IN VITRO ANTIVIRAL ACTIVITY OF ORTHOSIPHON STAMINEUS EXTRACT AGAINST DENGUE VIRUS TYPE 2

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ABSTRACT

In this study, *Orthosiphon stamineus* aqueous leaves extract was tested against dengue virus type 2. Cytotoxicity screening against Vero cells using MTT assay showed that the CC50 values for extract is 5 mg/ml. The antiviral activity towards Dengue virus type 2 (DENV-2) was investigated by observing the morphological changes, which were further confirmed the cellular viability evaluated by MTT technique. The selective indices (SI = CC50 / EC50) for the extract is 13. These results demonstrate that the extract prepared from *O. stamineus* possesses phytochemical compound that was non cytotoxic to the cell with potential antiviral activity. These species are good candidates for further activity-monitored fractionation to identify active principles.

Keywords: DENV-2, *Orthosiphon stamineus*; MTT assay; dengue virus 2; non cytotoxic.

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1. INTRODUCTION

1.1. Dengue Virus

Dengue virus (DENV) belongs to the family of Flaviviridae, which is a large family of viruses consisting of three genera; Flavivirus, Pestivirus and Hepacivirus. DENV is one of over 70 members of the genus Flavivirus causing severe disease and mortality in both humans and animals [1]. Dengue is the most important viral infection transmitted among humans by arthropod-borne [2-3]. The disease is endemic in more than 100 countries throughout Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific. The incidence of dengue has grown dramatically in the world in recent decades. It has been reported that over 2.5 billion people are now at risk for dengue infection [4].

In Malaysia, a 277% increase in cases was reported in early 2014 compared with the same period in 2013 [5]. There are currently no vaccines or specific therapeutical treatment. There are four distinct serotypes of dengue virus and each of these serotypes can cause disease symptoms ranging from self-limited febrile illness called dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [6-7]. Infection with one serotype confers protective immunity against that serotype, but not against other serotypes. In fact, several retrospective and prospective studies have revealed that secondary infection with a heterologous serotype is a risk factor for developing DHF/DSS [8-9].

1.2. *Orthosiphon Stamineus*

Orthosiphon stamineus Benth (family: Lamiaceae) or Misai Kucing (Malay for "Cat's Whiskers") is commonly used as Java Tea [10]. Misai Kucing is a native plant in South East Asia (Malaysia, Indonesia and Thailand) and some part of Tropical Australia. Orthosiphon stamineus has been used to treat urinary lithiasis, edema, eruptive fever, influenza, rheumatism, hepatitis and jaundice [11]. Moreover, it has been scientifically proven that *O. stamineus* exhibits a range of pharmacological properties such as anti-inflammatory, anti-oxidant, anti-bacterial, anti-angiogenesis properties and as hepatoprotective effects [12-14]. *O. stamineus* is rich in the active chemical compounds such as stereos, oleanolic acid, polyphenols, flavonoids and terpenoids. Polyphenol which is the most dominant compound in *O. stamineus* leaves prevents the formation of lipid peroxidation products in the biological

system, and has a considerable role in reducing oxidative stress. Furthermore, high amount of flavonoids such as eupatorin, sinensetin, rosmarinic acid and quercetin was also detected in different tissues of this plant. Rosmarinic acid are described as antioxidant, antimicrobial, antiviral and antiinflammatory while the biological activity of rosmarinic acid was reported to have antibacterial, antiviral and antioxidative [15].

2. METHODOLOGY

2.1. Plant Materials and Extraction

One hundred grams of *O. stamineus* powder was dissolved in 1 liter of water and kept on the automatic shaker for 24 hours for extraction of water-soluble compounds. The extract was filtered through Buchner funnel using vaccume pump connected to side arm flask. The filtrate thus collected was centrifuged at 3,000 rpm for 10 min to remove the particulate substances. The clear supernatant was freeze dried to obtain the fine powder. The freeze-dried powder was stored in the freezer till the use.

2.2. Cell Lines and Growth Conditions

Two types of cell lines were used in this study, C6/36 cells and Vero cells. C6/36 cells were maintained in L-15 medium (Sigma) supplemented with 5% fetal bovine serum (FBS) at 28 °C. Vero cells (ATCC CCL- 81) was initiated from the kidney of a normal adult African green monkey were maintained in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C with 5% carbon dioxide.

