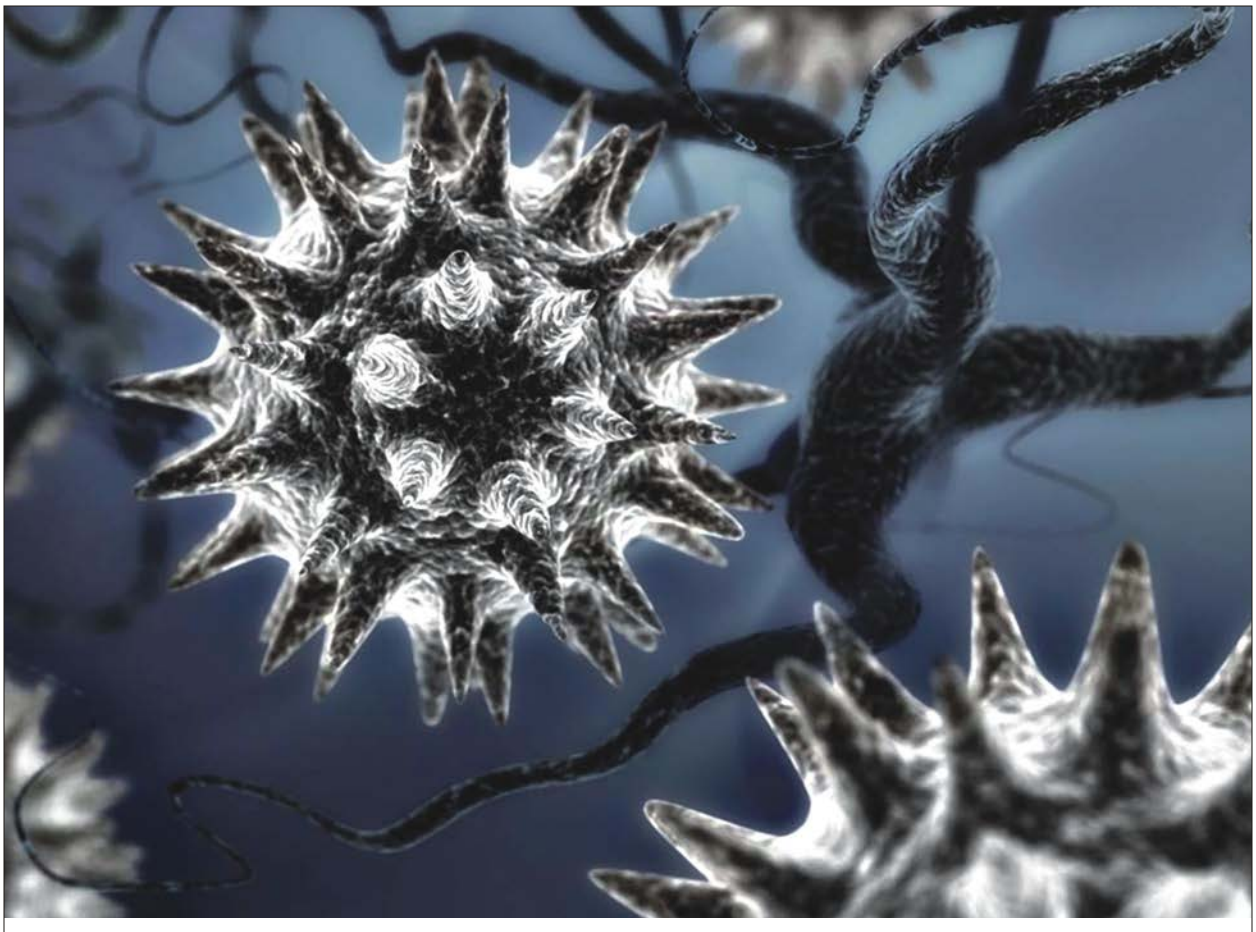

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology 10 (1): 17-27, 2014
ISSN 1816-4900 / DOI: 10.3923/ijv.2014.17.27

In vitro Primary Evaluation of Antiviral Activity of Crude Extract of *Quercus ilex* L. Against Amantadine Resistant *Orthomyxo virus*

[1]Omar M. Yousef, [2]Mohamed El-Raey, [3]Ahmed A. El Sanousi and [3]Mohamed A. Shalaby
[1]WHO Eastern Mediterranean Regional Reference Laboratory For Diagnosis of Polio and Enteroviruses, (VACSERA), Agouza, Cairo, Egypt
[2]Department of Phytochemistry, National Research Center, Dokki, Cairo, Egypt
[3]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza, Cairo, Egypt

Corresponding Author: Omar M. Yousef, WHO Eastern Mediterranean Regional Reference Laboratory For Diagnosis Of Polio And Enteroviruses, (VACSERA), Agouza, Cairo, Egypt

