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CYTOTOXICITY AND ANTIVIRAL ACTIVITY OF ANNONA MURICATA AQUEOUS LEAVES EXTRACT AGAINST DENGUE VIRUS TYPE 2

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ABSTRACT

In this study, *Annona muricata* aqueous leaves extract was tested against dengue virus type 2. Firstly, the cytotoxicity of *A. muricata* was evaluated using a cell viability assay. The cytotoxicity of *A. muricata* on Vero cells was tested and the value of cytotoxic concentration, CC50 was ~2.5 mg/ml and the 50% Effective Concentration, EC50 was ~ 0.20 mg/ml. Selectivity index of extract against DENV-2 was more than 10 indicating potential as antiviral agent. Cells were pre- and post-treated with the extract and the viral inhibitory effect was investigated by observing the morphological changes, which were further confirmed the cellular viability evaluated by MTT technique. The results revealed that the post-treatment was more effective in inhibiting viral replication compared to pre-treatment. The findings indicated that *A. muricata* has good potential for prospective nature-based antiviral drug.

Keywords: DENV-2, *Annona muricata*; Vero cells; MTT technique; antiviral drug.

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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]. Some of the most important emerging as well as resurging diseases worldwide can be allocated to the genus of the mosquito-borne flaviviruses.

DENV circulates as four serotypes (DENV 1-4) and is mainly transmitted by mosquitoes *Aedes aegypti* and *Aedes albopictus*. It accounts for the highest disease and mortality rates amongst flaviviruses [2]. The four serotypes are closely related and share around 65% identity in their genome, which makes diagnosis difficult since they cross-react extensively in serological tests [3]. Although they are closely related, infection with one serotype only provides lifelong immunity for that specific serotype but does not provide cross-protective immunity against another serotype [4]. In contrast, subsequent infection with another serotype has been reported to be a risk factor for developing Dengue hemorrhagic fever (DHF) or Dengue shock syndrome (DSS) [5].

1.2. *Annona muricata*

Annona muricata L. (Magnoliales: Annonaceae) is a tropical plant species known for its edible fruit which has some medicinal merits, but also some toxicological effects. Traditional medicinal uses of *A. muricata* have been identified in tropical regions to treat diverse ailments such as fever, pain, respiratory and skin illness, internal and external parasites, bacterial infections, hypertension, inflammation, diabetes and cancer. More than 200 chemical compounds have been identified and isolated from this plant; the most important being alkaloids, phenols and acetogenins [6].

2. METHODOLOGY

2.1. Plant Materials and Extraction

One hundred grams of *A. muricata* 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 freezedried 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 200μL 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

A cytotoxicity test was performed to determine the maximum nontoxic dose of the plant extracts. 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 *A. muricata* aqueous leaves 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 *A. muricata* 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

The leaves, bark, fruit and seed of *A. muricata* have been subject of countless medicinal uses [7]. Natives of Malaysia used *A. muricata* leaves to treat cutaneous (external) and internal parasites [8]. Interest in a large number of traditional natural products has increased [9-12]. It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral and antitumor agents [13]. *A. muricata* was shown to exhibit antiviral activity against Herpes simplex virus-1 and Herpes simplex virus-2 [14-15]. *A. muricata* also showed antibacterial activity against Gram positive and Gram negative bacteria. Currently, there is no anti dengue compound known to be isolated from *A. muricata*. The plant is known to contain flavonoids, alkaloids, saponins, tannins and glycosides involved in antimicrobial bioactivity. The mechanism of action is probably due to a synergism of these compounds [16]. In this study, the potential use of this plant to inhibit in vitro DENV-2 replication was investigated.

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 ~ 2.5 mg/ml.

