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Research Article
In vitro study, Antiviral Activity of Styrylpyrone Derivative Against Dengue Virus Type 2

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Abstract

Background and Objective: Secondary metabolites derived from *Goniothalamus umbrosus* were found to have a lot of beneficial bioactive effects on human health, such as antibacterial, antivirus, anti-tumor and anti-malaria. In this study, Styrylpyrone Derivative (SPD) extracted from *G. umbrosus* root was tested against dengue virus type 2 (DENV-2) replication. **Materials and Methods:** In the present study, cytotoxicity of SPD was evaluated by a cell viability assay using 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) assay. Focus forming unit reduction assays were carried out to evaluate the antiviral activity of SPD against DENV-2. These include post-treatment, pre-treatment and virucidal assays. **Results:** Cytotoxicity test of SPD in Vero cells showed the concentration of 50% of cytotoxicity (CC_{50}) was 42.5 μM (8.5 μg mL^{-1}), this indicates that SPD is non-cytotoxic. High antiviral activity was observed in post-treatment with 100% reduction of DENV-2 foci at 25 μM of SPD. In the pre-treatment assay, more than 50% foci reduction was observed using the same concentration of SPD was observed. Whereas, more than 50% foci reduction was observed at 25 μM SPD in the virucidal assay. **Conclusion:** The findings indicated that the SPD from *G. umbrosus* has good potential for a prospective nature-based antiviral drug.

Key words: Cytotoxicity, MTT, Styrylpyrone Derivative, *Goniothalamus umbrosus*, Dengue Virus Type 2, antiviral activity, focus forming unit reduction assay

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dengue infection is caused by the dengue virus (DENV). DENV belongs to the genus Flavivirus in the family Flaviviridae. Dengue infections caused by the four antigenically distinct dengue virus. Despite these variations, infection with each of the dengue serotypes results in the same disease and range of clinical symptoms[1]. The virion contains a positive-sense, single-stranded RNA molecule of approximately 11 kb in length. Three are structural proteins: the capsid (C), envelope (E) and membrane (M) proteins. Seven are nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. These nonstructural proteins play roles in viral replication and assembly[2]. DENV is transmitted principally by *Aedes aegypti* mosquito[3]. Dengue virus infections may be asymptomatic or may lead to undifferentiated fever, Dengue Fever (DF) or Dengue Hemorrhagic Fever (DHF) with plasma leakage that may lead to hypovolemic shock (dengue shock syndrome, DSS)[4]. At present, there is no specific antiviral treatment currently available for dengue fever[5]. Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection by inhibiting the replication of several viruses[6]. However, very little is known about the potential of plants against the dengue virus.

Secondary metabolites derived from *Goniothalamus* spp. were found to have numerous useful bioactive effects on human health, such as anti-tumor, anti-aging, anti-stress and anti-malaria[7,8]. Earlier studies showed that SPD has the potential to be evaluated for further development as antibacterial and antiviral drugs[9-12]. SPD inhibits during early virus replication[13]. SPD was tested against DENV-2 in post-treatment, pre-treatment and virucidal assays to determine the mode of action during the viral infection. In this study, styrylpyrone derivative (SPD), which was extracted from *Goniothalamus umbrosus*, was tested against dengue virus type 2 (DENV-2).

MATERIALS AND METHODS

Study area: The present study was carried out in the postgraduate laboratory of Faculty of Medicine, Universiti Sultan Zainal Abidin, Malaysia. The total duration of this study was from 9 September, 2016- 9 January, 2019.

Cells and virus: 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, derived from the kidney of an African green monkey, were maintained in Dulbecco's Modified Eagle Medium (DMEM) at 37°C with 5% carbon dioxide. 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 inoculating70-80% confluent C6/36 cells with 200 µL virus stock diluted in 2 mL of medium supplemented with 1% FBS. After 1.5 h 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. The virus titer was determined by focus forming unit reduction assay using Vero cells.

Cytotoxicity test: As for the extract cytotoxicity evaluation, the Vero cells (2.5×10^5 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 SPD. Cells with only growth medium (DMEM) were used as a negative control. After incubation of about 72 h, the growth medium was discarded and replaced with 100 µL of 3(4,5-dimethylthiazol-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 sulfoxide (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.