ABSTRACT

In this study, the antiviral effect of holm oak wood extract was investigated against (H9N2) virus, which belongs to the group of influenza (A) viruses that cause serious diseases to human and animal, this *in vitro* study was carried out in comparison to Amantadine HCl (AMN) in addition to extracts of green tea leaves and pomegranate fruit rinds and arils, as these substances have shown previously a considerable antiviral properties against influenza (A) viruses. Initially, cytotoxicity examination was conducted for the crude aqueous methanol extracts (CAMEs) of *Quercus ilex* L. (wood), *Camellia sinensis* (leaves) and *Punica granatum* (rind), besides crude ethanolic extract (CEE) of *P. granatum* (arils) on MDCK cell line. This was followed by evaluation for their *in vitro* antiviral activities against AMN resistant (H9N2) virus infection on the same cell line using MTT based colorimetric assays. Holm oak wood extract Showed visible antiviral effect against (H9N2) *influenza virus* on MDCK cells, where the virus inhibition percentage exceeded 97% at the viral dose of (50 TCID50) and was equivalent for large extent to the antiviral effect shown by green tea extract against the same virus, despite the comparatively more toxic effect of green tea extract on MDCK cells. On the other hand, the pomegranate extracts unexpectedly exhibited much less *Influenza virus* inhibitory effects than the former extract. Based on the results revealed in this study, the virus inhibitory effects shown by the holm oak wood extract, suggest this plant as promising one that need more research to find out its active components that may be useful in the development of new effective antiviral agents.

Key words: Antiviral, *Quercus ilex*, *Punica granatum*, *Camellia sinensis*, *Amantadine*, H9N2

INTRODUCTION

Regarding human and animal health, *Influenza* type A Viruses (IAV) are the most dangerous pathogens among the five genera of *influenza viruses* that belong to the family Orthomyxoviridae (Palese and Shaw, 2007), they are responsible for seasonal flu epidemics as well as serious pandemics that are associated with high morbidity and mortality rates (Memoli *et al.*, 2008; Zimmer and Burke, 2009).

The binding of IAV viral hemagglutinin (HA) to sialic acid receptor on the host cell surface initiates the infection course, followed by receptor mediated endocytosis of the virus particles, with subsequent viral uncoating inside cells, After fusion, the virus shuts off cell replication and cell protein synthesis, consequently, infected cells die by apoptosis or cytolysis (Wu *et al.*, 2010).

The M2 inhibitor, Amantadine (AMN) blocks the ion channel activity of the M2 protein, thus interfering with viral uncoating inside cells; hence, it is one of the very few drugs approved by the FDA to treat *Influenza A virus* infection (Palese 2004; De Clercq, 2006).

Unfortunately, the long history of AMN use was frequently associated with emergence of drug-resistant *influenza* viral variants (Linhares *et al*., 1989). By the end of the last decade, the Centers for Disease Control and Prevention (CDC) reported that 100% of the seasonal H3N2 virus isolate tested were resistant to (AMN) and 99.6% of the seasonal H1N1 viruses tested were resistant to oseltamivir, both antiviral drugs are currently in use for both prophylactic and therapeutic treatments of *Influenza viruses* (CDC, 2009).

Therefore, there has been a continuous need for new antiviral agents to overcome the increasing problem of viral resistance to existing antiviral drugs.

Plants are appropriate candidates for antiviral research programs because they produce a large number of phytochemical substances to adapt themselves to environmental stressors including attack by pathogens and they also have a long history of effective and harmless use as traditional medications against different illnesses including infectious diseases (Dixon, 2001; Guo *et al*., 2006).

Green tea (*C. sinensis*) extracts and derived compounds displayed an evident *in vitro* antiviral activity against different *Influenza viruses* (Song *et al*., 2005, 2007), rotavirus, enterovirus (Mukoyama *et al*., 1991) in addition to Human *Papillomavirus* (HPV) (Gross *et al*., 2007; U.S. FDA, 2008).

It was reported that pomegranate (*P. granatum*) components possess an *in vitro* antiviral activity against HIV-1 (Neurath *et al*., 2004) and more recently reported to inhibit *Influenza virus* replication (Haidari *et al*., 2009; Sundararajan *et al*., 2010).

On the other hand, the reported antibacterial activities exposed by the extracts of *Q. ilex* leaves (Gulluce *et al*., 2004) and *Q. ilex* bark (Berahou *et al*., 2007), is thought to be attributable to the rich polyphenolic content of *Quercus* species extracts including proanthocyanidins, acylated flavonoid glycosides and tannins (Zhentian *et al*., 1999; Meng *et al*., 2001; Ito *et al*., 2002), However, no information has been reported including antiviral activity of *Q. ilex* extracts against *Influenza viruses*.

In this study, we aimed to evaluate the antiviral effect of *Q. ilex* (wood) extract against H9N2 *Influenza virus* reference strain (*Influenza A/turkey/Wisconsin/1/1966*) infection in (MDCK) cells, compared to Amantadine HCl as a Standard anti-*Influenza A antiviral* drug besides the extracts of *C. sinensis* and *P. granatum* which both are two edible plants of previously reported anti-Influenza virus capabilities.

