A Preliminary Study on the Anti-dengue Viral Activity of *Munronia pinnata*

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Abstract

Dengue infection is a major health care problem in tropical and subtropical countries. The recently approved dengue vaccine has limitations, and there is no antiviral drug for treatment at present. For centuries plants and plant extracts have been used in traditional medicine for the treatment of various infections. The whole plant of *Munronia pinnata*, which has been used for treating fever patients in Sri Lankan traditional medicine, was tested for anti-dengue viral activity.

The cytotoxicity assay of *M. pinnata* on Vero cells using 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) revealed Maximum Nontoxic Dose (MNTD) as 125 µg/ml and 50% cytotoxic concentration (CC₅₀) as 428.9 ± 21.55 µg/ml. Plaque reduction antiviral assay performed on dengue-4 virus infected Vero cells demonstrated half-maximal inhibitory concentration (IC₅₀) as 26.12 ± 0.91 µg/ml. The selectivity index (SI) of *M. pinnata* was 16.42.

Based on the selectivity index, *Munronia pinnata* appears to be a viable candidate for identifying biologically active compounds with anti-dengue viral activity.

**Keywords:** Dengue, Munronia pinnata, Antiviral, Plaque reduction Assay, Vero Cells

1. Introduction

The arbovirus dengue (DENV) is transmitted to humans via female mosquitoes of the *Aedes* species. Although recent (2021) global dengue cases are still not published, there were 104,771,911 dengue cases reported globally in the year 2017 (Zeng et al., 2021). In addition, the number of deaths due to dengue increased from approximately 16,957 cases in 1990 to 40,467 in 2017 (Zeng et al., 2021). The
geographical range of dengue is expected to further expand due to climate change, urbanization and travel (Messina et al., 2019).

Four distinct dengue serotypes are known: DENV-1, DENV-2, DENV-3 and DENV-4. DENV has a single-stranded RNA as its nuclear material, and it is cleaved into three structural proteins, capsid, membrane and envelope, and seven non-structural proteins. These structural and non-structural proteins are needed for the virus life cycle and seem to enhance the pathogenicity of the virus (Zybert et al., 2010).

DENV infections range from subclinical to dengue fever and severe dengue. Dengue fever could present with or without warning signs and later progress into severe dengue, which manifests with fatal complications due to plasma leakage, severe bleeding and organ impairment (World Health Organization, 2020). However, the fatality rate of dengue ranges from 1% to 15% (European Centre for Disease Prevention and Control, 2020).

Vector control strategies have not been effective so far in controlling dengue infection. The most promising dengue vaccine Dengvaxia (CYD-TDV, Sanofi Pasteur) increases the risk of getting severe dengue in the seronegative population at the time of first vaccination (Flasche et al., 2019). Although dengue is a significant health problem, there is no specific antiviral agent for treatment. Clinical trials were conducted with repurposed drugs and were not successful as expected (Low et al., 2014; Whitehorn et al., 2016).

Medicinal plants have been used as remedies for fever from ancient times. Among these plants, Carica papaya and Euphorbia hirta, are found to be the most widely used anti-dengue viral remedies in folk medicine (Saleh et al., 2021). Previous studies have confirmed the action of plant-derived substances in preventing dengue virus attachment, penetration into cells, viral replication as well as having virucidal potential (Lim et al., 2020).

In Sri Lankan traditional medicine, Munronia pinnata (Family: Meliaceae; Common name: ground neem/king bitter; Local name: Bin Kohomba,) is used as a treatment for fever, purification of blood, dysentery, diabetes, tuberculosis, cough, stomachache, sores, malaria, skin diseases (Hapuarachchi et al., 2013) and brain, lung cancers (Kuruppu et al., 2019).

M. pinnata is rich in phytochemicals such as phytosterols, fatty acids, sesquiterpenes (Napagoda et al., 2014), and limonoids (Yang et al., 2019), saponins, alkaloids, tannins, flavonoids, steroids and glycosides (Dharmadasa et al., 2013). Previous studies report that M. pinnata has hypoglycemic (Hapuarachchi et al., 2011), antioxidant, antiinflammatory (Hapuarachchi et al., 2015), antibacterial (Keerawelle et al., 2019), antifungal (Kaliyadasa et al., 2020), antimalarial (Dharmadasa et al., 2012), and hepatoprotective (Hapuarachchi et al., 2013) effects.

