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RESEARCH ARTICLE

IN VITRO SCREENING OF ANTIVIRAL ACTIVITY OF LYOPHILIZED EXTRACTS OF *AZHADIRACHTA INDICA* AND *QUERCUS LUSITANICA* ON DENGUE 1 & 3 SEROTYPES

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ABSTRACT

Dengue fever has become a global concern and can lead to a severe life threatening illness. The development of dengue vaccines is complicated by the antibody dependent enhancement effect. Thus the development of a plant based antiviral preparation promises a more potential alternative in combating dengue disease. The present study investigated the antiviral effects of standardized lyophilized aqueous, aqueous-ethanolic and ethanolic extracts *of Azhadirachta indica* and *Quercus lusitanica* on Dengue 1 & 3 strains in Vero cell line and compared with known antiviral drug Ribavirin. The antiviral activity of lyophilized aqueous extract of *Azhadirachta indica* showed better activity for Dengue 1 & 3 at a Maximum nontoxic dose concentration of 500µg/ml and the ethanolic extracts are partially inhibited at the concentration of 500µg/ml to dengue 1 but not in dengue 3. Whereas the aqueous-ethanolic extract of neem and three extracts of *Quercus lusitanica* did not show any inhibition on dengue 1 & 3. The antiviral activity of Ribavirin exhibited 15.6µg/ml & 7.5µg/ml for dengue 1 & 3 respectively. These data suggest that the lyophilized aqueous extracts of *Azhadirachta indica* possess the ability of inhibiting the activity of Dengue 1 & 3 *by in vitro* assays. This plant is worth to be further investigated and might be advantageous as an alternative drug for dengue treatment.

INTRODUCTION

Dengue disease, regardless of its serotypes is transmitted from person to person by *Ades aegypti* and *Ades albopictus* mosquitoes in domestic environment (Halstead, 1997) and is an endemic in more than 110 countries with two –fifths of the world population at risk (Guzman, 2004). The World Health Organization (WHO) estimates that 50-100 million dengue infection occurs each year and that almost half the world's population lives in countries where dengue is endemic. Currently close to 75% of the global population exposed to dengue are in the Asia Pacific region (Shasonk Sankar and Debashmita Dubey 2013). Similarly, a total of 4.6 fold increase in dengue cases has also been reported in America over the three decades (Gibbons, 2010). Dengue appears in two forms: the first class is dengue fever with symptoms that range from mild fever to high fever with retro-orbital pain, severe headache, maculopapular rashes, muscle and joint pain. The other more severe form, Dengue Haemorrhagic fever (DHF) and Dengue Shock syndrome (DSS) may present with abdominal bleeding, hemorrhage and circulatory failure, which is fatal if without prompt and proper management (Gubler, 1998). There are four serologic types of dengue virus, DENV-1, 2, 3 and 4. A primary infection with any of the four serotypes results in a lifelong immunity to that serotype, and temporary immunity to the others. However, this temporary immunity usually wanes after 6 months, at which point an individual is susceptible to the other three DENV serotypes (Murrel *et al*., 2011). The primary infection is most often asymptomatic, but sequential infections in the presence of heterologous dengue antibodies often lead to a more severe secondary infection causing DHF or DSS. Murrel *et al*. (2011) attributed this due to the antibody-dependent enhancement (ADE) effect. Studies of the outbreaks in endemic areas, such as South East Asia revealed that a primary infection with DENV-1 or DENV-3 frequently resulted in a more severe disease than if DENV-2 or DEV-4 where the primary infection (Vaughan *et al*., 1997). Hence this study focused foremost on to Dengue 1 and 3 serotype for remedies.

Early studies have shown that extracts from different parts of plants could provide good antiviral results as compared to their synthetic analogues (Chiang *et al*., 2005). As such, the development of a plant-based antiviral preparation promises a more potential alternative in combating dengue disease. "Let food be your medicine and let medicine be your food" was the advice of the father of medicine, Hippocrates, over two millennia ago (Wang MY *et al*., 2002). Herbal and natural products of folk medicine have been used for Centuries in every culture throughout the world. Scientists and medical

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professionals have shown increasing interest to know the truth about the benefits of these remedies.

