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Cytotoxic and Antiviral Activities of *Clinacanthus nutans* Crude Extract

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Clinacanthus nutans, also known as snake grass, is a local medicinal plant that displays antimicrobial, antiviral, and anticancer immunomodulatory activities. Whole dried plants were macerated with methanol for crude extract preparation. The focus of this study was to assess the cytotoxicity and antiviral activities of the *C. nutans* crude extract. Cytotoxicity screening against Vero cells using MTT assay showed that the CC_{50} value of the crude extract of *C. nutans* was 1.625 mg/mL. Plaque reduction assays were carried out to evaluate the antiviral activity of the extract against herpes simplex virus type 1 (HSV-1). These included post-treatment, pre-treatment, and virucidal assays. The selective indices ($SI = CC_{50} / EC_{50}$) of the extract in post-treatment, pre-treatment, and virucidal assays were 11, 6, and 3, respectively. Our results indicate that *C. nutans* has a promising potential to be explored as a source of potent antiviral products.

Key words: *Clinacanthus nutans*; herpes simplex virus type 1 (HSV-1); cytotoxicity; plaque assay

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Clinacanthus nutans (Burm.) Lindau (Family: Acanthaceae) is commonly known as snake grass. Species from this family are mainly distributed in Malaysia, Indonesia, Thailand, Vietnam, and parts of China's forests [1]. It has been used traditionally to treat skin infection, insects and snake bites, dysentery, burns, scalds, fever, gout, and diabetes [2-3]. Recent studies have shown that *C. nutans* has notable antiviral activities against herpes simplex virus type-2, antitumor, antioxidant properties, and anti-neuroinflammatory effects [4-6].

Herpes simplex virus type-1 (HSV-1) is a common causative agent for cold sores or common cold and orolabial infection. The most common sites of infection are of the mucosal epithelia, which include labial herpes keratitis, gingivostomatitis, and genital herpes [7]. However, a chronic syndrome can develop after an acute infection when infection disseminates from the mucosal epithelia to other tissues with slow healing. The impact has more adverse outcomes in immunocompromised patients [8], which include newly born children, transplant patients or HIV patients who are lack of susceptibility, immunesuppressive drug regimens and prolonged toxicity and prophylaxis, respectively [9].

HSV infection can be treated with acyclovir, an acrylic purine nucleoside analogue, but the emergence of drug resistance to acyclovir has created an obstacle for the treatment, mainly in immunocompromised individuals [10]. Acyclovir resistance has been progressively reported and is caused by mutations in either the thymidine kinase or the DNA polymerase genes [11]. Consequently, replacement therapies for patients with documented resistance are needed to decrease the clinical effect of drug resistance towards herpes viruses [12]. Therefore, in order to combat this resistant HSV-1 strain, new antiviral agents with a distinct mode of action are urgently needed.

MATERIALS AND METHODS

Plant Material: *C. nutans* plants were collected, washed, and dried at 60°C. The plant authentication was performed by a competent botanist from the Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin. Dried whole plants consisting of leaves, stems, and roots were cut into smaller segments and ground into powder using a grinder. The powder (200 g) was soaked in 500 mL of methanol for three days. The extract was filtered and the solvent was evaporated under reduced pressure using a rotary vacuum evaporator (Eyela). Finally, the extract was blow-dried to give 25 g of extract, and then stored in a refrigerator at 4°C until further use.

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Cells and Virus: HSV-1 clinical strain and Vero cells used in this study were obtained from stocks available at the Virology Laboratory, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 5% Fetal Bovine Serum (Nacalai Tesque), penicillin/streptomycin (100 U/L), and non-essential amino acids. The cell culture was maintained in an incubator at 37°C and humidified 5% CO_2. HSV-1 was propagated in Vero cells and incubated until cytophatic effects developed. The titer of the virus was estimated and stored at -80°C until used.

