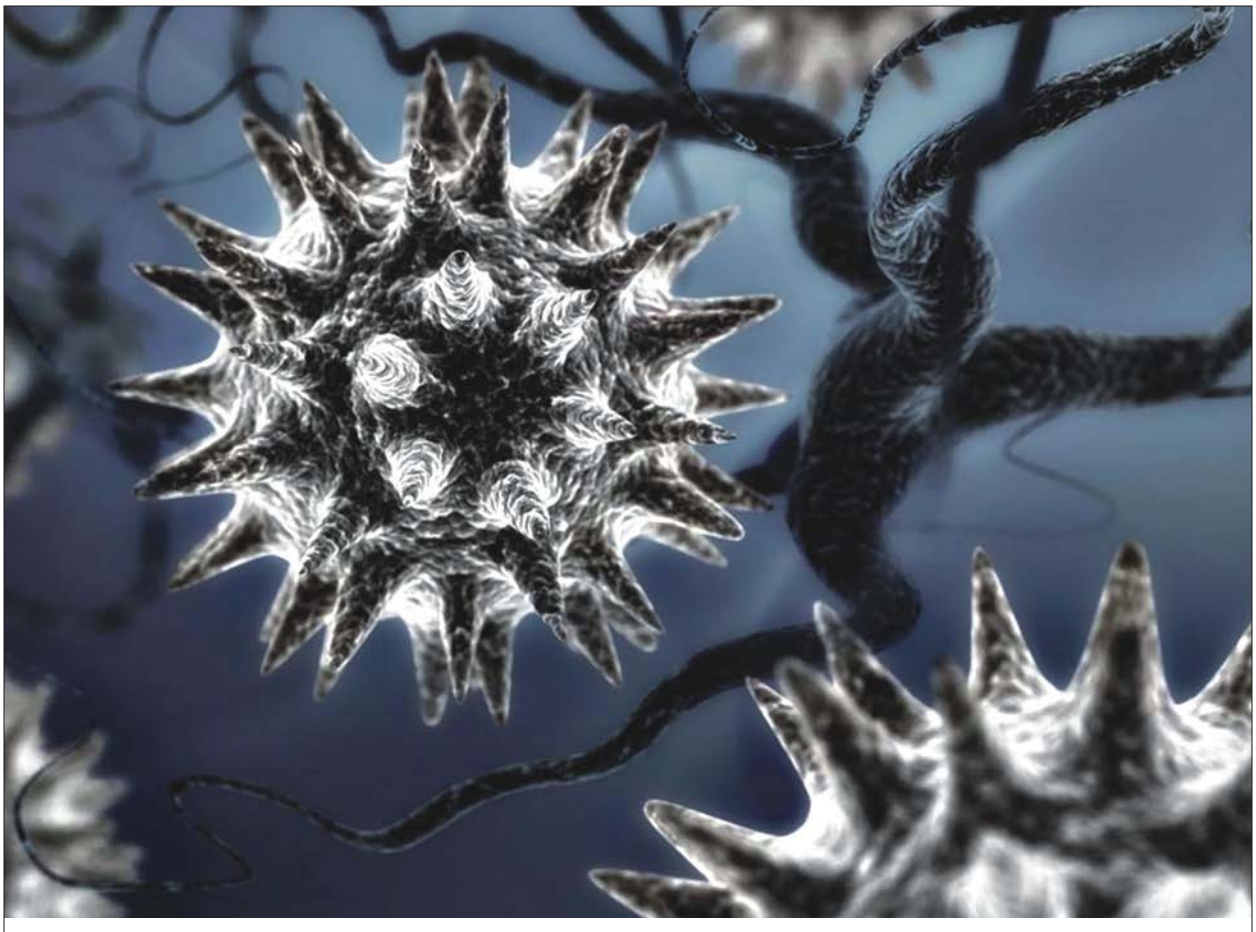

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Primary Screening of Antiviral Potential of *Quercus ilex* (Wood) Extract Against BVDV: A Pestivirus Model of Hepatitis C Virus

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ABSTRACT

In this study, we investigated the *in vitro* antiviral potential of *Quercus ilex* (wood) extract against Bovine Viral Diarrhea Virus (BVDV), a pestivirus model of Hepatitis C Virus (HCV), in comparison to extracts of green tea (*Camellia sinensis*) and pomegranate (*Punica granatum*) which are previously reported to possess antiviral properties. The Crude Aqueous Methanol Extracts (CAMEs) of *Q. ilex* (wood), *P. granatum* (rind) *and C. sinensis* (leaves), besides crude ethanolic extract (CEE) of *P. granatum* (arils) were firstly evaluated for cytotoxicity on MDBK cell line. Afterward, evaluated for their *in vitro* antiviral activities against (BVDV) by means of (MTT/ XTT) based colorimetric assays. The *Q. ilex* (wood) CAME exhibited an evident anti-BVDV activity which was found to be more pronounced than the other tested plant extracts on MDBK cells with inhibitory percentage (IP)>97% at virus dose of 10 and 100 $TCID_{50}$. *Q. ilex* (wood) extract has shown antiviral activity, thus suggesting this plant extract as a candidate for further activity-monitored fractionation to recognize its active constituents.

