INHIBITORY POTENTIAL OF *QUERCUS LUSITANICA* EXTRACT ON DENGUE VIRUS TYPE 2 REPLICATION

Sylvia Y Muliawan[1], Lam Sai Kit, Shamala Devi[2], Onn Hashim[3] and Rohana Yusof[3]

[1]Department of Microbiology, Faculty of Medicine, Trisakti University, Jakarta, Indonesia; [2]Department of Medical Microbiology, [3]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract. This study reports the *in vitro* inhibitory potential of crude extract of *Quercus lusitanica* (*Q. lusitanica*) seeds on the replication of dengue virus type 2 (DEN-2). *In vitro* antiviral activity of *Q. lusitanica* extract, assessed in C6/36 cells (cloned cells of *Aedes albopictus* larvae) employing a virus inhibition assay, showed dose-dependent inhibition. The *Q. lusitanica* extract at its maximum non-toxic 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 NS1 is a glycoprotein present in all flaviviruses and appears essential for virus viability. To further evaluate *Q. lusitanica* extract as an antiviral compound, we investigated the effect of *Q. lusitanica* extract on the NS1 protein expression of infected C6/36 cells through proteomics technique. The result showed downregulation of NS1 protein expression of infected C6/36 cells after treatment with this extract. In conclusion, we found that *Q. lusitanica* extract has a good inhibitory effect on the replication of dengue virus type 2, both in conventional cell culture and proteomics technique.

INTRODUCTION

Mosquitoes of the genus *Aedes* transmit dengue virus and predominantly cause a debilitating illness known as dengue fever. Mosquito control programs are difficult to implement and maintain, thus making the development of new antiviral drugs and a safe vaccine imperative. New antiviral agents of plant origin can be easily accepted, being non-toxic and inexpensive. Several studies have investigated whether commercially available plant extracts have an inhibitory effect on certain diseases. The present study investigated whether *Q. lusitanica* had an inhibitory effect on dengue virus type 2 (DEN-2) replication. *Q. lusitanica*, often known as *Q. infectoria*, is a small tree or shrub from the Mediterranean area, mainly present in Greece, Asia Minor, Syria, and Iran. The galls of *Q. lusitanica* have great medicinal value and have pharmacologically been deciphered to be adstringent, antidiabetic, antitremorine, local anesthetic, an antipyretic used over the centuries, and antiparkinsonian (Dar *et al*, 1976; Dar and Ikram, 1979; Hwang *et al*, 2000). In Asian countries, the galls of *Q. lusitanica* have been used for centuries in oriental traditional medicine for treating inflammatory diseases (Galla, 1911). However, there have been no reports on the antiviral activity of *Q. lusitanica* against DEN-2. This led us to study the effect of *Q. lusitanica* for anti-dengue virus 2 activity. In a previous study, we investigated dengue virus type 2 nonstructural NS1 protein expression in C6/36 cells (Muliawan *et al*, 2003; Muliawan, 2005) using proteomics technique. In the present study, we characterized the profile of NS1 protein expression using the same technique, to clarify the inhibitory effect of *Q. lusitanica* on DEN-2 replication.

Correspondence: Sylvia Y Muliawan, Department of Microbiology, Faculty of Medicine, Trisakti University, Jakarta 11440, Indonesia.
Tel: (62)(21)-5672731, -5655786;
Fax: (62)(21)-5660706
E-mail: sylviaym2003@yahoo.com

MATERIALS AND METHODS

Preparation of *Q. lusitanica* extract

In this study, we used a methanol extract *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 the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia, and maintained in RPMI-1640 supplemented with 10% fetal calf serum. Confluent monolayers of C6/36 cells were grown in 24-well microtiter (MT) Linbro plates for performing *in vitro* antiviral efficacy assays of the *Q. lusitanica* extract.

Virus

Dengue virus 2 (DEN-2) New Guinea C strain,

obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia was adapted in C6/36 cell culture and day-old suckling mice for *in vitro* testing. C6/36 cell culture antigen and 20% mouse brain antigen were titrated to determine the tissue culture infective dose 50% ($TCID_{50}$) for use in *in vitro* testing as per the method described by Schmit (1979).