MATERIALS AND METHODS

Cell line: A continuous cell line of MDCK (Madin-Darby Canine Kidney cells), was obtained from cell culture department of the holding company for biological products and vaccines (VACSERA), Agouza, Cairo, Egypt. Cells were grown in DMEM supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B. The cells were incubated at 36°C in 5% CO_2 humidified atmosphere and were subcultured twice a week.

Virus: A low pathogenic *Avian influenza* A virus H9N2 reference strain (*Influenza A/turkey/Wisconsin/1/1966*) was used with stock solution infective titer of $10^{4.8}$

$TCID_{50}$ mL^{-1} (50% tissue culture infective doses), it was kindly provided by Prof. Ahmed EL-Sanousi, Prof. of virology, Faculty of veterinary medicine, Cairo University, Cairo, Egypt. Virus was stored in small aliquots in -70°C freezer until use.

Reagents: DMEM, Dulbecco's Modified Eagle's Medium, (Lonza®, Belgium), was used as: Growth medium (+10% Fetal Bovine Serum, FBS), or as maintenance medium (+2% FBS); FBS, (Gibco®, invitrogen™, USA); Antibiotic mix 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B. (SERVA® Electrophoresis GmbH, Germany).

MTT, [3-(4,5-dimethylthiazol-2ol)-2,5 diphenyl tetrazolium bromide)], (SERVA® Electrophoresis GmbH, Germany). Crystalline Trypsin = 2500 USP U mg^{-1}, solid, (from bovine pancreas), (Sigma Aldrich® Co., St. Louis, USA).

DMSO, distilled Dimethyl SulfOxide (SERVA® Electrophoresis GmbH, Germany).

Standard antiviral: Amantadine Hydrochloride obtained from (Sigma Aldrich® Co., St. Louis, USA) used as a standard antiviral drug against *Influenza A virus* at dose of 25 μg mL^{-1} (AI-Jabri *et al*., 1996).

Plant materials: Holm oak (*Quercus ilex*), a whole branch of the evergreen tree of *Q. ilex* grown in El-Orman National Herbarium, El-Orman Gardens, Dokki, Cairo, Egypt.

Green tea, (*Camellia sinensis*), commercial EL-MAABAD® Chinese pure green tea, purchased from the famous HARRAZ herbal shop, Bab El-Khalck, Cairo, Egypt. Pomegranate, (*Punica granatum*), the whole fruits, available at conventional fruit shops, Cairo, Egypt.

Extract preparation: The crude aqueous extracts of *Q. ilex*, *C. sinensis* and pomegranate rinds were prepared as follows:

Fifty grams of the grinded dried part of the plant (Table 1) was firstly dissolved in 250 mL of de-ionized bi-distilled water and then boiled for 2 h, the outcomes were filtered on filter paper and evaporated to dryness under vacuum at 40°C using rotatory evaporator, the dried aqueous solutions were re-extracted by methanol to get rid of dust residues and undesirable compounds, then extracts were preserved at 4°C until use.

In case of *Pomegranate arils*, extraction process was done as follows:

Fifty grams of the detached arils were soaked in 250 mL of absolute ethanol to get rid of free sugars for one day, filtered using filter paper, dried under vacuum, preserved at 4°C until use.

Different plant extracts were redissolved later (just before use) in sterile PBS (or in dist. DMSO [max. concentration = 0.2%] in case of *Q. ilex* wood extract) and subsequently diluted in cell culture medium (Table 1) and sterilized by filtration using (0.22 mm filter) just before testing.

Table 1: Four plant extracts used in antiviral assay

Latin name	Family	Common name	Part used	Extract
Camellia sinensis	Theaceae	Green tea	leaves	Aqueous methanolic
Punica granatum	Lythraceae	Pomegranate	Fruit arils	Ethanolic
Punica granatum	Lythraceae	Pomegranate	Fruit rinds	Aqueous methanolic
Quercus ilex	Fagaceae	Holm oak	Wood	Aqueous methanolic

Virus titration: Colorimetric H9N2 virus Titration was done on MDCK cells according to Levi *et al.* (1995) with minor modifications. Briefly, Cells were seeded in growth medium in 96-well flat bottom Microtiter cell culture plates (cell star®, greiner bio-one®, Germany), at a density of 15000 cells/well and then incubated at 36°C in a humidified atmosphere containing 5% CO_2 for 24-48 h, then ten-fold serial dilutions of virus stock were prepared, when cells showed 70-90% confluence, growth medium was removed and cells were infected with 50 μL of each dilution of the virus, after 1-2 h adsorption period, 200 μL of maintenance media (devoid of FBS and supplemented with 2.5 μg mL^{-1} of Crystalline Trypsin) were added to each well and plates were put in incubator again.

Uninfected cells incubated only with DMEM were used as negative control cells. The cytopathic effects were observed daily for 5-6 days, then MTT assay was performed as described by Mosmann (1983), Changes occurred to the monolayers of cells were detected by measuring the formed (MTT) formazan crystals Optical Density (OD) within each well using microplate ELISA reader (Biotech® international, USA) at a wavelength of 540 nm.