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

INTRODUCTION

Undoubtedly, infectious diseases caused by different viral pathogens particularly, chronic and emerging viruses represent a growing worldwide anxiety, in human health as well as in veterinary field, since important viruses can be inactivated by only a few numbers of prophylactic and therapeutic agents (Vanden Berghe *et al*., 1986; Okeke *et al*., 2005).

Hepatitis C Virus (HCV) chronically infects hundreds of millions of patients worldwide (Shepard *et al*., 2005) and it shares virological and molecular properties with BVDV, therefore BVDV is regarded as a surrogate model for HCV especially in antiviral testing procedures (Buckwold *et al*., 2003).

Bovine Viral Diarrhea Virus (BVDV), is a Pestivirus of the family Flaviviridae which can cause lifelong persistent infection in cattle, BVDV has two biotypes: Non-cytopathogenic (ncp) strains and cytopathogenic (cp) strains (which induce cytopathic effects (CPE) via apoptosis) (Zhang *et al*., 1996; Hoff and Donis, 1997).

Verma *et al.* (2008) mentioned that due to the growing problem of viral resistance to available antiviral drugs and viral latency there is a need to search for new compounds with antiviral activity for treatment of viral infections.

Plants and its derived compounds are typically appropriate as antiviral candidates for many reasons (1) They have a long history of use as traditional medications against different diseases including infectious diseases, (2) Plants produce a large number of phytochemical substances to adapt themselves to environmental stressors including invasion by microorganisms and (3) Plants are natural, that's why, they may cause less damage to host cells infected by viruses, than do pharmaceutical antivirals (Dixon, 2001; Guo *et al.*, 2006).

Green tea (*Camellia sinensis*) extracts and derived compounds exhibited a marked *in vitro* antiviral activity against rotavirus and enterovirus (Mukoyama *et al.*, 1991), Human Papilloma Virus (HPV) (Gross *et al.*, 2007; U.S. FDA, 2008) and influenza virus strains (Song *et al.*, 2005).

Neurath *et al.* (2004) reported that pomegranate (*Punica granatum*) components inhibited *in vitro* HIV-1 viral infection and in more recent report inhibited influenza virus replication (Haidari *et al.*, 2009).

On the other hand, it has been reported that *Quercus* species extracts are rich in polyphenolic compounds such as proanthocyanidins, tannins and acylated flavonoid glycosides (Zhentian *et al.*, 1999; Meng *et al.*, 2001; Ito *et al.*, 2002). This is suggested to be the cause of the antibacterial activity shown by the extracts of *Quercus ilex* leaves (Gulluce *et al.*, 2004) and *Quercus ilex* bark (Berahou *et al.*, 2007).

However, no information including antiviral activity of *Quercus ilex* extracts has been reported.

The aim of this work was to evaluate the *in vitro* antiviral potential of *Quercus ilex* (wood) extract in comparison to extracts of two edible plants of known antiviral properties, *C. sinensis* and *P. granatum* against BVDV that represents a widespread threat for farm animal wealth besides it is considered as surrogate model for HCV.

MATERIALS AND METHODS

Plant materials: 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; Holm oak (*Quercus ilex*), a medium size branch of the evergreen tree of *Q. ilex* grown in El-Orman national herbarium, El-Orman gardens, Dokki, Cairo, Egypt.

Extract preparation: The crude aqueous extracts of *Quercus ilex*, *Camellia sinensis* and pomegranate rinds were prepared as follows:

- Fifty grams of the grinded dried part of the assigned 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

Table 1: Plant extracts used in cytotoxicity assay and antiviral assay

Latin name	Family	Common name	Part used	Extracted by	Solvent
Camellia sinensis	Theaceae	Green tea	Leaves	H_2O+Methanol	PBS+DMEM
Punica granatum	Lythraceae	Pomegranate	Arils	Ethanol	PBS+DMEM
Punica granatum	Lythraceae	Pomegranate	Rinds	H_2O+Methanol	PBS+DMEM
Quercus ilex	Fagaceae	Holm Oak	Wood	H_2O+Methanol	Dist. DMSO +DMEM

PBS: Phosphate buffer saline, DMEM: Dulbecco's modified eagle's medium, Dist. DMSO: Distilled dimethyl sulfoxide

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

- Fifty grams of the detached arils was 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 in sterile PBS (or dist. DMSO [max. concentration = 0.2%] in case of *Q.ilex*) and subsequently diluted in cell culture medium (Table 1) and sterilized by filtration using (0.22 mm filter) just before testing

