Antiviral Activity of an Extract from Leaves of the Tropical Plant Cynometra cauliflora

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ABSTRACT
Background: Cynometra cauliflora is a species of tree in the family Fabaceae and has been used in folk medicinal preparation. Objectives: In this study, Cynometra cauliflora methanolic leaves extract was tested against clinical isolate herpes simplex virus type-1 (HSV-1). Materials and Methods: The leaves of C. cauliflora plant was extracted using methanol extraction method. Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay. Plaque reduction assays were carried out to evaluate the antiviral activity of C. cauliflora extract against HSV-1. These include post-treatment, pre-treatment and virucidal assays. Results: The value of cytotoxicity concentration, CC50 of C. cauliflora extract was 36 mg/mL. High antiviral activity was observed in post-treatment. C. cauliflora extract treatment was found to not interfere directly to infectious particle and confer mild protection when given as prophylaxis. Conclusion: This study provides important novel insights on the phytomedicinal properties of C. cauliflora extracts on HSV-1. Key words: Herpes simplex virus type 1, Cynometra cauliflora, plaque reduction assay, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); virucidal.

INTRODUCTION
Medicinal plants of the Malaysian forest were reportedly rich in biological activities. Several interesting natural products were isolated from local medicinal plants such as styrlypyrone derivatives isolated from G. umbrosus have shown a potent antiviral activity against HSV-1 and dengue virus type 2 (DENV-2). Other in vitro studies also showed SPD is active against several cancer cell lines namely; HL-60 (leukemia), HepG2 (liver), PANC-1 and Hela cells. geraniin extracted from the rind of Nephelium lappaceum have shown antiviral activity against HSV-1 and dengue virus type 2 (DENV-2). C. cauliflora is a species of tree in the family Fabaceae and has been used in folk medicinal preparation. It is also commonly known as ‘Nam-Nam’ among native Malaysian as a tropical plant under the Fabaceae family. The fresh leaves parts were collected from the state of Terengganu, Malaysia. The leaves were cleaned with tap water to remove dirt and oven-dried at 60°C. Dried leaves powder of C. cauliflora was extracted with methanol. C. cauliflora leaves (100 g) was macerated with methanol (300 mL) to produce crude methanol extract. The extracts were filtered and solvent was evaporated under reduced pressure using rotary vacuum evaporator.

MATERIALS AND METHODS
Plant material
The fresh leaves parts were collected from the state of Terengganu, Malaysia. The leaves were cleaned with tap water to remove dirt and oven-dried at 60°C. Dried leaves powder of C. cauliflora was extracted with methanol. C. cauliflora leaves (100 g) was macerated with methanol (300 mL) to produce crude methanol extract. The extracts were filtered and solvent was evaporated under reduced pressure using rotary vacuum evaporator.

Cells and virus
Vero cell from American Type Culture Collection (ATCC) CCL-81 was used for both cytotoxicity and antiviral test. Dulbecco’s Modified Eagle’s Medium
(DMEM) (SigmaAldrich, USA) supplemented with 5% fetal bovine serum (FBS) (Sigma- Aldrich, USA) was used for cell maintenance throughout the experiment. Clinical strain of HSV-1 used was obtained from the stock culture of Faculty Science and Technology, Universiti Kebangsaan Malaysia.

**Cytotoxicity test**

Briefly, 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 3.13 mg/mL to 100 mg/mL. 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 using formula below:

\[
\text{Cell viability (\%) = } \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{cell}} - \text{OD}_{\text{blank}}} \times 100
\]

where \( \text{OD}_{\text{test}} \) = optical absorbance of cells treated with SPD, \( \text{OD}_{\text{blank}} \) = optical absorbance for well filled with DMSO and \( \text{OD}_{\text{cell}} \) = optical absorbance for cells without treatment with SPD. Nonlinear regression was done to obtain the CC50 value (cytotoxic concentration which killed 50% of cells).

**Antiviral assay**

Antiviral activity was also evaluated by the plaque assay method. Screening for antiviral activity was performed using 3 different treatments. 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-HSV-1 activity of extract, virus was inoculated to cells 24 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 50 PFU. The effectiveness of extract as an antiviral agent expressed as selectivity index (SI).

\[
\text{Selectivity Index (SI) = Cytotoxicity concentration (CC}_{50}^{50}) \div \text{Effective concentration (EC}_{50}^{50})
\]

**RESULTS**

**Cytotoxicity evaluation of C. cauliflora extract**

MTT assay was conducted to determine the cytotoxicity of C. cauliflora extract towards Vero cells. The cytotoxicity assay result, as presented in Figure 1, shows the percentage of cell viability versus C. cauliflora extract concentration. The estimated CC50 value towards the Vero cells was 36.0 mg/mL.