Positive wells were those with an OD value lower than the cutoff value which was determined as the mean OD of the uninfected control wells minus 2×SD (i.e., standard deviation), whereas, negative wells were those with an OD value = The cutoff value according to Levi *et al.* (1995). The 50% tissue culture infective dose (TCID50) per mL was calculated as described previously by Reed and Muench (1938).

Moreover, a confirmatory Cell culture Heamagglutination virus titration was done for H9N2 virus on MDCK cells as follows:

Cells were seeded in growth media in 96-well U-shaped bottom cell culture plates (cell star®, greiner bio-one®, Germany) at a density of 15000 cells/well and then incubated at 36°C in a humidified atmosphere containing 5% CO_2 for 24-48 h, when cells showed 70-90% confluence, growth media were removed, then tenfold serial dilutions of virus stock were prepared and cells were infected with 50 μL of each dilution of the virus, after 1-2 h adsorption period, 200 μL of maintenance media (devoid of FBS and supplemented with 2.5 μg mL^{-1} of Crystalline Trypsin) were added to each well and plates were put in incubator again.

Uninfected cells incubated only with DMEM were used as a negative control. Throughout incubation, the cytopathic effect was recorded daily for 5-6 days.

Then, 50 μL of 1% chicken RBCs solution was added to each well of the plate to detect the incidence of heamagglutinating influenza viruses within each well and then the plates were left for 15-20 min in room temperature, the end point was the virus dilution which induced (+ve) complete haemagglutination (lattice shape) or (-ve) no haemagglutination (button shape) of RBCs, the 50% tissue culture infective dose (TCID50) per mL was then calculated as described previously by Reed and Muench (1938).

Cytotoxicity assay: The assay was performed using 96-well microtiter plates seeded with 15000 MDCK cells/well and after 24-48 h of incubation at 36°C in a humidified 5% CO_2 atmosphere, cells were treated with decreasing concentrations of plant extracts (2 fold serially diluted).

Monolayers of cells incubated with DMSO were considered as solvent controls whereas, monolayers of cells incubated only with DMEM were used as cell controls.

Cytotoxic changes occurred to the monolayers of cells during the next 5-6 days were daily observed microscopically and subsequently detected colorimetrically using MTT assay by measuringthe formed MTT formazan crystals within each well using microplate reader at a

wavelength of 540 nm as described above to determine the maximum non-toxic concentration (MNTC) of each extract to MDCK cells.

Antiviral assay: MDCK Cells were seeded in growth media in 96-well flat bottom cell culture plates at a density of 15000 cells/well and then incubated at 36°C in a humidified atmosphere containing 5% CO_2 for 24-48 h.

A tenfold serial dilution of virus stock was carried out, when cells showed 70-90% confluence, growth media were removed and cells were infected with 50 µL of each dilution of the virus, after 1-2 h adsorption period, 200 µL of maintenance media containing the MNTC of each of the used extracts were added to each well (or maintenance media without extract were added as negative cell controls) and plates were put in incubator again for 5-6 days.

During incubation, the virus cytopathic effect (CPE) was daily observed microscopically and at the end of incubation period MTT assay was done according to Mosmann (1983). Where viral CPE was detected colorimetrically by measuring the formed XTT formazan crystals (i.e., OD) within each well using microplate reader at a wavelength of 540 nm.

Controls consisted of untreated infected cells (virus control), treated non-infected cells (extract control), untreated non-infected cells (cell control) and cells incubated only with DMSO were considered as (solvent control).

Viral inhibition rate (IP %) was calculated from the equation:

$$\left[\frac{(\text{Odtv} - \text{ODcv})}{(\text{ODcd} - \text{ODcv})}\right] \times 100\%$$

where, ODtv, ODcv and ODcd indicate the optical density of the extract treated infected cells, the optical density of the untreated virus infected control and the optical density of the cell control, respectively.

RESULTS

The results of Cytotoxicity assay of plant extracts on MDCK cells; showed that the maximum non toxic concentrations were 100 µg mL^{-1}, 12.5 µg mL^{-1}, 62.5 µg mL^{-1} and 250 µg mL^{-1} for *Q. ilex*, *C. sinensis*, pomegranate rinds and pomegranate arils, respectively (Table 2).

Remarkably, the solvent, DMSO at the used concentration (i.e., 0.2%) showed no toxicity on MDCK cells.

The titer of the used H9N2 virus was measured by two methods:

- The MTT microculture virus titration (MCVT) assay method (Fig. 1, 2), the result obtained was that the virus used in this experiment has a titer of $10^{4.732}$ $TCID_{50}$ mL^{-1}

Table 2: Result of cytotoxicity assay of different plant extract on MDCK cells

Plant extract	MNTC (µg mL^{-1})
Q. ilex (wood)	100.0
C. sinensis (Leave)	12.5
P. granatum (rinds)	62.5
P. granatum (arils)	250.0