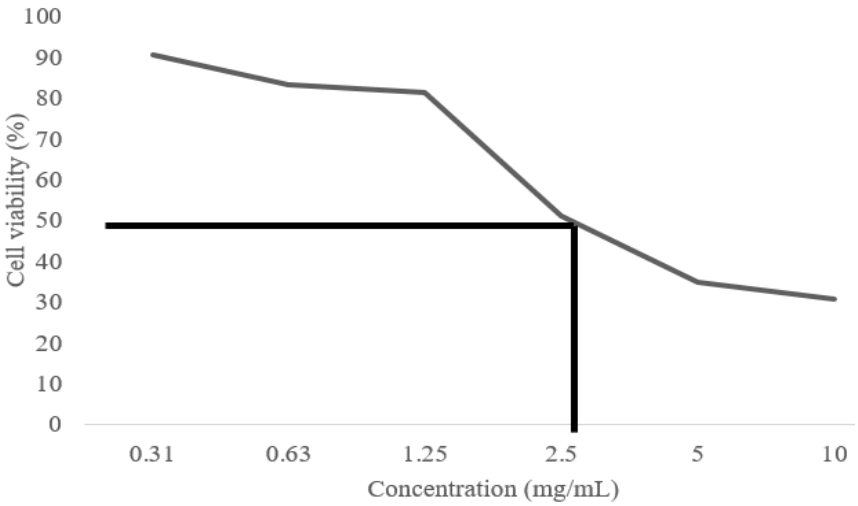

Fig.1. Cytotoxicity assay of *A. muricata* extracts against Vero cells. The assay was performed after 48 hours of treatment with various concentrations of the plant extracts

There is no signs of toxicity were observed in Vero cells when the extract were added at concentrations ranging from 0.31 to 1.25 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 0.20 mg/ml as shown in Fig. 2.

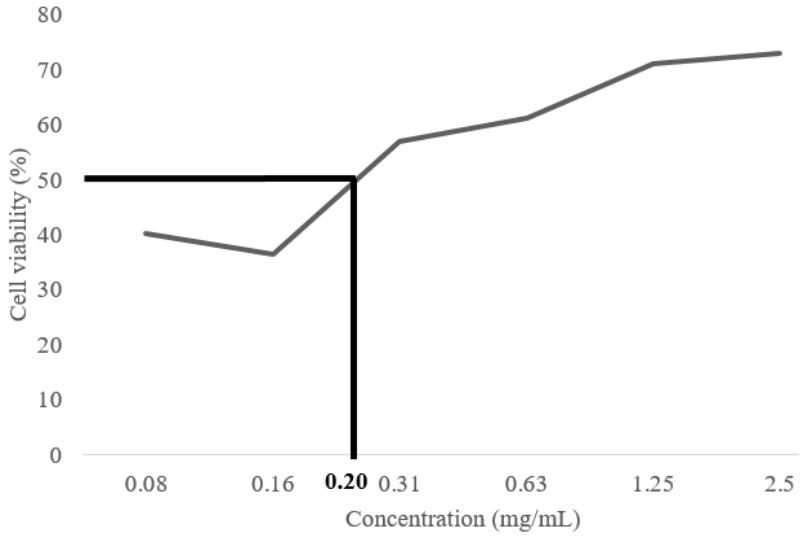

Fig.2. Determination of extracts effective concentration (EC50)

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 [17]. The effectiveness of the extract as an antiviral compound expressed as selectivity index (SI)

revealed that the extract had greater SI value of 12.5. Any antimicrobial compound that has SI values higher than 10 (SI > 10) ensures the potential to be developed as an agent of antiviral drug [18]. 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). 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 viral replication more in the post-treated cell compared to the pre-treated cell. The ability of *A. muricata* to confer protection to the cells before DENV-2 infection was tested by pre-treating the cells with the extract for 24h prior to viral infection. Protection could be conferred through extracellular mechanisms. The extract might interrupt the interaction of several envelope glycol proteins with cell surface receptors requires for fusion of the virion envelope with a cell plasma membrane, resulting in ineffective viral infection [19]. Interestingly, 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 post treatment was shown to be more effective antiviral activities than the pre-treatment assay. Similar findings have been reported [20-22], where significant antiviral activity against various viruses was observed when the cells were post-treated with compounds or plant extracts following viral infection. This strategy is of great importance because it could be administered once the virus infection has been established in the cell.

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

***A. Muricata* Aqueous Leaves Extract Concentration (mg/ml)**	**Post-Treated**	**Pre-Treated**
	% Cell Survival	
1.25	73	99
0.63	71	85
0.31	57	44

4. CONCLUSION

A. muricata aqueous leaves extract can be cytotoxic, but at lower concentration than the CC50 value, it has the potential to be an antiviral agent. The anti-DENV-2 activity occurred in the early stages of the replication, which was after the virus adsorption and penetration within the first 1.5 hours of infection. These species are good candidates for further activity-monitored fractionation to identify active principles.

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