Although some plants in the family Meliaceae revealed antiviral activity, including anti-dengue activity (Petrera, 2015), M. pinnata hasn't been previously studied for antiviral activity. Since M. pinnata is widely used as a fever remedy and consists of phytochemicals (g. Flavonoids) that have documented antiviral effects, the present study was carried out to identify the antiviral properties of M. pinnata against the dengue virus for the first time.

2. Materials and Methods

2.1. Collection of plant

M. pinnata was collected from the Pattipola Ayurveda Herbal Garden, Sri Lanka and authenticated at the National Herbarium, Peradeniya, Sri Lanka. A voucher specimen (6/01/H/03/No:02) was deposited at the same place.
2.1. Preparation of crude aqueous extracts

The plant was freshly collected and washed well in running tap water and cut into small pieces. Then it was washed with distilled water followed by deionized double distilled water and air-dried for 24-48 hours. A sample (30 g) of the plant was boiled in 960 ml of water until the volume was reduced to 120 ml (one-eighth of the original volume) according to the traditional method used in the preparation of decoctions (Hapuarachchi et al., 2014). The decoction was freeze-dried using a freeze dryer (Labocon, UK) and stored at -20°C until used.

2.2. Cells and viruses

African Green Monkey (Cercopithecus aethiops), Kidney cells (Vero cells), and Aedes albopictus larval cells (C6/36) were provided by Prof. N. Malavige, Centre for Dengue Research, University of Sri Jayewardenepura, Sri Lanka. DENV-4 virus was purchased from Genetech, Kitulwatte Road, Colombo 08, Sri Lanka.

Vero cells were cultured in complete growth medium (CGM) with 12 g/l Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma Aldrich, USA) supplemented with 5% fetal bovine serum (FBS; Sigma, USA), 1% L-glutamine (Sigma, USA), 1.2 g/l sodium bicarbonate (Sigma, USA), 1% Non-essential amino acid (Sigma, USA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma, USA) buffer 15 mM/l and incubated at 35°C. C6/36 cells were grown in CGM supplemented with 10% FBS and were incubated at 28°C.

DENV-4 virus was propagated in C6/36 cells at 28°C in the maintenance medium (MM). MM was prepared by replacing 10% FBS in CGM with 1.5% FBS and was supplemented with 1% penicillin-streptomycin (Sigma, USA). Following seven days of incubation, a cell culture supernatant containing the virus was collected and stored at -80°C. All flasks (Santa Cruz, USA) were incubated at 28 °C in a normal incubator (Daihan Labtech, Korea) with tightly capped lids.

The virus titration protocol of Talarico et al. (2005) was modified and used in the study. Vero cells (1x10^5 cells /well) were grown in 24 well plates (Santa Cruz, USA) for 24 hours until the cells were 80%
confluent. Tenfold dilutions of virus in MM were allowed to adsorb onto monolayers at 35°C for one hour. Control wells had uninfected Vero cells. Following adsorption, the virus was removed, and 1ml of overlay medium [OM; 1% methylcellulose (Sigma, USA) in MM] was added. After seven days of incubation at 35°C, the cell layer was fixed with 4% formaldehyde (Sigma, USA) overnight at room temperature. Wells were washed with tap water and stained with 1% crystal violet (Himedia, India) in 10% ethanol (Sigma, USA). Virus titration was carried out in triplicate wells, and the average plaque count was used to calculate the virus titre. Plates were sealed and placed in an incubator (Thermo Fisher Scientific, USA) without the provision of carbon dioxide (CO2).

2.3. Cytotoxicity Assay

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, USA) assay was carried out to determine 50% cytotoxic concentration (CC50) and maximum nontoxic doses (MNTD) of the plant. Vero cells (5 x 10^4 cells/well) were seeded on 96 well plates (Corning, USA) for 24 hours to obtain a monolayer. The plant extract was added at different concentrations (16.67-500 µg/ml) when cells were 80% confluent and incubated at 35°C for seven days. The supernatant was then removed. MTT (5 mg/ml) was added and incubated at 35°C for two hours in the dark. MTT was replaced with 100 µl of acidic isopropanol (Sigma, USA), and absorbance was read at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific, USA). Dose-response curves were plotted using GraphPad Prism (version 9.0.0) software to calculate CC50 and MNTD.