(MNTC): Maximum non toxic concentration

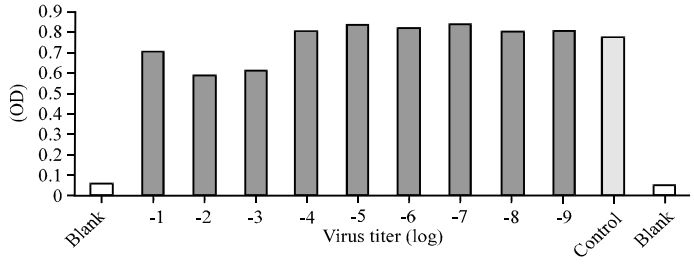

Fig. 1: Microplate reader mean absorbance results of virus titration assay of tenfold serially diluted H9N2 virus on MDCK cells (using MTT assay) (OD), absorbance

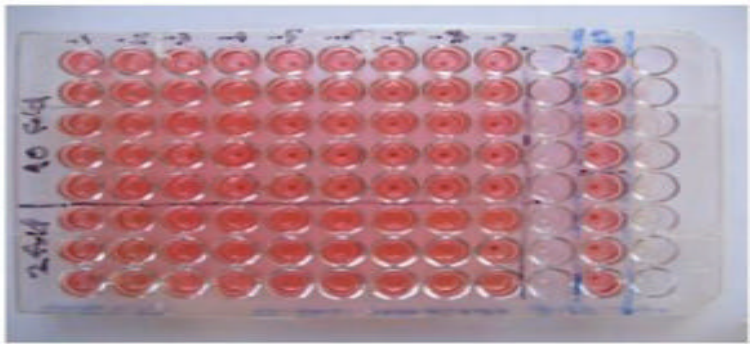

Fig. 2: Cell culture HA results of virus titration assay of tenfold serially diluted H9N2 virus on MDCk cells (using 1% RBCs solution), button shape (-ve) heamagglutination, lattice shape (+ve) heamagglutination

Table 3: Result of antiviral assay of different plant extracts and Amantadine against H9N2 *Influenza virus* reference strain (influenza A/turkey/Wisconsin/1/1966) infection in MDCk cells

	Virus inhibation percentage (IP %)	
Antiviral candidates	5 $TCID_{50}$	50 $TCID_{50}$
Q. ilex (wood)	-	++++
C. sinensis (Leave)	+	++++
P. granatum (rinds)	-	-
P. granatum (arils)	++++	-
Amantadine Hcl	-	-

-: IP<25%, +: IP = 25-50%, ++: IP = 50%, +++: IP = 90-96%, ++++: IP≥97%, $TCID_{50}$: 50% tissue culture infective doses

- The cell culture heamagglutination (CCHA) virus titration method (Fig. 1) the result obtained was that the virus used has a titer of $10^{4.8}$ $TCID_{50}$ mL^{-1}

However, an unexpected higher mean absorbance in case of 10^{-1} dilution compared to the 10^{-2} dilution and the next higher dilutions was seen (Fig. 1).

Regarding the antiviral assay, both *Q. ilex* (wood) extract and *C. sinensis* (leaves) extract showed potent antiviral effects (IP = 97 %) against H9N2 virus at virus dose of 50 $TCID_{50}$, (Table 3).

However, pomegranate arils extract demonstrated a potent antiviral effect too (i.e., IP = 97%) against H9N2 *Influenza A* virus, only shown at virus dose of 5 $TCID_{50}$ (Table 3).

In case of pomegranate rinds extract no touchable antiviral effect was observed (Table 3).

AMN used with the virus infected cells exhibited a very poor antiviral effect (i.e., IP<25%).

DISCUSSION

Although, Investigation of the antiviral potential of various promising plants was difficult in the past, in the last three decades, strategies for the *in vitro* evaluation of plant derived compounds with biological activity have progressed, along with the development of automated antiviral bioassay that depends on colorimetric quantification of the proliferating cell cultures (Mosmann, 1983; Weislow *et al*., 1989; Mukhtar *et al*., 2008).

Plant extracts used in herbal medicine were recently estimated by the WHO to cover the health needs of more than two thirds of the world's population, including the treatment of viral diseases (Robinson and Zhang, 2011) and they have been suggested to be useful to overcome the growing problem of viral resistance to the available synthetic antiviral drugs (Chung *et al*., 1995; Vlietinck *et al*., 1995).

Plant family Fagaceae includes the genus *Quercus* which contain so many species, some of which are reported to have anthelmintic, antibacterial, antioxidant and antiviral activities (Konig *et al*., 1994; Hussein *et al*., 2000; Andrensek *et al*., 2004; Gulluce *et al*., 2004; Muliawan *et al*., 2006; Berahou *et al*., 2007).

However, no information including antiviral activity of *Q. ilex* (wood) extracts has been reported against influenza viruses.

The aim of this work was to evaluate in a colorimetric way the *in vitro* antiviral potential of *Q. ilex* (wood) extract against H9N2 *Influenza virus* infection in (MDCK) cells.

The H9N2 virus titer revealed by (CCHA) virus titration method confirms the accuracy of the result of colorimetric MTT (MCVT) method as the titers revealed by both methods were nearly identical; this also prove the previously mentioned findings of Parida *et al*. (1999) who stated that MTT assay is superior to conventional MCVT method.

