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Research paper

Antiviral Activity of *Cynometra Cauliflora* Leaves Methanolic Extract Towards Dengue Virus Type 2

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Abstract

The present study is aimed at determining cytotoxicity and antiviral activities for methanolic extract obtained from leaves of *Cynometra cauliflora*. Cytotoxicity screening against Vero cells using MTT assay showed that the CC50 values for the extract was 36 mg/ml and the 50% Effective Concentration, EC50, was 2.19 mg/ml. The antiviral activity towards dengue virus type 2 (DENV-2) was determined using MTT method. Three treatments were used in the antiviral test; 1) post-treatment, 2) pre-treatment, and 3) virucidal. The results revealed that the post-treatment was more effective in inhibiting viral replication compared to pre-treatment and virucidal assay. The selectivity index (SI = CC50 / EC50) for the extract was 16. The findings indicated that the extract prepared from *C. cauliflora* was non cytotoxic to the cell with potential antiviral activity.

Keywords: *Cynometra cauliflora; methanol extract; antiviral activity; antiviral; dengue virus.*

1. Introduction

Therapeutic potential from medicinal plants as natural products nowadays seems profitable for international marketplace [1]. Establishing the therapeutic potentials from this source is more natural, inexpensive and without adverse effects. Vast antiviral screening from medicinal plants has been done worldwide to date. Interestingly, recent research has revealed that leaves methanolic extract from medicine plant may possess antioxidant, antibacterial and antivirus properties [2].

Current study shows that natural product represent a rich potential source of new anti-dengue compound. However, the most probable mode of infection can only be further confirmed by transcriptomic or proteomic studies.

Dengue virus (DENV) is a mosquito-borne member of the Flaviviridae family and is transmitted through the bites of *Aedes aegypti* and *Aedes albopictus* female mosquitoes. DENV are single-stranded, positive sense RNA viruses belonging to the Flaviviridae family. There are four distinct serotypes, dengue serotypes 1, 2, 3, and 4 [3]. Dengue appears in two forms, first classic dengue fever with symptoms that range from mild fever to high fever. The other more severe form, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [4]. All DENV serotypes are widespread geographically in tropical and subtropical regions of the world, causing life-threatening disease imposing considerable health and economic burden. There were no approved anti-viral drugs against dengue, and no approved dengue virus vaccines [5].

Malaysia has many traditional medicinal plants that have been reported on strong antiviral activity and some of them have already been used to treat people who were infected with viruses [6-8]. *Cynometra cauliflora* L. is classify under family of Fabaceae or Leguminosae and Caesalpiniaceae. It is typically found in eastern and northern parts of Peninsular Malaysia, Southeast Asia, and India. It is also commonly known by local as Nam-Nam or Buah Katak Puru in Malaysia [9]. The selection of the plant for evaluation was based on its traditional usage, every part of the plant exhibit pharmacological activities. *C. cauliflora* roots, leaves and fruits are used to treat various, antioxidant [10], anti-inflammatory, antiproliferative effects against human promyelocytic leukaemia cells [11] and anti-lipase [12] are the reported pharmacological activities. *C. cauliflora* leaves are traditionally used for treating diabetes and hyperlipidemia [13], whereas fruits are used as cure for appetite loss.

On the other hand, seed oil of plants is good for skin diseases [14]. Currently, there is no antidengue compound known to be isolated from *C. cauliflora*. The plant is known to contain terpenoids and tannins. Terpenoids play an important function to healing scar and wound. Tannins inhibition of enzyme activity by complexion with substrates of bacteria and fungi. Direct action of tannins on the microorganism metabolism, through the inhibition of oxidative phosphorylation, decreasing the availability of essential ions to the metabolism of the microorganisms [15].

2. Material and Methods

2.1. Preparation of Extracts

Powdered air-dried *C. cauliflora* leaves was extracted with methanol. The crude methanol extract was prepared by maceration of *C. cauliflora* leaves (100 g) with methanol (300 ml). The extract was filtered and evaporated to dryness in vacuum [16].