Reagents: DMEM, Dulbecco's Modified Eagle's Medium, (Lonza®, Belgium), used as: Growth medium (+10% fetal bovine serum, FBS), or as maintenance medium (+ 2% FBS); DMEM [without phenol red and without L-glutamine], (Lonza®, Belgium), used in the XTT assay; MTT, [3-(4,5-dimethylthiazol-2ol)-2,5 diphenyl tetrazolium bromide)], (SERVA® Electrophoresis GmbH, Germany); XTT based (TOX2) *in vitro* Toxicology Assay Kit, (Sigma Aldrich® Co., St. Louis, USA) (Catalog Number X4751): Consists of Sodium salt of XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt), to which added 1% phenazinemethosulfate (PMS); FBS, fetal bovine serum. (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); Porcine Trypsin/EDTA, (Sigma Aldrich® Co., St. Louis, USA); DMSO, Dimethyl sulfoxide. (SERVA® Electrophoresis GmbH, Germany).

Cell line: A continuous cell line of "ncp BVDV free" MDBK (Madin-Darby bovine kidney), was obtained from cell repository of 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 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 local (Iman 1) cytopathic BVDV strain propagated on MDBK cells and the infective titre of the stock solution was 10^6 $TCID_{50}$ mL^{-1} (50% tissue culture infective doses), kindly provided by Prof. Hussain Ghaly, Head of render pest department, veterinary serum and vaccine research institute, Abbasia, Cairo, Egypt. Virus was stored in small aliquots in -70°C freezer until use.

Virus titration: Colorimetric virus Titration was done on MDBK 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 $well^{-1}$ and then incubated at 36°C in a humidified atmosphere containing 5% CO_2 for 24-48 h, ten-fold serial dilutions of virus stock were prepared, when cells shown 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 maintenance media (supplemented with 2% FBS) were added and plates were put in incubator again.

Uninfected cells incubated only with DMEM were used as control cells.

The cytopathic effect was observed daily for 5-6 days, then MTT assay was performed as described by Mosmann (1983). Changes occurred to the Monolayer 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 2X (SD), standard deviation. Whereas, negative wells were those with an OD value = the cutoff value.

The 50% tissue culture infective dose ($TCID_{50}$) per ml was calculated as described previously by Reed and Muench (1938).

Cytotoxicity assay: The assay was performed using 96-well microtiter plates seeded with 15000 MDBK cells $well^{-1}$ 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 control, whereas, monolayers of cells incubated only with DMEM were used as a cell control.

Cytotoxic changes occurred to the monolayers of cells during the next 5-6 days were observed microscopically and subsequently detected colorimetrically by measuring the formed MTT formazan crystals within each well using microplate reader as described above to determine the Maximum Non-Toxic Concentration (MNTC) of each extract for MDBK cells.

Antiviral assay: Cells were seeded in growth media in 96-well flat bottom cell culture plates at a density of 10000 cells $well^{-1}$ 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 prepared and when cells shown 70-90% confluence, growth media were removed and cells were infected with 100 μL of each dilution of the virus, after 1-2 h adsorption period, excess virus was removed and 100 μL of maintenance media containing MNTC of different extracts or maintenance media without extract were added 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 XTT assay was done according to Chiang *et al.* (2003). 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 450 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:

$$\frac{ODtv\text{-}ODcv}{ODcd\text{ - }ODcv}\times 100\%$$

Table 2: Results of cytotoxicity assay and antiviral assay

Name of the plant	Max. non toxic concentration (MNTC) (μg mL^{-1})	Virus inhibition (IP%) 1 $TCID_{50}$	10 $TCID_{50}$	100 $TCID_{50}$
Quercus ilex	100	+	++++	++++
Camellia sinensis	50	-	-	-
Punica granatum (rind)	125	-	-	-
Punica granatum (arils)	250	-	++++	+

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

where, ODtv, ODcv and ODcd indicate the optical density of the extracts with virus infected cells, the optical density of the virus negative control and the optical density of the cell control, respectively.

RESULTS

In the present study we have demonstrated that no cytotoxicity on MDBK cells was observed at concentrations below 50 μg mL^{-1} in all examined extracts, the maximum non toxic concentrations found were 100, 50, 125 and 250 μg mL^{-1} in case of extracts of *Quercus ilex*, *Camellia sinensis*, pomegranate rinds and pomegranate arils, respectively (Table 2), where the pomegranate arils extract was found to have the least toxicity on MDBK cells while the highest relative toxicity was seen in case of *Camellia sinensis* extract.

Remarkably, Distilled DMSO (used as a solvent for *Quercus ilex* wood) at the used concentration was found not to be harmful to used cells.