Maximum non-toxic dose (MNTD)

Prior to screening the *Q. lusitanica* extract for inhibitory potential, it was subjected to toxicity studies to determine the MNTD to C6/36 cells (*in vitro*) system as per the method described by Van den Berghe *et al* (1978), and Schmidt (1979). Briefly, serial two-fold dilutions of this extract were added to 24-well Linbro plates containing confluent monolayer C6/36 cells with suitable cell control. The inoculated cells were observed daily up to day-4 for the appearance of toxicity in the cells

Determination of antiviral activity

***In vitro* virus inhibition assay.** The *in vitro* inhibitory potential of *Q. lusitanica* was evaluated in C6/36 cells by virus inhibition assay, as described by John and Mukandan (1978), and Premnathan *et al* (1996). Serial two-fold dilutions of non-toxic doses of *Q. lusitanica* (0.016-1 mg/ml) were prepared in RPMI-1640. Simultaneously, serial 10-fold dilutions (10^{-1}-10^{-3}) corresponding to 1,000-10 $TCID_{50}$ were prepared separately. Different doses of *Q. lusitanica* extract were added to Linbro plates using the technique described above, with added virus control. The plates were incubated for 1 hour at room temperature, then each virus dilution was added in equal proportions. The plates were then incubated at 28°C and observed daily up to day-4 for the development of cytopathic effect (CPE).

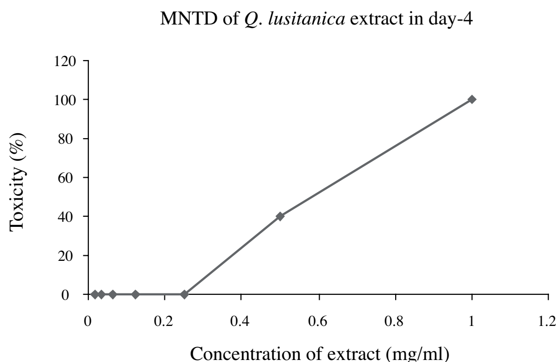

Fig 1- Relative toxicity and maximum non-toxic dose (MNTD) of *Q. lusitanica* extract *in vitro*.

Non structural NS1 protein expression. To demonstrate the inhibitory effect of *Q. lusitanica* extract against DEN-2 on NS1 protein expression, two-dimensional gel electrophoresis (2-DGE) was performed using the Multiphore II Electrophoresis System based on the recommended method by Amersham Biosciences (Berkelman and Stenstedt, 1998). The sample was diluted in buffer containing 8M urea, 0.5% (v/v), Triton X-100, 0.5% (v/v), IPG-phor buffer 3-10 and 12 mM dithiothreitol (DTT). Pre-cast immobilized dry strips pH 3-10 (Amersham Biosciences) were rehydrated overnight in the same solution. The strips were aligned in a tray filled with mineral oil (Amersham Biosciences). Isoelectric focusing was performed in three steps by varying the voltage as suggested by the manufacturer. For the second dimension, the strips were incubated for 15 minutes in a solution consisting of 6M urea, 1% (w/v) SDS, 30% (v/v) glycerol and 0.3M DTT that were dissolved in 0.05M Tris-HCl (pH 6.8). This was followed by incubation for another 15 minutes in a similar solution but containing 50 mM iodoacetamide instead of DTT. Strips were placed on a SDS-PAGE 10% homogenous gel (Amersham Biosciences) and electrophoresed for 100 minutes. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed according to Laemmli (1970). SDS-PAGE gels were developed by silver staining according to the method of Heukeshoven and Dernick (1988). In this study, 2-DGE was performed on C6/36 cells treated with low concentrations (0.006 to 0.013 mg/ml) and high concentrations (0.025 to 0.1 mg/ml) of *Q. lusitanica* extract followed by infection with varying dilutions of DEN-2, then the cells were harvested and 2-DGE was performed.

RESULTS

Evaluation of the inhibitory potential of *Q. lusitanica* on DEN-2 *in vitro* was preceded by cytotoxicity studies to determine the maximum non-toxic dose (MNTD) for virus inhibition assay. The maximum non-toxic dose (MNTD) of various preparations of *Q. lusitanica* is shown in Fig 1. The MNTD for *Q. lusitanica* extract was 0.25 mg/ml. *In vitro* inhibitory potential of *Q. lusitanica* extract on DEN-2 in C6/36 cells revealed inhibition of virus replication in a dose-dependent response, as shown in Fig 2. The *Q. lusitanica* extract at its maximum concentration (0.25mg/ml) showed 100% inhibition on replication of the whole range of virus concentrations employed in the present study, as indicated by the absence of CPE (Fig 2). The low dose of *Q. lusitanica*

(0.032 mg/ml) showed 100% inhibition with 10 TCID50 of virus, but only 50% and 25% inhibition of 100 and 1,000 TCID50 of virus, respectively (Fig 2). When comparative analysis was made between 2-DGE protein profiles of C6/36 cells infected by DEN-2 and uninfected C6/36 cells, it was noted that the NS1 protein expression spots, which migrated at the same molecular weight (50 kDa) but with slight difference on pI, were present in cells infected with DEN-2 (Fig 3a), but not in uninfected cells (Fig 3b) (Muliawan *et al*, 2003; Muliawan, 2005). To investigate the inhibitory effect of *Q. lusitanica* extract against DEN-2 on protein expression, 2-DGE was performed; the results showed that as the concentration of *Q. lusitanica* extract was increased, expression of the nonstructural NS1 was downregulated (Fig 4a, 4b). However, at a higher concentration of *Q. lusitanica* extract (0.1 mg/ml), the NS1 protein could not be detected (Fig 4b).