2.2 Cell Culture and Virus

Two types of cell lines were used in this study, C6/36 cells and Vero cells. The C6/36 mosquito cell line was grown as monolayer at 28ºC in L-15 medium (Sigma) supplemented with 5% fetal bovine serum (FBS) at 28 °C. The African green monkey kidney epithelial cells, Vero cell (ATCC CCL- 81) was grown as monolayer at 37 ºC with 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) (Sigma). Dengue virus type-2 (DENV-2) used in this study is a prototype of the New Guinea C strain. DENV-2 was propagated in C6/36 cell line for 7 days at 28 ºC in the absence of CO2. Culture supernatant was harvested and centrifuged at 1500 rpm for 10 min. The supernatant was collected and stored at 80 ºC as virus stock until use [17].

2.3. Cytotoxicity Assay

Cytotoxicity assay was done on monolayers Vero cells grown in 96 well plate utilizing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) for colorimetric analysis [18-19]. As for the *C. cauliflora* leaves extracts 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 extract, ranging from 100 mg/ml to 3.13 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 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, Austria) at 540 nm. Optical density (OD) of individual well was quantified using spectrophotometer at 540nm. Cells viability was calculated in (1) using formula below:

$$\text{Cell viability (\%)} = \text{ODtest} - \text{ODblank} / \text{ODcell} - \text{ODblank} \times 100 \quad (1)$$

where ODtest = optical absorbance of cells treated with SPD, ODblank = optical absorbance for well filled with DMSO and OD-cells = optical absorbance for cells without treatment with SPD. Nonlinear regression was done to obtain the CC50 value (cytotoxic concentration which killed 50% of cells).

2.4. Antiviral Assay

Antiviral activity was also evaluated by the MTT method. Screening for antiviral activity was performed using 3 different treatments according to [20] with some modification:

1) Post-treatment: To evaluate antiviral activity of extract against intracellular replication of DENV-2, cells were inoculated with virus 2 hour before treatment with extract.
2) Pre-treatment: In order to determine the prophylactic anti-dengue activity of extract, virus was inoculated to cells 5 hours after treatment with extract.
3) Virucidal: Direct virucidal effect of the extract was investigated by incubating virus with extract for 1 hour before it was inoculated on the cells.

For the antiviral tests, the extract concentration tested was twice lower than the CC50 value in order to reduce the possibility of toxicity towards the cells. The viral concentration used for cell inoculations was fixed at 200 FFU. The effectiveness of extract as an antiviral agent expressed as selectivity index (SI) calculated in (2).

$$\text{Selectivity Index (SI)} = \text{Cytotoxicity concentration (CC50)} / \text{Effective concentration (EC50)} \quad (2)$$

3. Results and Discussion

Several infectious viral diseases have been reported till date and newer ones are occurring frequently. Among emerging diseases, most of the diseases involve viruses such as HIV, Influenza, Herpes simplex virus (HSV), Dengue, Chikungunya, Zika, Hepatitis A (HSV), Hepatitis B (HSB), Hepatitis C (HCV), etc. [21-23]. Medicinal plants of the Malaysian forest were reportedly rich in biological activities such as antimicrobial, cytotoxic, antiviral, anti-inflammation, antioxidant, antitumor promoting, and antidiabetic activities [24-28]. Several interesting local medicinal plants such as *Goniothalamus* spp that was reported to have anti-HSV-1, anticancer activities and also induced apoptotic cell death [29-31], as well as both of *Annona muricata* and *Orthosiphon stamineus* were reported to have antidengue activities [20]. *Melastoma malabathricum* was reported capable in inhibiting HSV-1 and measles viruses during the early stages of viral replication [32]. *Polygonum minus* was reported to have anti-HSV-1 and anticancer activities [29, 33]. *Phyllanthus* species possess antiviral effects against herpes simplex (HSV) and dengue virus infections (DENV) [34].

In this study, the potential use *C. cauliflora* to inhibit in vitro DENV-2 replication was investigated. An MTT assay was carried out to determine the cytotoxicity and antiviral activity of *C. cauliflora* on Vero cell lines. Dengue virus is strictly a human pathogen and no appropriate animal model is available to study its pathogenesis. The cytotoxicity assay result, as presented in Figure 1, shows the percentage of cell viability versus extract concentration. The estimated CC50 value of the extract towards the Vero cells was 36 mg/ml. The EC50 value of the extract tested against the DENV-2 was 2.19 mg/ml, as shown in Figure 2. In the antiviral assay, extract at the concentration of more than the CC50 value of 36 mg/ml was also tested. No microscopic difference in the cell monolayers morphological was identified in the cells that were incubated for three days with extract at the CC50 value of more than 36 mg/ml when compared to untreated cells. The effectiveness of the extract as an antiviral compound expressed as (SI) revealed that the extract had SI value of 16.

