RE-EVALUATION OF *QUERCUS LUSITANICA* EXTRACT AS AN INHIBITORY AGENT AGAINST VIABILITY OF DENGUE VIRUS TYPE 2

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Abstract: The aim of this study was the reconfirmation of *Quercus lusitanica* extract as an antiviral compound against dengue virus type 2 (DV2) using conventional plaque assay technique. *In vitro* antiviral activity of *Q. lusitanica* extract was assessed in C6/36 cells employing virus inhibition assay. The result showed that *Q. lusitanica* extract, at its maximum nontoxic concentration of 0.25 mg/ml, completely inhibited 10-1,000 $TCID_{50}$ of virus as indicated by the absence of cytopathic effect (CPE). The low dose of *Q. lusitanica* (0.032mg/ml) showed 100% inhibition with 10 $TCID_{50}$ of virus, but only 50% and 25% inhibition with 100 and 1,000 $TCID_{50}$, respectively. The plaque assay result for the viral control at 10 $TCID_{50}$ of DV2 gave $13x10^4$ pfu/ml. After treatment, 0.032 mg/ml of this extract yielded 3.5×10^4 pfu/ml, while 0.063 mg/ml of this extract gave $1.5x10^4$ pfu/ml. The results showed that *Q. lusitanica* extract has a good inhibitory effect on viability of DV2, whether by post genomic technique or conventional method.

INTRODUCTION

Dengue fever (DF) or dengue hemorrhagic fever (DHF) has emerged as a public health problem worldwide (Gubler, 2002; Guzman and Kouri, 2003; Guzman *et al*, 2004). Dengue viruses are among the most important arboviruses because of the high morbidity they cause among humans who inhabit urban communities in the tropical and subtropical regions of the world (Gubler, 1998; Guzman and Kouri, 2002). It is estimated that two billion people live in areas at-risk for dengue virus transmission, and that as many as 100 million infections (Rigan-Perez *et al*, 1998; WHO, 2000; Shuenn-Jue *et al*, 2001), 500,000 hospitalizations, and 25,000-30,000 deaths occur annually, mostly among children (Gubler, 1999, 2002; Dussart *et al*, 2006). It is now endemic in more than 100 countries (the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific) and poses a threat to more than 2.5 billion people (Guzman and Kouri, 2003). The more severe forms of the disease, dengue hemorrhagic fever and dengue shock syndrome (DSS), have been reported in up to 5-10% of secondary infections, with case fatality rates as high as 10% (Halstead, 1988; Rigan-Perez *et al*, 1998; WHO, 2000; Shuenn-Jue *et al*, 2001). Dengue virus is a positive-polarity RNA virus in the family Flaviviridae, and there are four antigenically related serotypes of dengue viruses (DV1, DV2, DV3, and DV4) (Lindenbach and Rice, 2001). A primary infection with any of the four serotypes of dengue viruses usually results in subclinical or self-limited febrile disease (Shuenn-Jue *et al*, 2001).

Despite the antigenic relatedness of dengue viruses, two or more serotypes may sequentially infect one host (Alvarez *et al*, 2005). In a previous study, it was demonstrated the *in vitro* inhibitory effect of *Quercus lusitanica* seed extract on the replication of DV2 through the NS1 protein expression of infected C6/36 cells using proteomics technique (Muliawan *et al*, 2006). The results showed the downregulation of NS1 protein expression of infected C6/36 cells after treatment with this extract. The NS1 is a glycoprotein present in all flaviviruses and appears to be essential for virus viability. The

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aim of this study was the reconfirmation of *Q. lusitanica* extract as an antiviral compound used against DV2 by conventional plaque assay technique. Plaque reduction neutralization technique (PRNT) remains the standard for the titration of neutralizing antibodies. Standard methods for titration dengue virus and measuring the ability of antiviral to neutralize the virus are based on plaque assays that require 5-7 days to complete (Lambeth *et al*, 2005).

MATERIALS AND METHODS

Preparation of *Q. lusitanica* extract

We used a methanol extract of *Q. lusitanica* from the Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Malaysia.

Cell line

C6/36 (cloned cell line derived from larvae of *A. albopictus*) was obtained from Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia and as described previously (Muliawan *et al*, 2006).

Virus

Dengue virus 2 (DV2) *New Guinea C* strain was obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia as described previously (Muliawan *et al*, 2006).

The fifty-percent tissue culture infective dose ($TCID_{50}$) of dengue virus suspension

Tenfold dilutions of the virus suspension were prepared in RPMI-1640 maintenance medium. The virus dilution was added to the wells and incubated at 37°C for one hour (including wells without virus as control). The infected cultures were then incubated at 28ºC for four days and monitored for cytopathic effect (CPE). The formula to determine the virus infectivity was the Kärber method (Schmidt and Emmons, 1989).