2.3. Virus Stock

Dengue virus type-2 (DENV-2) used in this study is a prototype of the New Guinea C strain, a kind gift from the Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia. The virus stock was prepared in T75 cm^2 tissue culture flasks by inoculating 70-80% confluent C6/36 cells with $200 \mu L$ virus stock diluted in 2mL of medium supplemented with 1% FBS. After 1.5h of viral adsorption, a 1% FBS complete growth medium was added and the virus was allowed to propagate at 28 °C until cytopathic effects (CPE) were observed. The cells and the culture supernatant were then harvested by gentle pipetting followed by centrifugation at 1500 rpm for 10 min. The viral supernatant was

collected in 1 mL aliquots and was stored at -80 °C as a viral stock until further use.

2.4. Cytotoxicity Test

As for the extract cytotoxicity evaluation, the Vero cells (2.5×105 cells/mL) were seeded into 96-well plates and incubated overnight at 37°C. Upon 80% confluence, the cells were treated with several concentrations of extract, ranging from 10 mg/ml to 0.31 mg/ml. Cells with only growth medium (DMEM) were used as negative control. After incubation of about 72h, the growth medium was discarded and replaced with 100 μL of 3(4,5-dimethythiazol-2-yl)2,5-diphenyl tetrazolium bromide or MTT solution and incubated for 3h. After that, the MTT solution was discarded and formazan crystal was dissolved using 100 μL of dimethyl sulphoxide (DMSO) to lyse the cells. Colour development was detected using a microplate reader (TECAN Infinite 200 PRO) at 540 nm. The percentage of living cells was calculated by comparison with healthy untreated cells.

2.5. Determination of Median Tissue Culture Infective Dose (TCID50)

A total of 1.5×10^4 cells/well were seeded into 96-well plate and incubated at 37°C with 5% CO_2. After 24 hours, a total volume of 100 μL of ten-fold serially diluted DENV-1 was inoculated into each well with 10 replicates for each dilution. Plates were further incubated at 37°C for 5 days, after which cytopathic effect (CPE) was observed microscopically under inverted microscope. To determine the TCID50 based on Karber method [23], the presence of CPE in each well was marked as '+', while its absence was marked as '-'. The proportion of wells with CPE in each serially diluted DENV-1 was calculated and the TCID50 was estimated using the formula 'Log TCID50 = L - d (s - 0.5) ', whereby L = lowest dilution factor; d = difference between dilution steps; s = sum of proportion. The value of TCID50 determined was applied in the antiviral assay.

2.6. Antiviral Assay

Screening for antiviral activity was performed using two different treatments, pre-treatment and post-treatment. To screen for antiviral activity, Vero cell monolayer were grown in 96 well microtiter plates. Controls consisted of untreated infected, treated noninfected and untreated noninfected cells. The *O. stamineus* aqueous leaves extract concentration tested was twice lower than the CC50 value in order to reduce the possibility of toxicity towards the cells.

The cellular viability was evaluated by MTT technique.

2.6.1. Pre-Treatment of Plant Extracts on DENV-2 Infected Cells

Different concentrations of each extract were added in triplicate to Vero and C6/36 monolayer cells in 96-well plates for 24h. Subsequently, the extracts were removed by washing twice with PBS and the cells were challenged with 100 µL of DENV-2 at its TCID50. After viral infection for 1.5h, the cells were washed twice with PBS to remove any residual unbound viruses and were overlaid with culture medium containing 1% FBS complete growth medium. The cells were observed daily for any morphological changes and at day 7 post infection, the cellular viability was evaluated by MTT technique.

2.6.2. Post-Treatment of DENV-2 Infected Cells with Plant Extracts

In the post treatment assay, Vero cells grown in 96-well plates were infected with 100 µL of DENV-2 at its TCID50. After 1.5h of viral infection, the cells were washed twice with PBS to remove any residual unbound viruses. This was followed by the addition of serial dilutions of plant extracts in triplicate. Seven days after infection, the cellular viability was evaluated by MTT technique.

2.7. Data Analysis

The 50% cytotoxic concentration (CC50) and the 50% inhibitor concentration of the viral effect (EC50) for each extract were calculated from concentration-effect-curves after linear regression analysis. The therapeutic index or selective index is defined as CC50 over EC50.