According to the World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs. About 80 % of individuals from developed countries use traditional medicine, However, such plants should be investigated to better understand their properties, safety and efficiency (Arunkumar and Muthuselvam 2009). The world is now looking towards India for new drugs to manage various challenging diseases because of its biodiversity of medicinal plants and an abundance of traditional knowledge, such as Sidha, Ayurvedha etc. (Chitravadiva *et al*., 2009). Over the years, the World Health Organization (WHO) advocated that countries should interact with traditional medicine with a view to identify and exploit aspects that provide safe and effective remedies for ailments of antivirals. In addition, there are still no antiviral drugs being tested against dengue disease in any clinical trials. As such the present study is aimed to screen and determine the anti-dengue activity of lyophilized aqueous, aqueous-ethanolic and ethanolic extracts of *Azhadirachta indica* and *Quercus lusitanica* on dengue serotypes 1 & 3.

MATERIALS AND METHODS

Plant chosen for the study

Azhadirachta indica

Azhadirachta indica, also known as Neem, is a tree in the mahogany family Meliaceae. It is one of two species in the genus Azhadirachta, and is native to India, Pakisthan, Bangladesh which is growing in tropical and subtropical regions. Products made from neem trees have been used in India for over two millennia for their medicinal properties. Neem products are believed by Ayurvedhic, practitioners to be antihelmintic, antifungal, antidiabetic and antiviral. It is considered as a major component in Ayurvedhic and Unani medicine and is particularly prescribed for skin diseases.

Quercus lusitanica

*Quercus lusitanica***,** also known as *Quercus infectoria* is a small tree or shrub belonging to the Fagaceae (Quercaceae) family. They are found in the Mediterranean area, mainly in Greece, Asia Minor, Syria and Iran (Noorsaadah *et al*., 2006). The galls are commonly known as Gall Oak, Lusitanian Oak, or Dyer's Oak. These galls are produced by the infection from the insect *Cynips gallae tinctoriae*.. In Tamil it is called 'maasikkaay' and is used by Tamils for more than 2000 years.

Collection of plant parts

The healthy plant parts of medicinally potential plants were collected and shade dried. The accuracy of the plant species was ascertained with Department of plant Biology & Biotechnology, Presidency College (Aut), Chennai. The names of plants and parts of the plant used are shown in Table -1

Preparation of plant extracts

The healthy parts were surface sterilized with 70% ethanol. The dry leaves of *Azhadirachta indica* were ground into powder where as the galls of the *Quercus lusitanica* were surface sterilized after breaking and cleaning the insects inside the galls. The fine powder was collected by sieving. 20 g of the dry powder and homogenized paste of fresh leaves were soaked as follows

Table 1. Plants used in the study

Botanical name	Common name	Part used	Traditional use	Extracts taken
Azhadirachta indica. Juss.	Neem	Leaves	Skin infections	Aqueous/aqueous ethanolic/ethanolic
Quercus lusitanica Lam.	Gall Oak, Lusitanian Oak, or Dyer's Oak	Galls	Inflammatory diseases	Aqueous/aqueous ethanolic/ethanolic

Aqueous Extract

20 g of the specified powdered plant part was soaked with 100 ml water and stored overnight at 4ºC. Filtered and centrifuged to get clarified extract. This method was repeated 3 times more, all extracts pooled together, concentrated, filtered through a 0.22µm pore sized Millipore filter and lyophilized and stored till use.

Aqueous -ethanolic Extract

20 g of the specified powdered plant part was soaked in 50 ml Ethanol–50 ml water (1:1) and stored overnight at 4ºC. Filtered and centrifuged to get clarified extract. This method was repeated 3 times more, all extracts pooled together and concentrated, filtered through a 0.22µm pore sized Millipore filter and lyophilized and stored till use.

Ethanolic (95%) Extract

20 g of the specified powdered plant part was soaked with 100 ml Ethanol and stored overnight at 4ºC. Filtered and centrifuged to get clarified extract. This method was repeated 3 times more, all extracts pooled together and concentrated, filtered through a 0.22µm pore sized Millipore filter and lyophilized and stored till use. The Physio-chemical characteristics of three lyophilized extracts of two plants were tabulated in Table 2.