However, an unexpected higher mean absorbance in case of 10^{-1} dilution compared to the 10^{-2} dilution and the next higher dilutions was seen (Fig. 1), which could be attributable to the presence of large amount of defective interfering particles (Von Magnus particles) that interfere with the infective H9N2 virions leaving the MDCK monolayer more intact than in the next higher virus dilution as mentioned previously by Nakajima *et al*. (1979).

The pomegranate arils extract was found to have the least toxicity on MDCK cells (Table 2). agreeing with the recent report of Sundararajan *et al*. (2010) who reported the relative safety of pomegranate extracts on MDCK cells, while on the other hand the highest relative toxicity was seen in case of *C. sinensis* extract which may be attributable to its different chemical structure, especially tannin content.

Indeed, the extract of pomegranate rinds resulted in more toxicity on MDCK cells in comparison to pomegranate arils extract, this may be due to its higher tannins content, in agreement with Tzulker *et al*. (2007) who found that pomegranate rinds are much abundant in hydrolysable tannins (mainlypunicalagin) content compared to pomegranate arils.

Remarkably, the solvent, DMSO at the used concentration (i.e., 0.2%) showed no toxicity on MDCK cells.

According to Hussein *et al.* (2000) and Simoni *et al.* (2007) viral inhibition percentage, (IP)≥97% is regarded as potent antiviral activity; (IP between 90-96%) is regarded as reasonable antiviral activity and (IP less than 90%) is regarded as poor antiviral activity.

The *Q. ilex* (wood) extract showed a potent antiviral effect (IP≥97%) against H9N2 virus at virus dose of 50 $TCID_{50}$, (Table 3), which perhaps attributable to this extract content of polyphenolic compounds (mostly ellagitannin), lignans and other organic compounds, which are abundant in oak wood as previously reported (Conde *et al.*, 1997; Fernandes *et al.*, 2009; Michel *et al.*, 2011).

Similarly, *C. sinensis* extract showed an output equivalent to that of *Q. ilex* as it showed a potent antiviral effect (i.e., IP≥97%) against H9N2 virus at challenging dose of 50 $TCID_{50}$, (Table 3), this antiviral effect apparently agrees with previous studies that described the antiviral properties of green tea against influenza A viruses on MDCK cells (Shimamura and Hara, 1991; Nakayama *et al.*, 1993; Song *et al.*, 2005, 2007).

Notably, despite of showing a potent antiviral effect against H9N2 *Influenza A* virus equivalent to that of *C. sinensis* extract, the (wood) extract of *Q. ilex* has an edge over the former as it exhibited much less cytotoxicity on MDCK cells (Table 2).

However, pomegranate arils extract demonstrated a potent antiviral effect too (i.e., IP≥97%) against H9N2 *Influenza A* virus, agreeing with the recent reports of Haidari *et al.* (2009) and Sundararajan *et al.* (2010) who described the antiviral effects of pomegranate extract on MDCK cells against influenza A viruses, but in actual fact, this antiviral effect is unlike that of *Q. ilex* and *C. sinensis* extracts against the same virus as it was only shown at lower H9N2 virus dose = 5 $TCID_{50}$ (Table 3), this could be caused by different antiviral mode of action of each of these plant extracts which may be caused by their different extract constituents.

Unfortunately, in case of pomegranate rinds extract no antiviral effect was observed (Table 3) disagreeing with the report of Jassim and Naji (2003) who reported the antiviral effect of pomegranate rind extract.

On the other hand, the AMN used with the virus infected cells exhibited a very poor antiviral effect (i.e., IP<25%) demonstrating that the H9N2 virus used in this work was reasonably resistant to AMN, this apparently agrees with the report of Weinstock and Zuccotti (2009) who mentioned that influenza viruses have a confirmed capacity to develop resistance to the AMN and other available anti-influenza medications in addition to the recent report of CDC (2009) which declared that 100% of the seasonal H3N2 virus isolates tested were resistant to the Adamantanes including AMN.

CONCLUSION

This preliminary work is the first report on *Q. ilex* L (wood) extract antiviral activity against H9N2 *Influenza A* virus which was found to be as effective as green tea (leaves) extract, while being less toxic to MDCK cells, further research is highly recommended to explore the active components of this plant which may be valuable in the development of new antiviral agents effective against influenza viruses.

ACKNOWLEDGMENTS

We are grateful to Prof. Hamdallah Zidan, Dr. Laila Bassiony and Dr. Eman El Maamon Nasr, (VACSERA), Agouza, Cairo (Egypt), for facilities and support they offered to us during this study.

REFERENCES

AI-Jabri, A.A., M.D. Wigg and J.S. Oxford, 1996. Initial *in vitro* Screening of Drug Candidates for their Potential Antiviral Activities. In: Virology Methods Manual, Mahy, B.W. and H.O. Kangro (Eds.). Academic Press, New York, pp: 293-308.

Andrensek, S., B. Simonovska, I. Vovk, P. Fyhrquist, H. Vuorela and P. Vuorela, 2004. Antimicrobial and antioxidative enrichment of oak (*Quercus robur*) bark by rotation planar extraction using ExtraChrom®. Int. J. Food Microbiol., 92: 181-187.