3. RESULTS AND DISCUSSION

Novel therapeutic options are urgently needed to improve global treatment of virus infections. Natural products from higher plants and marine organisms represent an almost inexhaustible resource of antiviral drug leads [16-17]. Several natural compounds including polysaccharides, flavonoids, terpenes, alkaloids, phenolics and amino acids showing antiviral activity have been isolated from plants used in traditional medicine [18-19]. These compounds may be involved in the defense of the plants against invading pathogens such as insects, bacteria, fungi and viruses [20]. *O. stamineus* is a traditional medicinal herb with various and dynamic medicinal properties [21-22]. *O. stamineus* was shown to exhibit antiviral activity against

HIV virus through their high content rosmarinic acid [23-24]. *O. stamineus* also showed antibacterial activity against Gram negative bacteria [25]. Currently, there is no anti dengue compound known to be isolated from *O. stamineus*.

In this study, the potential use of this plant to inhibit in vitro DENV-2 replication was investigated. In this study, *O. stamineus* aqueous leaves extract were examined for their antiviral activity against DENV-2. The antiviral activity and cytotoxicity of plant extract were determined using MTT method by measuring the absorbance value at wavelength 540 nm. The cytotoxicity of these extracts were further evaluated by MTT method to determine the non-toxic concentration in Vero cell. Evaluation of cytotoxicity is an important part of the assessment of the potential antiviral agent since the beneficial extracts should be selective for virus-specific processes with little or no effects on metabolism of host cells [26].

To determine the nontoxic dose, Vero cells were exposed to two fold serially diluted extract at concentrations ranging from 0.31 to 10 mg/mL. The cytotoxicity assay result, as presented in Fig. 1 shows the percentage of cell viability versus extract concentration. The estimated CC50 value towards the Vero cells was ~ 5 mg/ml.
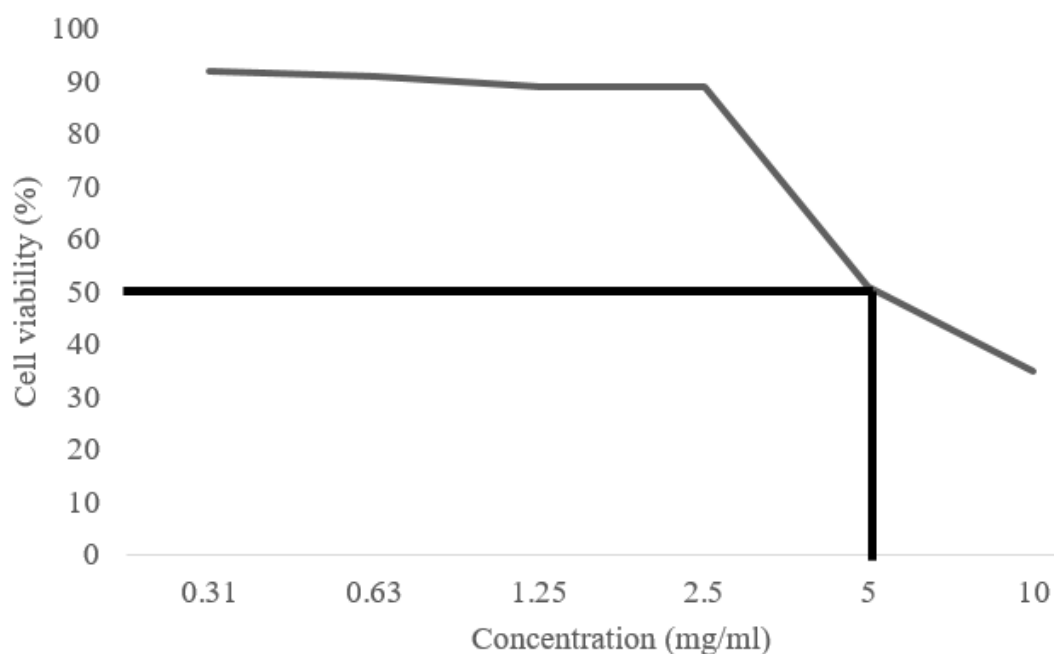

Fig.1. Cytotoxicity assay of *O. stamineus* extracts against Vero cells. The assay was performed after 48 hours of treatment with various concentrations of the plant extracts