According to [35], any antimicrobial compound that has SI values higher than 10 (SI > 10) ensures the potential to be developed as an agent of antiviral drug. 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 result demonstrated that the extract was capable to control viral infection after 2 h post-infection which according to [36], is the early stage of replication particularly during viral attachment.

This outcome suggested that the extract was effective in controlling virus post infection. The virucidal action of extract against DENV-2 was evaluated. The viruses were exposed simultaneously to various concentrations of extract. Extract at 12.5 mg/ml was effective as an anti-DENV-2 at early stage of the entry of virus into the host. This observation showed that extract was effective at early viral replication.

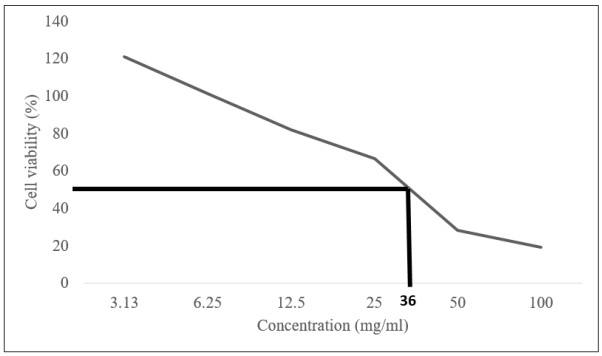

Fig. 1: Cytotoxicity assay of *C. cauliflora* extracts against Vero cells.

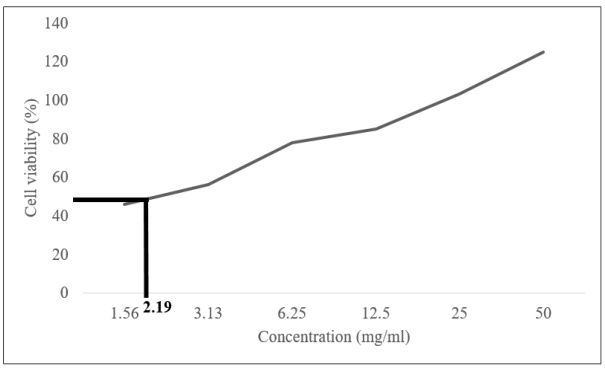

Fig. 2: Determination of extracts effective concentration (EC50).

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

Extract Concentration (mg/ml)	Pre-Treatment	Post-Treatment	Virusidal
	% Cell Survival		
12.5	49	78	58
6.25	26	56	32
3.13	18	46	25

C. cauliflora showed antiviral activity with different modes of action against DENV-2. Extract was effective in inhibiting cell death by 200 FFU DENV-2 inoculated cells using post-treatment. Interestingly, post-treatment assay was conducted in Vero cells 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 than the pre-treatment and virusidal assay. The ability of *C. cauliflora* to confer protection to the cells before DENV-2 infection was tested by pretreating the cells with *C. cauliflora* methanol extracts for 5h prior to viral infection. Protection could be conferred through extracellular mechanisms.

Extractmight interrupt the interaction of several envelope glycoproteins with cell surface receptors requires for fusion of the virion envelope with a cell plasma membrane, resulting in ineffective viral infection [37]. *C. cauliflora* extracts presented low to less prophylactic effects on Vero cells, perhaps due to the presence of various plant alkaloids in the crude extract of *C. cauliflora*, which may act synergistically to decrease the effective interaction of the active compounds. Simultaneous addition of virus and test extract to cell represents the capability of the extract either to modify cellular receptors for viral attachment or the viral attachment sites hence preventing successful attachment and penetration. Therefore, extract was mild efficient in directly affecting the virus particles.

4. Conclusion

On the basis of the present finding, *C. cauliflora* might be a good candidate in the search for a natural antimicrobial agent. This study provides scientific understanding to further determine the antimicrobial values and investigate other pharmacological properties. Treatment of cells with extract after virus inoculations showed better antiviral effect than treatment before viral inoculations. This suggests that extract was more effective in inhibit replication following infection of the cells with the virus.

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