Maximum non-toxic dose (MNTD)

Prior to screening the *Q. lusitanica* extract for their inhibitory potential, the plant extract was subjected to toxicity studies to find out the maximum dose that would be nontoxic to C6/36 cells (*in vitro*) system as per the method described by van den Berghe *et al* (1978), Schmidt (1979), and Muliawan *et al* (2006). The maximum nontoxic dilution (MNTD) of the antiviral compound was then used in the antiviral testing.

Determination of antiviral compound testing based on presence or absence of CPE on DV2-infected C6/36 cells

A tenfold dilution of virus stock was prepared in RPMI-1640 maintenance medium. Serial dilutions of antiviral compound were prepared in RPMI-1640 maintenance medium. Cells were added to Linbro plates, and then antiviral compounds were added. As a control, uninfected cells, infected cells, and an antiviral compound were added to the test. The cells were incubated at 37ºC for two hours. After incubation, the virus dilutions were added to the Linbro plates (1,000 $TCID_{50}$ to 10 $TCID_{50}$) and incubated at 28ºC for four days. The presence or absence of CPE was monitored daily. Any alteration in morphology, ranging from focal clumping and granulation of the cells to complete destruction of the cell sheet, was considered a CPE.

Plaque assay

Tenfold serial dilutions of virus stock were prepared in L-15 2% fetal bovine serum. Pig spleen (PS) cells were then resuspended in L-15. These cells were added to each well of a 24-well Linbro plate. In the next step, virus dilutions were added to wells in duplicate. Two wells were kept as uninfected controls (cell control). The cells were incubated at 37°C for 3-4 hours until adherent. The wells were then overlaid gently with 1.5% CMC agarose, and incubated at 37°C for six days. Cells were then stained with naphthalene black on Day 6.

RESULTS

Evaluation of the inhibitory potential of *Q. lusitanica* on DV2 *in vitro* was preceded by cytotoxicity studies to determine the MNTD for virus inhibition assay. The MNTD for *Q. lusitanica* extract was 0.25 mg/ml, as shown previously by Muliawan *et al* (2006). *In vitro* inhibitory potential of *Q. lusitanica* extract on DV2 in C6/36 cells showed inhibition of virus replication in a dose-dependent response, as shown previously by Muliawan *et al* (2006).

Inhibitory effect of *Q. lusitanica* extract on DV2 replication

Before screening the inhibitory potential of *Q. lusitanica* extracts on C6/36 cells, the 50% of tissue culture infective dose ($TCID_{50}$) was determined. The 50% endpoint titer was a 10^{-4} dilution of DV2 stock (harvested from mice brain). The maximum dilution of DV2 that produced 100% cytopathic effects (CPEs) was determined and found to be 10^{-3} (10 $TCID_{50}$), and this value was employed as the highest dilution of virus used in the antiviral compound assay *in vitro*. To determine the effect of antiviral compounds against DV2 replication, the criterion was the presence or absence of CPE.

To evaluate the effect of *Q. lusitanica* extract as an antiviral compound against DV2 replication in C6/36 cells, confluent monolayers of C6/36 cells were treated with *Q. lusitanica* extract prior to infection by DV2, and then cytopathic effects were evaluated. Untreated C6/36 cells infected by DV2 were used as a control. From the result obtained, it could be seen that cells not infected by DV2 did not show any cytopathic effect (CPE) (Fig 1, panel A). CPE was however detected in a culture of C6/36 cells infected by DV2 (Fig 1, panel B). When C6/36 cells were treated with *Q. lusitanica* extract at various concentrations prior to infection by DV2, it was apparent that there was protection against virus infection as evidenced by the reduction in cytopathic effect. Fig 1 (panel C) shows the inhibition of CPE in a 1,000 $TCID_{50}$ concentration of DV2 on C6/36 cells treated with 0.25 mg/ml of *Q. lusitanica* extract.