2.4. Antiviral assay

The antiviral activity was evaluated using plaque reduction assays with DENV-4 and Vero cells. Vero cells (1 x 10^5 cells /well) were cultured for 24 hours to adhere. The resulting monolayers were infected with 50 Plaque Forming Units (PFU) /well of DENV-4 virus, except for plant and cell controls. Following 1 hour of virus adsorption at 35°C, OM containing MNTD of plant extracts and doubling dilutions were added to each well. Plates were sealed and placed in an incubator without carbon dioxide. After seven days of incubation at 35°C, the wells were fixed with 4% formaldehyde overnight and stained using 1% crystal violet in 10% ethanol. Plaques were counted, and the half-maximal inhibitory concentration (IC50) was calculated from dose-response curves as the plant extract concentration required for reducing virus plaques by 50%. Assays were performed twice, and each plant was tested in duplicate wells. Selectivity index (SI) defined as the CC50/IC50 ratio was calculated.

3. Results

MTT assay was carried out to investigate the cytotoxicity of plant extracts on Vero cells revealed that the values of *M. pinnata* for MNTD and CC50 ± Standard error (SE) were 125 µg/ml and 428.9 ± 21.55 µg/ml (Figure. 2) respectively. MNTDs and doubling dilutions of MNTDs of *M. pinnata* were used for antiviral assay against DENV-4. The IC50 ± SE value of *M. pinnata* was 26.12 ± 0.91 µg/ml (Figure. 3), and the SI was 16.42.
Figure 2: log(inhibitor) vs. response -- Variable slope dose-response curve \[ Y=\frac{100}{(1+10 \log IC50-X) \times \text{HillSlope}} \] was plotted using non-linear regression analysis for cell viability of Vero cells after the treatment with different concentrations of aqueous extracts of *M. pinnata*. Data points are expressed as mean ± SE of triplicates.

Figure 3. log(inhibitor) vs. response -- Variable slope dose-response curve\[ Y=\frac{100}{(1+10^{((\log IC50-X) \times \text{HillSlope})})} \] was plotted using non-linear regression analysis for Inhibition of Dengue virus type 4 on Vero cells treated at nontoxic concentrations of *M. pinnata* aqueous extract. Data points are expressed as mean ± SE of two individual experiments.

The photomicrograph of uninfected cells shows healthy polygonal cells with well-defined nuclei in the cytoplasm (Figure 4; A), while infected cells show dead cells, elongated cells, and increased vacuole formation and multinucleated cells, suggesting syncytial formation (Figure 4; B). Although the majority of cells are healthy cells, some of the cells in *M. pinnata* treated with infected cells in Figure 4; C shows multinucleated cells and vacuoles in the cytoplasm. However, there are no plaques present in DENV-4 infected wells treated with MNTD of *M. pinnata* (Figure 4; F). Infected untreated wells (Figure 4; E) show clear plaques. When the *M. pinnata* concentrations for infected wells were gradually reduced, cytopathic effects appeared, and plaque count increased.
4. Discussion

*M. pinnata* is one of the most widely used medicinal plants to treat dengue infections in Sri Lankan traditional medicine (Siriwardhene et al., 2020). This study was carried out to investigate the antiviral activity of *M. pinnata* against the dengue virus. Cytotoxicity assay shows MNTD and CC\(_{50}\) of *M. pinnata* as 125 µg/ml and 428.9 ± 21.55 µg/ml, respectively, following seven days of incubation. *M. pinnata* demonstrated antiviral activity against the DENV-4, with an IC\(_{50}\) of 26.12 ± 0.91 µg/ml and an SI of 16.42.

In a previous study, it was reported that Vero cells had an EC\(_{50}\) value of 2691.3 ± 5.3 µg/ml on cytotoxicity when incubated for 24 hours with *M. pinnata* (Senanayake et al.,2020). Trujillo-Correa et al arbitrarily considered plants with an SI value greater than 10 as having high selectivity (Trujillo-Correa et al., 2019). As such, *M. pinnata*, which has an SI of 16 could be considered to have high selectivity when compared with other reported anti-dengue viral compounds and crude extracts. However, active compounds of *M. pinnata* should be purified and further investigated individually against anti-dengue viral activity to elucidate SI values since the value of the crude extract depends on cytotoxicity and mode of viral inhibition of each component and their synergistic contribution to antiviral mechanisms. Furthermore, the presence of non-antiviral compounds also can contribute to the inhibition of viral growth by other mechanisms (eg: enhancing absorption of active ingredients).