Berahou, A., A. Auhmani, N. Fdil, A. Benharref, M. Jana and C.A. Gadhi, 2007. Antibacterial activity of *Quercus ilex* bark's extracts. J. Ethnopharmacol., 112: 426-429.

CDC, 2009. 2009-2010 Influenza season week 50 ending. December 19, 2009. http://stomachflusymptomsguide.blogspot.com/2009/12/fluview-week-50.html

Chung, T.H., J.C. Kim, M.K. Kim, S.C. Choi and S.L. Kim *et al.*, 1995. Investigation of korean plant extracts for potential phytotherapeutic agents against B-virus hepatitis. Phytotherapy Res., 9: 429-434.

Conde, E., E. Cadahia, M.C. Garcia-Vallejo, B.F. de Simon and J.R.G. Adrados, 1997. Low molecular weight polyphenols in cork of *Quercus suber*. J. Agric. Food Chem., 45: 2695-2700.

De Clercq, E., 2006. Antiviral agents active against *Influenza A* virus. Nat. Rev. Drug Discov., 5: 1015-1025.

Dixon, R.A., 2001. Natural products and plant disease resistance. Nature, 411: 843-847.

Fernandes, A., I. Fernandes, L. Cruz, N. Mateus, M. Cabral and V. de Freita, 2009. Antioxidant and biological properties of bioactive phenolic compounds from *Quercus suber* L. J. Agric. Food Chem., 57: 11154-11160.

Gross, G., K.G. Meyer, H. Pres, C. Thielert, H. Tawfik and A. Mescheder, 2007. A randomized, double-blind, four-arm parallel-group, placebo-controlled Phase II/III study to investigate the clinical efficacy of two galenic formulations of Polyphenon E in the treatment of external genital warts. J. Eur. Acad. Dermatol. Venereol., 21: 1404-1412.

Gulluce, M., A. Adiguzel, H. Ogutcu, M. Sengul, I. Karaman and F. Sahin, 2004. Antimicrobial effects of *Quercus ilex* L. extract. Phytother. Res., 18: 208-211.

Guo, J.P., J. Pang, X.W. Wang, Z.Q. Shen, M. Jin and J.W. Li, 2006. *In vitro* screening of traditionally used medicinal plants in China against enteroviruses. World J. Gastroenterol., 12: 4078-4081.

Haidari, M., M. Ali, S.W. Casscells III and M. Madjid, 2009. Pomegranate (*Punica granatum*) purified polyphenol extract inhibits *Influenza virus* and has a synergistic effect with oseltamivir. Phytomedicine, 16: 1127-1136.

Hussein, G., H. Miyashiro, N. Nakamura, M. Hattori, N. Kakiuchi and K. Shimotohno, 2000. Inhibitory effects of sudanese medicinal plant extracts on hepatitis C virus (HCV) protease. Phytother. Res., 14: 510-516.

Ito, H., K. Yamaguchi, T.H. Kim, S. Khennouf, K. Gharzouli and T. Yoshida, 2002. Dimeric and trimeric hydrolyzable tannins from *Quercus coccifera* and *Quercus suber*. J. Nat. Prod., 65: 339-345.

Jassim, S.A. and M.A. Naji, 2003. Novel antiviral agents: A medicinal plant perspective. J. Applied Microbiol., 95: 412-427.

Konig, M., E. Scholz, R. Hartmann, R. Lehmann and H. Rimpler, 1994. Ellagitannins and complex tannins from *Quercus petraea* bark. J. Nat. Prod., 57: 1411-1415.

Levi, R., T. Beeor-Tzahar and R. Arnon, 1995. Microculture virus titration-a simple colourimetric assay for *Influenza virus* titration. J. Virol. Methods, 52: 55-64.

Linhares, R.E., M.D. Wigg, M.H. Lagrota and C.M. Nozawa, 1989. The *in vitro* antiviral activity of isoprinosine on simian rotavirus (SA-11). Braz. J. Med. Biol. Res., 22: 1095-1103.

Memoli, M.J., D.M. Morens and J.K. Taubenberger, 2008. Pandemic and seasonal influenza: Therapeutic challenges. Drug Discovery Today, 13: 590-595.

Meng, Z., Y. Zhou, J. Lu, K. Sugahara, S. Xu and H. Kodama, 2001. Effect of five flavonoEid compounds isolated from *Quercus dentate* Thunb on superoxide generation in human neutrophils and phosphorylation of neutrophil proteins. Clin. Chim. Acta, 306: 97-102.

Michel, J., M. Jourdes, M.A. Silva, T. Giordanengo, N. Mourey and P.L. Teissedre, 2011. Impact of concentration of ellagitannins in oak wood on their levels and organoleptic influence in red wine. J. Agric. Food Chem., 59: 5677-5683.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.

Mukhtar, M., M. Arshad, M. Ahmad, R.J. Pomerantz, B. Wigdahl and Z. Parveen, 2008. Antiviral potentials of medicinal plants. Virus Res., 131: 111-120.