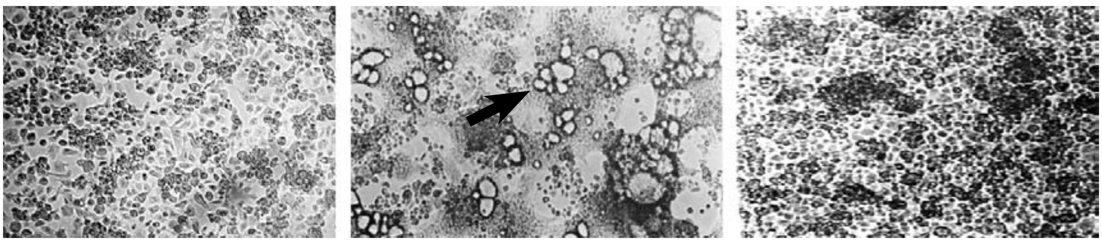
Plaque formation

From the *in vitro* inhibition assay (CPE), it was shown that *Q. lusitanica* extract inhibited infection by DV2 at concentrations of 0.032 to 0.25 mg/ml. To evaluate *Q. lusitanica* extract further as an antiviral compound at low concentrations, a viral plaque assay was performed. Control cells were inoculated with a virus titer of 10^{-3} (10 $TCID_{50}$).

Fig 2 shows the differences between the uninfected monolayer of pig spleen cells (panel A) and DV2-infected monolayer of pig spleen cells by DV2 (panel B). Infected cells show cytopathic changes in the form of plaques.

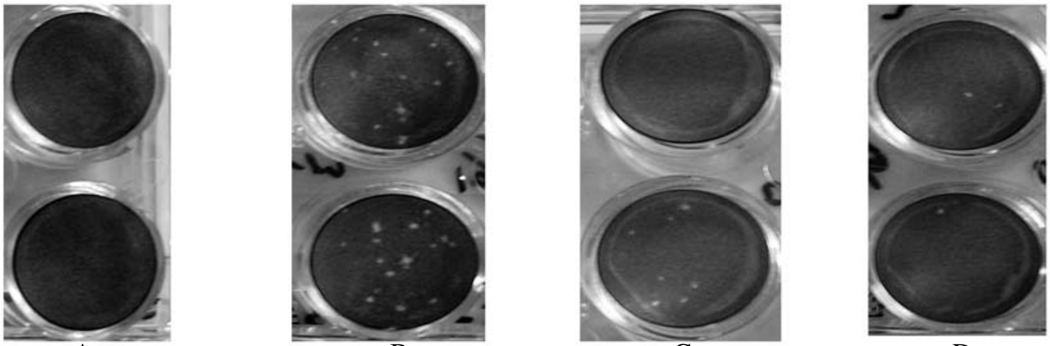
Monolayers of pig spleen cells that were

A B C



C6/36 cells | Before treatment | After treatment

Fig 1- Virus inhibition assay (CPE).



A	B	C	D
Uninfected	10 $TCID_{50}$	0.032 mg/ml extract (10 $TCID_{50}$)	0.063 mg/ml extract (10 $TCID_{50}$)

Fig 2- Plaque assay after and before treatment with *Q. lusitanica* extract.

treated with 0.032 and 0.063 mg/ml of *Q. lusitanica* extract were infected with 10 $TCID_{50}$ dilutions of DV2. This was followed by overlaying with nutrient agar medium and then incubating for six days. For visualization, plaques were stained with naphthalene black. Plaques were counted on the second day after their appearance, when they were clearer and better defined. The viral control at 10^{-3} dilution (10 $TCID_{50}$) of DV2 gave a result of 13 x 10^4 pfu/ml. The results also indicated that 10^{-3} dilution of DV2 treated with 0.032 mg/ml of *Q. lusitanica* extract yielded 3.5 x 10^4 pfu/ml (Fig 2, panel C), while 0.063 mg/ml *Q. lusitanica* extract gave 1.5 x 10^4 pfu/ml (Fig 2, panel D).

DISCUSSION

Q. lusitanica has been used in Oriental traditional medicine for treating inflammatory diseases (British Pharmaceutical Codex, 1911), and as an astringent and anti diabetic (Hwang *et al*, 2000). In this study, the protective efficacy of this extract was demonstrated by the inhibition of DV2 replication, as indicated by the relative absence or reduction of CPE in virus inhibition assays in the concentration 0.032 to 0.25 mg/ml. This technique had also been employed by Premnathan *et al* (1996), Parida *et al* (2002), and Muliawan *et al* (2006). Plaque assay remains the standard for the titration of neutralizing antibodies. Standard methods for titration of dengue virus and measuring the ability of antiviral to neutralize the virus are based on plaque assays that require 5-7 days to complete (Lambeth *et al*, 2005). In the present study, the plaque assay technique was a reliable way to see the ability of *Q. lusitanica* as an antiviral to neutralize the DV2 onto pig spleen cells. We hope future researchers will continue this line of research using this plant extract. In conclusion, *Q. lusitanica* extract has inhibitory potential against DV2 viability in a dose-dependent manner.

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