Two sesquiterpenes, β-caryophyllene and caryophyllene oxide have been reported in the n-hexane extracts of *M. pinnata* (Napagoda et al., 2014). β-caryophyllene and caryophyllene oxide had demonstrated high binding capacity against dengue viral proteins during an *in-silico* study (Pájaro et al.,2015). β-caryophyllene inhibited NS1 protein production of DENV-2 in HepG2 cells and it was
revealed that the β-caryophyllene is more potent in inhibiting NS1 levels in the early stages of the DENV life cycle. Further, it was able to inhibit surface protein E of all four dengue viruses in Vero cells (Flechas et al., 2017). Limonoids isolated from S. macrophylla inhibited DENV-2 in Huh-7 cells (Cheng et al., 2014). Six limonoids, namely munropin A-F have been isolated from the aerial parts of M. pinnata (Yang et al., 2019). The presence of these compounds in M. pinnata could be associated with antiviral activity demonstrated by M. pinnata in the present study. Further, it suggests that M. pinnata could restrict the early stages of the dengue virus life cycle (Flechas et al., 2017) as it contains β-caryophyllene.

M. pinnata is a potent hepatoprotective agent (Hapuarachchi et al., 2015), and it is an additional benefit when used as an anti-dengue agent. Senecrassidiol isolated from M. pinnata has shown antiinflammatory activity and was able to inhibit carrageenan-induced acute inflammation in Wistar rats. (Hapuarachchi et al., 2018). The use of potential anti-dengue viral candidates with multi pharmacological activities could provide better therapeutic efficacy via reducing the inflammatory activity and other complications to decrease the disease severity of dengue.

Certain improvisations were made in the present study to carry out cell culture assays. Since it is recommended as a good practice to maintain cells just below the optimal temperature as overheating causes cell death (Advancing Cell Culture, 2015), the temperature was maintained in Vero cells and the Vero cell-based assays at 35°C just below the optimal temperature of 37°C. In addition, this temperature did not alter the required pH and was suitable for maintaining the required pH in the medium. Plates were sealed and placed in an incubator without the provision of CO₂ and HEPES was added to regulate pH. The buffering activity of HEPES in the medium was adequate to maintain pH to buffer the acidic environment generated by cellular metabolism and dissolved environmental CO₂. Since CO₂ is not a requirement for cell growth, NaHCO₃/HEPES buffer system was used to maintain pH in the present study without supplying CO₂ gas (UK Health Security agency, 2021). The conditions applied in cell culture techniques in the present study did not affect the cell growth, and it was user friendly. The antiviral activity of drugs/natural products and their secondary metabolites can be assayed, especially in resource-limited settings.

The present study only revealed the anti-DENV-4 activity of M. pinnata. Talarico et al (2005) have shown the inhibitory activity of dengue viruses is serotype dependent. Therefore, further studies with all four dengue virus serotypes are essential to investigate the capacity of M. pinnata as a pan serotype inhibitor.

In this preliminary assay, IC₅₀ of DENV-4 infected M. pinnata treated Vero cells and CC₅₀ of M. pinnata treated Vero cells was 26.12 ± 0.91 µg/ml and 428.9 ± 21.55 µg/ml respectively. Bioassay-guided fractionation is recommended to identify anti-dengue viral compounds present in M. pinnata.

5. Conclusions

Based on in-vitro studies under improvised conditions, we report M. pinnata as a viable candidate for further studies towards identifying biologically active compounds with anti-dengue viral activity.
Acknowledgments

The research work reported here was funded by the University of Sri Jayewardenepura grant (ASP/01/RE/MED/2015/42), and a Research grant (001/2018) from the Centre for Plant Materials and Herbal Products Research, University of Sri Jayewardenepura, Sri Lanka. We acknowledge The Centre for Dengue Research, University of Sri Jayewardenepura for providing cells, Prof. E.B. Damonte, Universidad de Buenos Aires, Argentina for providing protocols for plaque reduction assay and Mr. Ranga Tudugala for assistance in statistical analysis.

Author contributions

KJ: laboratory work, literature search, writing manuscript
PS: Supervision of cytotoxicity assays, review of the article
SS: Supervision of collection and identification of plants and fractionation, review of the article
CG: Supervision of cytotoxicity assays, review of the article
KG: Funding, conceptualization, designing, supervision of virus assays, review of the article

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