DISCUSSION

Q. lusitanica has been used in Oriental traditional medicine for treating inflammatory diseases (Galla, 1911), as an adstringent, anti-diabetic, etc (Dar *et al*, 1976; Dar and Ikram, 1979; Hwang *et al*, 2000). In this study, the protective efficacy of this extract was proven by the inhibition of DEN-2 replication, as indicated by the relative absence or reduction of CPE in virus inhibition assay in concentrations of 0.032-0.25 mg/ml. This technique had also been employed by John and Mukandan (1978), Premnathan *et al* (1996), and Parida *et al* (2002). The nonstructural NS1 is a glycoprotein present in all flaviviruses, and appears to be essential for virus viability. Intracellular NS1 has also been shown to be involved in the early stage of viral replication (Mackenzie *et al*, 1996; Muylaert *et al*, 1996, 1997) in agreement with its retention in intracellular organelles of the infected cell (Mason, 1989; Post *et al*, 1990) and its ability to interact with membranes (Winkler *et al*, 1989; Flamand *et al*, 1992). In the present study, NS1 was used to indicate dengue infection and to evaluate the inhibitory potential of *Q. lusitanica* extract against DEN-2 replication. This study showed that *Q. lusitanica* extract has the ability to downregulate NS1 protein expression, and this could be related to the reduction or absence of cytopathic effect on DEN-2-infected C6/36 cells in antiviral testing. Since there were no previous reports of studies employing this technique and/or material, we could not obtain comparative data to support our results. Therefore, it is hoped that future researchers will continue this line of research. In conclusion, *Q. lusitanica* extract has inhibitory potential against DEN-2 replication in a dose-dependent manner.

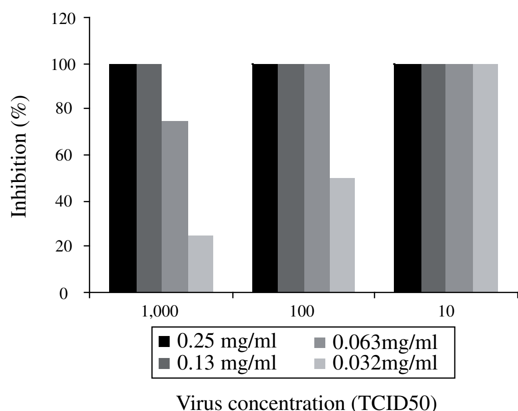

Fig 2- Inhibitory potential of *Q. lusitanica* extract on DEN2.

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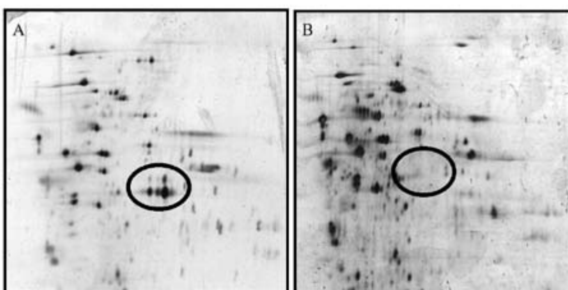

Fig 3- Protein profile of C6/36 cells with silver staining. (A) infected cells with DEN2 and (B) uninfected cells.

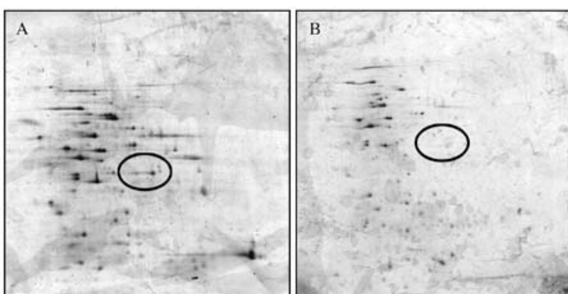

Fig 4- Protein profile of infected C6/36 cells after treatment with *Q. lusitanica* (silver staining). (A) low concentration (0.006-0.013 mg/ml) and (B) high concentration (0.025-0.1 mg).

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