In this study, the *Quercus ilex* (wood) extract was found to have a potent antiviral activity against BVDV with viral inhibition percentage (IP = 97%) at virus doses of 100 $TCID_{50}$ and 10 $TCID_{50}$ (Table 2).

Furthermore, pomegranate arils extract exhibited a potent antiviral effect against BVDV (i.e., with IP value = 97%) but only at virus dose of 10 $TCID_{50}$ (Table 2).

On the other hand, in case of *Camellia sinensis* and pomegranate rinds extracts no touchable antiviral effect was noticed (Table 2), although, both extracts at the used concentrations (i.e., MNTC) caused an unexpected cytotoxicity to the MDBK cells infected with BVDV.

DISCUSSION

The World Health Organization (WHO) reported that the traditional utilization of plant derived materials to combat diseases (including infectious diseases) is done by about 70-95% of the world's population, especially in developing countries (Robinson and Zhang, 2011).

Aqueous and alcoholic extracts from plants used in traditional medicine has been suggested to be potential sources of new antiviral agents to overcome the problems met with the available synthetic antiviral drugs especially the problem of viral resistance (Chung *et al*., 1995; Vlietinck *et al*., 1995),

Quercus species of Family (Fagaceae) are numerous and are believed to be a rich source of polyphenolic compounds and other bioactive phytochemicals (Zhentian *et al*., 1999; Meng *et al*., 2001; Ito *et al*., 2002), this could explain the reported biological activities such as antioxidant, anthelmintic, antibacterial and antiviral activities of some *Quercus* species (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 *Quercus ilex* (wood) extracts has been reported.

The aim of this work was to evaluate in a colorimetric way the *in vitro* antiviral potential of *Quercus ilex* (wood) extract against BVDV (as a surrogate model for HCV).

Regarding cytotoxicity on MDBK cells, pomegranate arils extract was found to have the least toxicity on this type of cells while the highest relative toxicity was seen in case of *Camellia sinensis* extract (Table 2) which may be due to its different nature and higher content of polyphinolics (tannins).

These results could be explained in the light of the recent report of Carreras *et al.* (2012) who stated that plant polyphenols may be free radical scavengers or generators, depending on their nature and concentration which may contribute to their influence on cell viability.

The OD values detected in case of some extract concentrations which were higher than that of cell control are indicating variable degrees of cytoprotective effect of the extract components (mainly polyphenolic content) on MDBK cells which may be attributable to antioxidant effects via., free-radical scavenging and metal chelation capacity, as reported formerly by Kulkarni *et al.* (2007), Sestili *et al.* (2007) and Tzulker *et al.* (2007).

Notably, DMSO (used as a solvent for *Quercus ilex* wood) at the used concentration showed no toxic effect on MDBK cells, agreeing with the previous report of Vijayan *et al.* (2004) who found that DMSO at a concentration of 0.2% has no harmful effect on cultured 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.

Previous reports revealed that extracts of *Q. infectoria*, a member of the plant family (Fagaceae) showed potential to inhibit members of the virus family (Flaviviridae) such as HCV (Hussein *et al.*, 2000) and dengue virus type 2 (Muliawan *et al.*, 2006).

In this study the *Quercus ilex* (wood) extract was found to have a potent (IP≥97%) antiviral activity against BVDV at virus doses of 10 and 100 $TCID_{50}$.

In fact, this antiviral effects may be due to the *Quercus ilex* extract content of phytochemicals such as hydrolyzable tannins and low molecular weight phenolic compounds (ellagitannin), lignans and other bioactive compounds which was previously described to be the main components of oak wood (Conde *et al.*, 1997; Fernandes *et al.*, 2009; Michel *et al.*, 2011).

Remarkably, results of cells treated with DMSO only (used as solvent control) confirmed that the observed antiviral activity of *Quercus ilex* extract was belonging totally to the extract itself not to its solvent.

Although, pomegranate arils extract exhibited a potent antiviral effect against BVDV (i.e., IP≥97%) but in fact, this antiviral effect is dissimilar to that of *Quercus ilex* extract against BVDV as it was only shown at lower virus dose of 10 $TCID_{50}$ (Table 2), this could be attributable to different antiviral mode of action of each of the two plants which may be caused by their different extract constituents.

On the other hand, in case of *Camellia sinensis* and pomegranate rinds extracts no touchable antiviral effect was noticed and these unexpected disappointing findings disagree with previous work of (Hussein *et al.*, 2000), who reported that pomegranate rind methanol extract inhibited the HCV protease activity by (IP≥89.9%).

CONCLUSION

This preliminary work is the first report on *Q. ilex* (wood) extract antiviral activity against BVDV. Further *in vitro* and *in vivo* research is needed to expose the active constituents of this plant which may be useful in the development of new effective antiviral agents against BVDV and possibly HCV.

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