There is no signs of toxicity were observed in Vero cells when the extract were added at concentrations ranging from 0.31 to 2.5 mg/mL. The cells showed a healthy monolayer

similar to the healthy control with no loss of monolayer and no obvious rounding, granulation or shrinking of cells was observed. However, the antiviral assay was pursued further as the concentration used in this assay was lower than the CC50 value. The EC50 value of the extracts tested against the DENV-2 was ~ 0.36 mg/ml, as shown in Fig. 2. Cytotoxicity and anti-DENV-2 activity of plant extracts on Vero cells expressed as CC50 and EC50. The therapeutic index or selective index is defined as CC50 over EC50 [27]. The effectiveness of the extract as an antiviral compound expressed as selectivity index (SI) revealed that the extract had greater SI value of 13. Any antimicrobial compound that has SI values higher than 10 (SI > 10) ensures the potential to be developed as an agent of antiviral drug [28]. The extracts were further evaluated for their prophylactic effect (pre-treatment) and the ability to inhibit replication following infection of the cells with the virus (post-treatment).
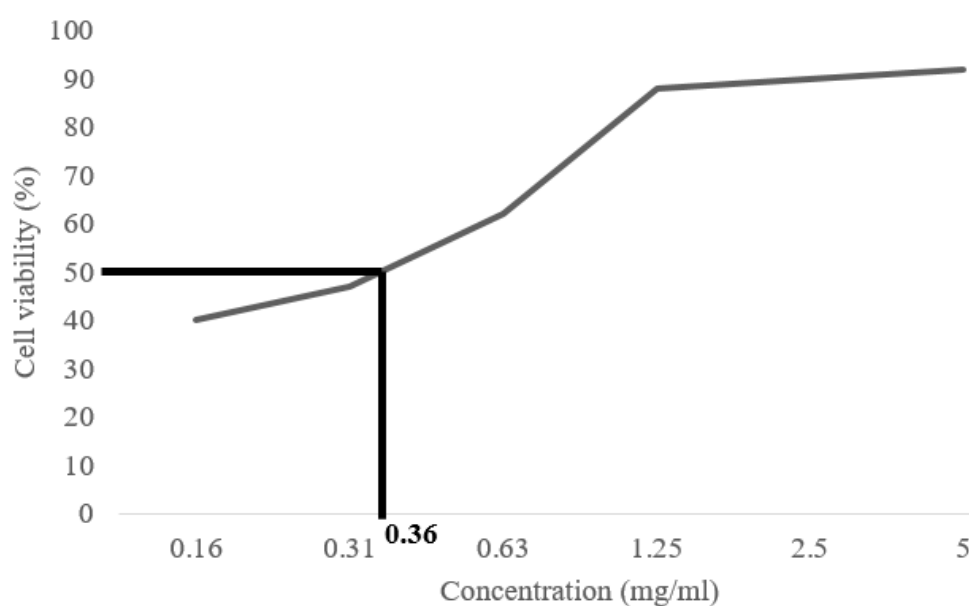

Fig.2. Determination of Extracts effective concentration (EC50)

Table 1 shows the comparisons in the cell survival when infected with DENV-2 and either pre-treated or post-treated cell with extract. The extract showed the capability to decrease DENV-2 replication more in the pre-treated cell compared to the post-treated cell. Cells that were treated for one day with the extract before virus was added, its TCID50 showed control to viral replication implying the ability to act as a prophylactic substance against viral infection (Table 1). Extract provided better protection to the cells in the pre-treatment protocol, suggesting that the potential sites of activity may include inhibition of virus binding and/or

entry which can be mediated by a number of cellular receptors such as CD46 present on Vero cells. Both host and viral surface receptors that have the carbohydrate components, focusing on the surface interactions in the early stage of flavivirus entry. The extract might interrupt the interaction resulting in ineffective viral infection [29]. Extract was found not to have effective effect in inhibiting cell death in post-treatment mode. This suggested that virus inoculated cells were not able to overcome viral infection when treated with the extract. Further purification of the extract will allow the determination of compounds able to inhibit DENV-2 replication.

Table 1. Comparison between the infected DENV-2 pre-treated and post-treated cells with extract

O. Stamineus Aqueous Leaves Extract Concentration (mg/ml)	Post-Treated	Pre-Treated
	% Cell Survival	
0.63	18	81
0.31	20	88
0.16	23	72

4. CONCLUSION

Studies on *O. stamineus* that showed anti-dengue properties should be further conducted extensively. Isolation, purification and characterization of the active compounds in order to discover the potential anti-dengue compounds should be carried out.

5. ACKNOWLEDGEMENTS

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