Antiviral assays: The DENV-2 was isolated from the infected Vero cells using standard protocols[14]. Screening for antiviral activity was performed using three different treatments[15]. 1) Post-treatment (C+V)+E: The Vero cells (C) were infected with the virus (V) and underwent 1h of incubation, then the mixture of SPD (E) and DMEM were inoculated on the plate and incubated for 4 days. 2) Pre-treatment (C+E)+V: The cells (C) were incubated with SPD (E) for 5h before they were inoculated with a virus (V). 3) Virucidal C+(E+V): Virus (V) with SPD (E) was incubated for 1h before it was inoculated on the cells (C). The SPD concentration tested was twice lower than the CC_{50} value in order to reduce the possibility of toxicity towards the cells. Antiviral activities of the compounds were

determined by calculating the percentage of foci reduction (% RF) compared against the controls maintained in parallel using the following formula:

$$RF\ (\%) = (C\text{-}T) \times 100/C$$

where, C is the mean of the number of foci from triplicates treatment without compound added and T is the mean of the number of foci from triplicates of each treatment measures with the respective compound.

The effectiveness of SPD as an antiviral agent expressed as selectivity index (SI) calculated as below:

$$\text{Selectivity Index (SI)} = \frac{\text{Cytotoxicity concentration } (CC_{50})}{\text{Effective concentration } (EC_{50})}$$

RESULTS

Cytotoxicity of SPD. To determine the nontoxic dose, Vero cells were exposed to twofold serially diluted SPD at concentrations ranging from 100 to 3 µM. The cytotoxicity assay result, as presented in Fig. 1, shows the percentage of cell viability versus SPD concentration. The estimated CC_{50} value towards the Vero cells was 42.5 µM (8.5 µg mL^{-1}). From this value, SPD can be considered to have low cytotoxicity as the CC_{50} value was lower than 50 µM (10.0 µg mL^{-1})[16]. However, the antiviral assay was pursued further as the concentration used in this assay was lower than the CC_{50} value. The 50% cytotoxic concentration (CC_{50}) was defined as the SPD concentration (µM) required for the reduction of cell viability by 50%, which were calculated by regression analysis. Tangent was drawn in the determination of SPD cytotoxic concentration (CC_{50}) graph to indicate the CC_{50} value of SPD.

Anti-DENV-2 activity of SPD: Focus Forming Unit Reduction Assays (FFURA) was done to screen for anti-DENV-2 activity using SPD with different concentrations. Fig. 2 shows the percentage of foci forming reduction in post-treatment, pre-treatment and virucidal assays, respectively. The results from post-treatment assay showed that 100% foci reduction was achieved at a concentration of 25 µM. In the pre-treatment assay, more than 50% foci reduction was observed at 25 µM. For the virucidal assay, SPD at a concentration of 25 µM enough to gain more than 60% inhibition of DENV-2 foci.

The effectiveness of certain compounds or extracts can be evaluated by using selective index (SI). The evaluation considered the toxicity value as well as the antiviral concentration for a specific cell line. Therefore, the SI was calculated for SPD in post-treatment, pre-treatment and virucidal assay. Comparison of SI for SPD between post-treatment, pre-treatment and virucidal is shown in Table1. SI for post-treatment of SPD showed high SI values compared to those obtained from pre-treatment and virucidal assays.

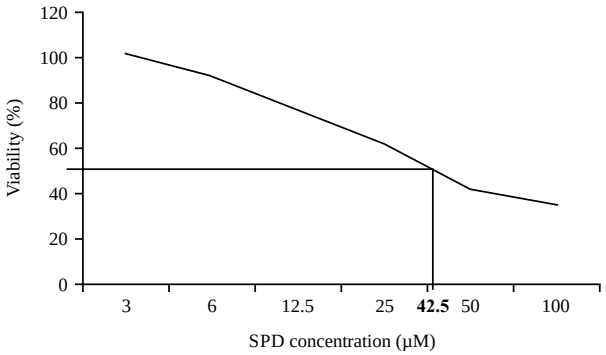

Fig. 1: Determination of SPD cytotoxic concentration (CC_{50})

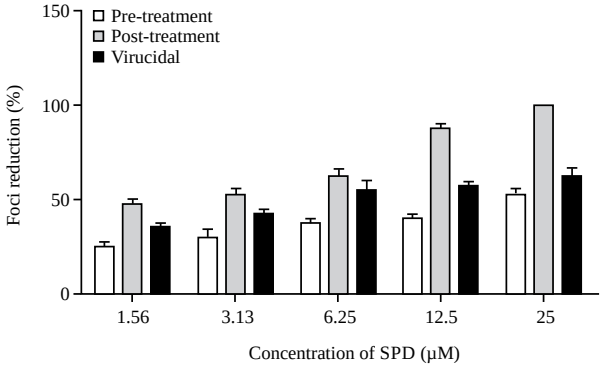

Fig. 2: Antiviral activities of SPD against DENV-2 via post-treatment, pre-treatment and virucidal assay