Virus stocks

Dengue viral strains (D1 and D3) were obtained from the National institute of virology, (NIV) Pune was reconstituted in 5 ml of 2% Minimum essential medium and 100 µl of the reconstituted virus was added to the confluent Vero cell line which is procured from National Centre for Cell Sciences (NCCS), Pune, India. Viral stocks were obtained by inoculating monolayer of Vero cells in a 25 cm^2 tissue culture flask with virus diluted 1:5 in 1ml of maintenance medium containing 2% FBS. After an hour, 4 ml of maintenance medium was added and the cells were cultured for 5 days. Cells and supernatant were then harvested by gentle pipetting. Cell debris was

removed by centrifugation at 3000 RPM for 10 minutes and the viral supernatant was elected in 2% Fetal Bovine Serum and stored at -80°C.

***In vitro* cytotoxicity assay to determine the maximal cytotoxic free concentration of extracts in comparison to the known antiviral drug ribavirin**

A monolayer of the Vero cell line was taken and the medium was discarded and the wells were given a gentle wash with Phosphate buffered Saline. Cells were trypsinised with Trypsin Phosphate versene Glucose. 10 ml of 10% minimum essential medium was added to the flask and the cells were removed. 100 µl of dislodged cells were plated into the 96 well microtitre plate and 100 µl of 10% minimum essential medium was added into each wells. The microtitre plate was then incubated at 37°C for 12 hrs under 5% C atmosphere.

About 1 mg of the three lyophilised extracts of two plants, and Ribavirin (Sigma Aldrich, India) was weighed separately and dissolved in 1 ml of water /ethanol based on the solubility and 200 µl of the extract /Ribavirin was added into the wells of first column of microtitre plate and were diluted into two fold manner till the last well. 100 µl from the last well was discarded. 100 µl from the respective dilutions were added into the respective wells containing cells. 100 µl of 2% MEM was added into the wells and control wells with cells were maintained. The plates incubated at 37°C in 5% CO_2 atmosphere for 72 hrs and was observed under inverted phase contrast microscope for determination of toxic free concentration.

Estimation of TCID 50 (Reed and Meunch., 1938)

The Dengue serotype 1 & 3 viral stock was removed from -80°C freezers and immediately thawed. Then the viral stock was diluted by 10 fold serial dilutions (10^{-1} – 10^{-5}) in maintenance medium About 100 µl of the diluted virus was suspended in 900 µl of 2% MEM and it was serially diluted and 100 µl of the diluted virus was added to the respective columns and incubated at 37°C for 1 hour and about 100 µl of 2% minimum essential medium was added. After 72 hrs, the cytopathic effect in the wells of each row was counted and TCID 50 (Tissue culture infective dose50%) was calculated according to the methods of Reed and Muench 1938.

***In vitro* antiviral assay**

In vitro inhibitory potential of plant extracts and Ribavirin was evaluated in Vero cell line using a virus inhibition assay as described by Premanathan *et al*. (1996). Briefly, plant extracts were prepared in aqueous, aqueous-ethanolic and ethanolic extracts and was carried out from Maximum nontoxic concentration. Simultaneously, a series of 10-fold dilutions (10^{-1}-10^{-4}) corresponding to 1000-1 TCID 50 of Dengue 1 & 3 viral stocks were prepared separately. About 100 µl of the estimated concentrations of virus was added to the established cell lines in 96 well plate and incubated for 1 hour at 37°C. After adsorption of the virus, the diluted and maximum nontoxic concentration of the drug was added to the adsorbed virus and incubated at 37°C. After 72 hrs, the Minimum inhibitory concentration of the drug was estimated by observing the presence of cytopathic effects.

RESULTS

Herbal medicines are potential sources for the development of new antiviral drugs, since they can be selected on the basis of their ethnic medicinal use, for example, against infection. These plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources to control viral infection. In this study, three extracts of *Azhadirachta indica* and *Quercus lusitanica* were examined for their antiviral activity against dengue 1 & 3 in comparison with Ribavirin was preceded by cytotoxic studies to determine the maximum nontoxic dose (MNTD) for *in vitro* antiviral assay. The Physio-chemical characteristic studies of three plant extracts were tabulated in Table 2. The MNTD of three extracts of *Azhadirachta indica* and *Quercus lusitanica* is shown in Table 3. The cytotoxicity of the three extracts of *Azhadirachta indicia* against Dengue 1 & 3 were evaluated, showing that the extracts were not toxic to Vero cells from 500µg/ml onwards where as the MNTD of *Quercus lusitanica* were estimated as aqueous (125µg/ml), aqueous-ethanolic (250µg/ml) and ethanolic (125µg/ml) showed toxic free in Vero cell line. In order to screen the anti-dengue properties of lyophilized aqueous, aqueous- ethanolic and ethanolic extracts of *Azhadirachta indica* and *Quercus lusitanica*, *in vitro* antiviral assay was conducted using the MNTD of each plant extracts against TCID 50 of dengue 1 & 3. Vero cells grown in the tissue culture flask with MEM supplemented with 10% FBS would form a monolayer sheet of cells. The morphology of Vero cells was clearly visualized using an inverted