**Anti-HSV-1 activity of C. cauliflora**

Plaque reduction assays were done to screen for anti-HSV-1 activity using C. cauliflora extract with different concentrations. Figure 2A, 2B and 2C shows the percentage of plaque reduction in post-treatment, pre-treatment and virucidal assays, respectively. The results from post-treatment assay showed that 100% plaque reduction was achieved at the concentration of 18 mg/mL. In pre-treatment assay, more than 50% plaque reduction was observed at 9 mg/mL. Meanwhile, C. cauliflora extract at any concentrations had no virucidal effect on HSV-1.

Effectiveness of certain compounds or extracts can be evaluated by using selective index (SI). In post-treatment assay, C. cauliflora extract exhibited potent antiviral activity against HSV-1 with EC50 = 2.14 mg/mL and with SI value of 16.8 (Table 1). Pre-treatment of Vero cells with C. cauliflora extract exhibited the prophylactic activity of extract against HSV-1 infection with EC50 = 8.5 mg/mL and with SI value of 4.23 (Table 1). C. cauliflora extract when added simultaneously with the virus not showed any anti-adsorption activity against HSV-1 (Table 1). Result revealed that C. cauliflora extract had greater SI value in post-treatment. Any antimicrobial compound that has SI values higher than 10 (SI>10) ensures the potential to be developed as an agent of antiviral drug. Selectivity index of C. cauliflora extract against HSV-1 was more than 10 indicating potential as antiviral agent.
Table 1: CC_{50}, EC_{50} and SI values of all extracts in post-treatment assay, pre-treatment assay and virucidal assay.

|                | CC_{50} (mg/mL) | EC_{50} (mg/mL) | SI (CC_{50}/EC_{50}) |
|----------------|----------------|----------------|----------------------|
| Post-treatment | 36.0           | 2.14           | 16.8                 |
| Pre-treatment  | 36.0           | 8.5            | 4.23                 |
| Virucidal      | 36.0           | -              | -                    |

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

Based on phytochemical analyses the findings in previous study, C. cauliflora leave extract has been reported to be rich in secondary metabolites such as tannin, flavonoid, saponins, cardiac glycosides and terpenoids. Lyu and collaborators reported the elucidation of the mechanism of the antitherpetic (HSV-1) activity in vitro via plaque reduction assay of flavonoid. Similarly, Sieniaw ska demonstrated that tannins and related compounds, exhibit antitherpetic activity in vitro. In addition, Perez reported that saponins inhibit the replication of HSV-1 and poliovirus type 2 as shown by inhibition of cytopathic effect and reduction of virus production. Thus, the richness of secondary metabolites in C. cauliflora plant may contribute to anti-HSV-1 properties. In this study, we investigated whether C. cauliflora methanolic extracts could confer protection to cells before or after the initiation of HSV-1 infection. The ability of the extract to act directly against HSV-1 virion particle was observed in virucidal assay. This antiviral analysis was performed on Vero cells as a model of infection in mammalian cells.

Screening for antiviral activity involves post-, pre- and virucidal treatment to determine the best mode for antiviral administration. In this part of the study, C. cauliflora extract treatment was found to not interfere directly to infectious particle and confer mild protection when given as prophylaxis. Instead, this study showed that extract-HSV-1 treatment most effective when administered as post-treatment. The ability of C. cauliflora to confer protection to the cells before HSV-1 infection was tested by pretreating the cells with C. cauliflora. Pre-treatment was done to study the effect of the extract as prophylactic agent in protecting the cell from HSV-1 adsorption and penetration. C. cauliflora extracts presented low to mild prophylactic effects, 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. Additionally, the results are presented as some of the antiviral compounds in these extracts may be present at low levels in a non-cytotoxic dilution of the extract. Therefore, extract can act as partial prophylactic agent to protect Vero cells against HSV-1 infection. Virucidal agents are chemical substances that attack and inactivate the extracellular viral particles by damaging the cell surface receptors requires for fusion of the virion envelope with a cell plasma membrane, resulting in ineffective viral infection. Pre-treatment was done to study the effect of the extract as prophylactic agent in protecting the cell from HSV-1 adsorption and penetration. C. cauliflora extracts presented low to mild prophylactic effects, 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. Additionally, the results are presented as some of the antiviral compounds in these extracts may be present at low levels in a non-cytotoxic dilution of the extract. Therefore, extract can act as partial prophylactic agent to protect Vero cells against HSV-1 infection. Virucidal agents are chemical substances that attack and inactivate the extracellular viral particles by damaging the cell surface receptors requires for fusion of the virion envelope with a cell plasma membrane, resulting in ineffective viral infection. Virucidal agents are chemical substances that attack and inactivate the extracellular viral particles by damaging the cell surface receptors requires for fusion of the virion envelope with a cell plasma membrane, resulting in ineffective viral infection.

CONCLUSION

As a conclusion, our findings suggest that crude extract prepared from C. cauliflora contains antiviral active compounds and could be potential antiviral agent.

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CONFLICTS OF INTEREST

None.

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GRAPHICAL ABSTRACT

Mechanism of action of C. cauliflora extract antiviral activity against HSV-1

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