Table 1: CC_{50}, EC_{50} and SI values of SPD in post-treatment, pre-treatment and virucidal assays

Treatments	CC_{50} (µM)	EC_{50} (µM)	SI
Post-treatment	42.5	2.18	19.5
Pre-treatment	42.5	22.8	1.9
Virucidal	42.5	5.0	8.5

CC_{50}: Cytotoxic concentration of SPD; EC_{50}: Effective concentration of SPD; SI: Degree of selectivity

DISCUSSION

Natural products and traditional medicines have their incomparable advantages and recently getting significant attention in global research[17]. *Goniothalamus umbrosus* J. Sinclair, or locally known as kenerak, is a tropical plant under

the Annonaceae family. This plant is traditionally used as an anti contraceptive, treating fever, malaria and as postpartum treatment[18].

Recently, SPD isolated from *G. umbrosus* was shown to exhibit antiviral activity against Herpes Simplex Virus type-1 (HSV-1)[19]. In this study, the potential use of SPD isolated from *G. umbrosus* to inhibit *in vitro* DENV-2 replication was investigated. Currently, there is no anti-dengue compound known to be isolated from *G. umbrosus*. The plant is known to contain naphthalene derivatives, eudesma-4(14),7(11)-diene, 1butyl-2-cyclohexene-1-ol, benzaldehyde and globulol[20]. These compounds were proposed to exert antibacterial and anticancer potential. Another group of phytochemicals in *Goniothalamus* species is styrylpyrone derivatives. Styrylpyrone derivatives were among styryl lactones being isolated from several *Goniothalamus* species[21].

In this study, SPD was investigated whether these secondary metabolites could confer protection to cells before or after the initiation of DENV-2 infection. The ability of SPD to confer protection to the cells before DENV-2 infection was tested by pre-treating the cells with SPD for 5h prior to viral infection. Post-treatment assay was conducted to investigate whether the intracellular activities, such as DENV-2 viral RNA replication or viral protein translation and assembly in infected cells, could be affected. The virucidal assay was performed to exert significant virucidal activity against extracellular DENV-2 particles.

The SPD showed the capability to decrease viral replication more in the post-treated cell compared to the pre-treated cell. This result demonstrated that the SPD could control viral infection after 1 h, which had been the early stage of replication involving the attachment of virus. This outcome suggested that the SPD was effective in controlling virus post-infection. The effectiveness of the SPD as an antiviral compound expressed as a selectivity index (SI). Post-treatment of SPD has the potential to be exploited where the SI values were more than 10. Antiviral agent with an SI value of more than 10 can be considered to possess high potential to be developed as antiviral drug[22]. However, the result of SI for pre-treatment suggests that SPD is not suitable to be used as a prophylactic agent. Results showed that SPD exerts virucidal activity against DENV-2. It is a big advantage for an antiviral drug to exhibit virucidal activity as well, as it would directly affect the virus without affecting the cells. It is also important to have antiviral drugs that possess different antiviral mechanisms because of the unavailability of safe vaccines and effective antiviral drugs against the dengue virus. This study provides important novel insights into the phytomedicinal properties of SPD on the dengue virus. In the future, a more in-depth study will be necessary to elucidate the mechanisms of inhibition OF SPD towards DENV-2 replication.

CONCLUSION

This study demonstrates the ability of SPD extracted from the root of *G. umbrosus* as a novel anti-DENV-2 agent via post-treatment, pre-treatment as well as a virucidal assay. All the conducted assays proved a high percentage of focus reduction in infected cells after exposure to SPD. SPD exhibited lower cytotoxicity effect towards Vero cells. This implies that SPD might be more suitable to be used as a long-term regime in combating DENV infections. Although focus reduction assays exhibited promising antiviral properties of SPD.

SIGNIFICANCE STATEMENT

This study discovered the antiviral properties of SPD isolated from *G. umbrosus* root. The result generated from this study can be used to supports further investigations directed on anti-DENV drug development and clinical evaluation for the treatment of DENV-2 infection. There is an urgent need to explore new therapy for DENV-2 infection because there is no specific antiviral treatment currently available for dengue infection. Alternative therapy using plant-based products is hoped to offer an alternative chemotherapy regiment hopefully with lower side effects.

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