Table 2. Physico-Chemical Characteristics of the Lyophilized Extracts of two medicinal Plants

Plants	aqueous extract		aqueous ethanolic extract		ethanolic extract	
	p H	Texture	p H	Texture	p H	Texture
Azadirachta indica	7.0	Dark brownish flakes	7.0	Dark brownish crystals	7.5	Greenish Pasty
Quercus lusitanica	7.0	Brown pasty	7.0	Brown pasty	7.0	Brown pasty

Table 3. Maximum Toxic free concentration of three lyophilized extracts of two medicinal plants and Ribavirin on Dengue 1 & 3

Extracts	Ribavirin (µg/ml)	*Azhadirachta indica*			*Quercus lusitanica*		
		aqueous extract (µg/ml)	aqueous ethanolic extract (µg/ml)	ethanolic extract (µg/ml)	aqueous extract (µg/ml)	aqueous ethanolic extract (µg/ml)	ethanolic extract (µg/ml)
Dengue 1 Serotype	500	500	500	500	125	250	125
Dengue 3 Serotype	500	500	500	500	125	250	125

Table 4. Antiviral activity of three lyophilized extracts of two medicinal plants on Dengue 1 with Ribavirin

Non toxic concentration	Ribavirin	*Azhadiracta indica*			*Quercus lusitanica*		
		aqueous	aqueous-ethanolic	ethanolic	aqueous	aqueous-ethanolic	ethanolic
500	+	+	-	+/-			
250	+	-	-	-		-	
125	+	-	-	-	-	-	-
62.5	+	-	-	-	-	-	-
31.2	+	-	-	-	-	-	-
15.6	+	-	-	-	-	-	-
7.5	-	-	-	-	-	-	-
3.7	-	-	-	-	-	-	-

+ Presence of inhibition: - Absence of inhibition: +/- Partially inhibited

Table 5. Antiviral activity of three lyophilized extracts of two medicinal plants on Dengue 3 with Ribavirin

Non toxic concentration	Ribavirin	*Azhadiracta indica*			*Quercus lusitanica*		
		aqueous	aqueous-ethanolic	ethanolic	aqueous	aqueous-ethanolic	ethanolic
500	+	+	-	-			
250	+	-	-	-		-	
125	+	-	-	-	-	-	-
62.5	+	-	-	-	-	-	-
31.2	+	-	-	-	-	-	-
15.6	+	-	-	-	-	-	-
7.5	+	-	-	-	-	-	-
3.7	-	-	-	-	-	-	-

+ Presence of inhibition: - Absence of inhibition

microscope. The CPE includes synctia formation and cell lysis with blebbing of cells, which referred to small detached remnants of apoptotic bodies that have undergone programmed cell death.

The anti-dengue potency of the active plant extracts were determined by *in vitro* virus inhibition assay. The presence of

CPE or inhibition of Dengue 1 & 3 in extract or in extract free control wells after 72 hours of incubation at 37°C with 5 % CO_2 were recorded. Inhibition of Dengue 1 & 3 was observed in the aqueous extracts from *Azhadirachta indica* was at a concentration range of 500 µg/ml against tested However, aqueous-ethanolic and ethanolic extract of *Azhadirachta indica* and all the three extracts of *Quercus lusitanica* did not prevent CPE or cell death from the effects of Dengue 1 & 3. Hence that extract doesn't show any anti-dengue activity, even in the maximum nontoxic concentration tested i.e 500 µg/ml.