Mukoyama, A., H. Ushijima, S. Nishimura, H. Koike, M. Toda, Y. Hara and T. Shimamura, 1991. Inhibition of rota virus and entero virus infections by tea extracts. Jpn. J. Med. Sci. Biol., 44: 181-186.

Muliawan, S.Y., L.S. Kit, S. Devi, O. Hashim and R. Yusof, 2006. Inhibitory potential of *Quercus lusitanica* extract on dengue virus type 2 replication. Southeast Asian J. Trop. Med. Public Health, 37: 132-135.

Nakajima, K., M. Ueda, and A. Sugiura, 1979. Origin of small RNA in von magnus particles of *Influenza virus*. J. Virol., 29: 1142-1148.

Nakayama, M., K. Suzuki, M. Toda, S. Okubo, Y. Hara and T. Shimamura, 1993. Inhibition of the infectivity of *Influenza virus* by tea polyphenols. Antiviral Res., 21: 289-299.

Neurath, A.R., N. Strick, Y.Y. Li and A.K. Debnath, 2004. *Punica granatum* (Pomegranate) juice provides an HIV-1 entry inhibitor and candidate topical microbicide. BMC Infect. Dis., Vol. 4. 10.1186/1471-2334-4-41

Palese, P., 2004. Influenza: Old and new threats. Nat. Med., 10: 82-87.

Palese, P., and M.L. Shaw, 2007. Orthomyxoviridae: The Viruses and their Replication. In: Fields Virology, Knipe, D.M. and P.M. Howley (Eds.). Lippincott, Williams and Wilkins, Philadelphia, PA., pp: 1647-1689.

Parida, M.M., G. Pandya, R. Bhargava, R. Bhattacharya and A.M. Jana, 1999. Comparison of two colorimetric assays to determine viral infectivity *in micro* culture virus titration. Indian J. Exp. Biol., 37: 1223-1226.

Reed, L.J. and H. Muench, 1938. A simple method of estimating fifty percent endpoints. Am. J. Epidemiol., 27: 493-497.

Robinson, M. and X. Zhang, 2011. The World Medicines Situation 2011 -Traditional Medicines: Global Situation, Issues and Challenges. 3rd Edn., WHO Press, Geneva, Switzerland.

Shimamura, T. and Y. Hara, 1991. Preventive and curative medicament against infection with *Influenza virus*, containing tea or tea polyphenols. European Patent EP 417385 (A2).

Simoni, I.C., A.P.S. Manha, L. Sciessere, V.M.H. Hoe, V.H. Takinami and M.J.B. Fernandes, 2007. Evaluation of the antiviral activity of Brazilian cerrado plants against animal viruses. Virus Rev. Res., Vol. 12.

Song, J.M., K.H. Lee and B.L. Seong, 2005. Antiviral effect of catechins in green tea on *influenza virus*. Antiviral Res., 68: 66-74.

Song, J.M., K.D. Park, K.H. Lee, Y.H. Byun and J.H. Park *et al.*, 2007. Biological evaluation of anti-influenza viral activity of semi-synthetic catechin derivatives. Antiviral Res., 76: 178-185.

Sundararajan, A., R. Ganapathy, L. Huan, J.R. Dunlap, R.J. Webby, G.J. Kotwal and M.Y. Sangster, 2010. *Influenza virus* variation in susceptibility to inactivation by pomegranate polyphenols is determined by envelope glycoproteins. Antiviral Res., 88: 1-9.

Tzulker, R., I. Glazer, I. Bar-Ilan, D. Holland, M. Aviram and R. Amir, 2007. Antioxidant activity, polyphenol content, and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. J. Agric. Food Chem., 55: 9559-9570.

U.S. FDA, 2008. Veregen (kunecatechins) ointment 15%. http://www.fda.gov/cder/foi/label/2006/021902lbl.pdf.

Vlietinck, J., L. van Hoof, J. Totte, A. Lasure, D.V. Berghe, P.C. Rwangabo and J. Mvukiyumwami, 1995. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J. Ethnopharmacol., 46: 31-47.

Weinstock, D.M. and G. Zuccotti, 2009. The evolution of influenza resistance and treatment. JAMA, 301: 1066-1069.

Weislow, O.S., R. Kiser, D.L. Fine, J. Bader, R.H. Shoemaker and M.R. Boyd, 1989. New soluble-formazan assay for HIV-1 cytopathic effects: Application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Nat. Cancer Inst., 81: 577-586.

Wu, S., K.B. Patel, L.J. Booth, J.P. Metcalf, H.K. Lin and W. Wu, 2010. Protective essential oil attenuates *Influenza virus* infection: An *in vitro* study in MDCK cells. BMC Complementary Alter. Med., Vol. 10. 10.1186/1472-6882-10-69

Zhentian, L., J. Jervis and R.F. Helm, 1999. C-Glycosidic ellagitannins from white oak heartwood and callus tissues. Phytochemistry, 51: 751-756.

Zimmer, S.M. and D.S. Burke, 2009. Historical perspective-emergence of *Influenza A* (H_1N_1) viruses. N. Engl. J. Med., 361: 279-285.