DISCUSSION

Despite the significant disease burden caused by various members of the genus Flavivirus, no specific antiviral therapy is currently licensed for treatment. Also, it is relevant to mention that this is the first study on the efficacy of a lyophilized aqueous, aqueous-ethanolic & ethanolic extracts of *Azhadirachta indicia* (leaves) and galls of *Quercus lusitanica* on dengue 1 & 3 serotype. Since these two medicinal plants have many medicinal values, for example, neem leaves are being traditionally used as curative against fungal and bacterial diseases and in spite of the enormous antimicrobial potential of this tree with versatile attribute, the research on the antiviral properties of neem is confined to only a few viruses' vice-Chikungunya, measles and HSV. Neem has been exploited for chemoprophylaxis of different viral infections viz-chicken pox, HSV HIV, Measles and Chikungunya (Kaij-e Kamb *et al*., 1992) There are many reports of plant extracts of *Quercus lusitanica* possessing relatively good potential to inhibit viruses (Van *et al*., 1978). The galls of the *Q.lusitanica* have been shown to have many medicinal properties such as Astringent, anti-diabetic, anti-pyretic and anti-Parkinson activities (Dar *et al*., 1976) The chemical constituents of the galls have been reported to comprise a large amounts of tannins, Gallic acid, syringic aicd, ellagic acid, Beta sitosterol, methyl betulate and methyl oleanate (Hwang *et al*., 2000).

The anti-dengue activity of each extract of two medicinal plants was shown in Table 4 & 5 for dengue 1 & 3 respectively. Parida *et al*., 2002 has reported the antiviral effect of the whole aqueous extract of *Azhadirachta indica* leaves on the dengue 2 virus in C6/36 cell line at a concentration of 1.897 mg/ml. Also, they separated Azhadirachtin as pure compound which did not show any inhibitory activity of dengue virus. Whereas in the present study the inhibitory activity of lyophilized aqueous extract (dry leaf extract) of the neem is appreciable which showed the effective concentration of 500µg/ml in Vero cell line for dengue 1 & dengue 3.

Though the desirable MIC of a potential drug is below 100µg/ml for a pure compound (Paul cos *et al*., 2006), the MIC of the lyophilized extract of *Azhadirachta indica* is 500 µg/ml, which is moderate activity because the lyophilized extract is not a pure compound but also contains some organic and inorganic materials that are inactive against viruses. Hence it is pertinent to continue for isolation and identification of active molecule and its safety. Ribavirin is an approved drug for treatment of respiratory synctial virus infection, as well as orally, together with alpha interferon, for treatment of hepatitis

C virus (HCV) infections (Ratree *et al*., 2006). Jhinhong *et al*., 2011 has studied the antiviral activity of imino sugar derivatives on dengue virus. They stated that the combinational therapy with a clinically approved broad spectrum antiviral compound, ribavirin greatly improved the antiviral activity of the imino sugar than the monotherapy of imino sugar by *in vitro* and *in vivo* on dengue 2. Hence, in this, the present study has chosen the Ribavirin (a known antiviral drug) as a standard drug for comparison and to determine the efficaciousness of the procedure. And also the effective concentration of Ribavirin on dengue 1 & 3 is about 15.6µg/ml & 7.5µg/ml, respectively in Vero cell line (Table 4 & 5). The earlier study has reported the antiviral activity of ribavirin on dengue 2 viruses at the concentration of 11.41µg/ml in LLC-MK2 cell line (Ratree *et al*., 2006). Noorsaadah *et al.* 2006 has reported the antiviral activity of crude methanolic extracts of *Quercus lusitanica* galls on the dengue 2 virus of 180µg/ml on C6/36 cell line. They also purified methyl gallate from the crude methanolic extract which showed a better inhibition at a concentration of 100µg/ml. Hence the present research has also focussed to find out any activity of aqueous, aqueous-ethanolic and ethanolic extracts in a lyophilized form to acquire better results, but these extracts didn't show any significant activity on dengue 1 & 3 which is undesirable.

Conclusion

On the basis of this study report on three extracts (aqueous, aqueous-ethanolic & ethanolic) of *Azhadirachta indica* and *Quercus lusitanica* on Dengue 1 & 3 viral strains, the aqueous extracts of *Azhadiracta indica* exhibited a substantial activity on both Dengue 1 & 3 serotypes. *In vitro* studies on these extracts showed anti-dengue properties which are pertinent to continue for isolation and identification of activity guided fractionation of active molecule and its safety that might provide an active drug to control dengue infection. The present study has revealed the importance of lyophilized aqueous extract of dried leaves of *Azhadirachta indica* on dengue virus which are being threat to human health and for the development of alternate safe and effective medicines.

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