Cytotoxicity Test: Cytotoxicity test was performed using MTT to evaluate the maximum non-toxic dose of the plant extract. Confluent Vero cells (2.0×10^4 cell/ well) grown in 96-well microtiter plates were treated with different concentrations of the extract with a starting concentration of 2.5 mg/mL. Two-fold serial dilutions were done on the extract until a final concentration of 0.078 mg/mL. After 48 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added into each well and the cells were further incubated for 3 h. Excess MTT was removed and 100 µL of dimethylsulfoxide (DMSO) was added. Absorbance was measured at 540 nm by using a microplate reader (Tecan Infinite 200 Pro). Percentage of cell viability was calculated and the CC_{50} (50% cytotoxicity concentration) value was obtained directly from the graph of cell population versus extract concentration. CC_{50} is defined as the sample concentration that is able to reduce 50% of cell viability compared to the untreated cells. Percentage of cell viability was determined using the formula below:

$$\text{Cell Viability (\%)} = [(OD_{Test} - OD_{Blank}) / (OD_{Cell} - OD_{Blank})] \times 100$$

Antiviral Assay: Antiviral assays were determined by plaque assay. The antiviral assays composed of post-treatment, pre-treatment, and virucidal assays [13].

Post-treatment assay: Vero cells (1.0×10^5 cell/well) were seeded into 24-well plates and incubated overnight. Upon confluency, the cells were inoculated with 200 µL of virus at ~50 PFU (plaque forming unit). The cells were incubated for 2 h to allow virus adsorption. After adsorption period, the cells were washed twice with phosphate-buffered saline to remove any residual unbound viruses. This was followed by the addition of serial dilutions of the plant extract, in triplicate, mixed with DMEM + methylcellulose (2.5 mg/mL to 0.078 mg/mL). The cells were then incubated for 48 h. After incubation, the cells were stained using crystal violet and plaques were counted via manual-based visual counting.

Pre-treatment assay: Vero cells (1.0×10^5 cell/well) were seeded in 24-well plates and incubated overnight. The cells were pre-treated with six concentrations of the extract, 2.5 mg/mL to 0.078 mg/mL for 24 h before being removed and infected with 200 µL of virus at ~50 PFU. After adsorption period, DMEM + methylcellulose was added and the cells were incubated for 48 h. After incubation, the cells were stained using crystal violet and plaques were counted via manual-based visual counting.

Virucidal assay: Direct virucidal effect of the extract was investigated by incubating the virus with the extract (2.5 mg/mL to 0.078 mg/mL) for 1 hour, before it was inoculated onto the cells. After adsorption period, DMEM + methylcellulose was added and the cells were incubated for 48 h. After incubation, the cells were stained using crystal violet and plaques were counted via manual-based visual counting.

RESULTS

Cytotoxicity of *C. nutans* extract. Cytotoxicity of the *C. nutans* extract on Vero cells was evaluated using the MTT assay. To determine the non-toxic dose, Vero cells was exposed to two-fold serially diluted *C. nutans* methanol extract at concentrations ranging from 0.078 to 2.5 mg/mL. The related CC_{50} was then calculated using Graph Pad Prism for Windows, version 5 (Figure 1). In this assay, the CC_{50} value of the *C. nutans* extract was determined at 1.625 mg/mL.

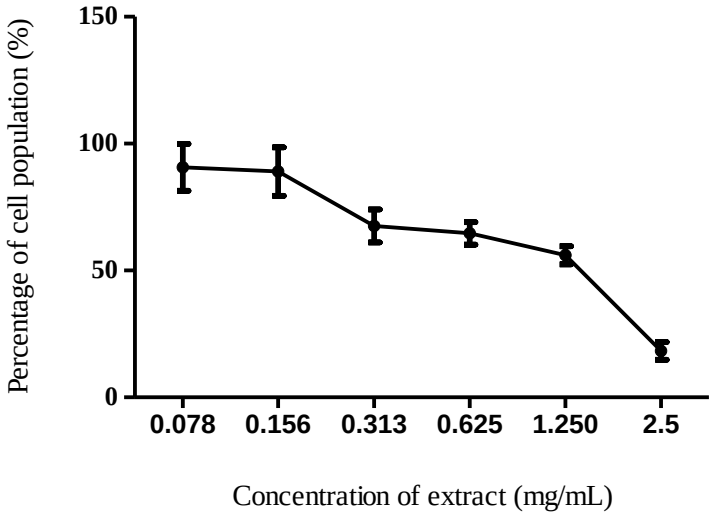

Figure 1. Effect of different concentrations of *C. nutans* extract towards the population of Vero cells.

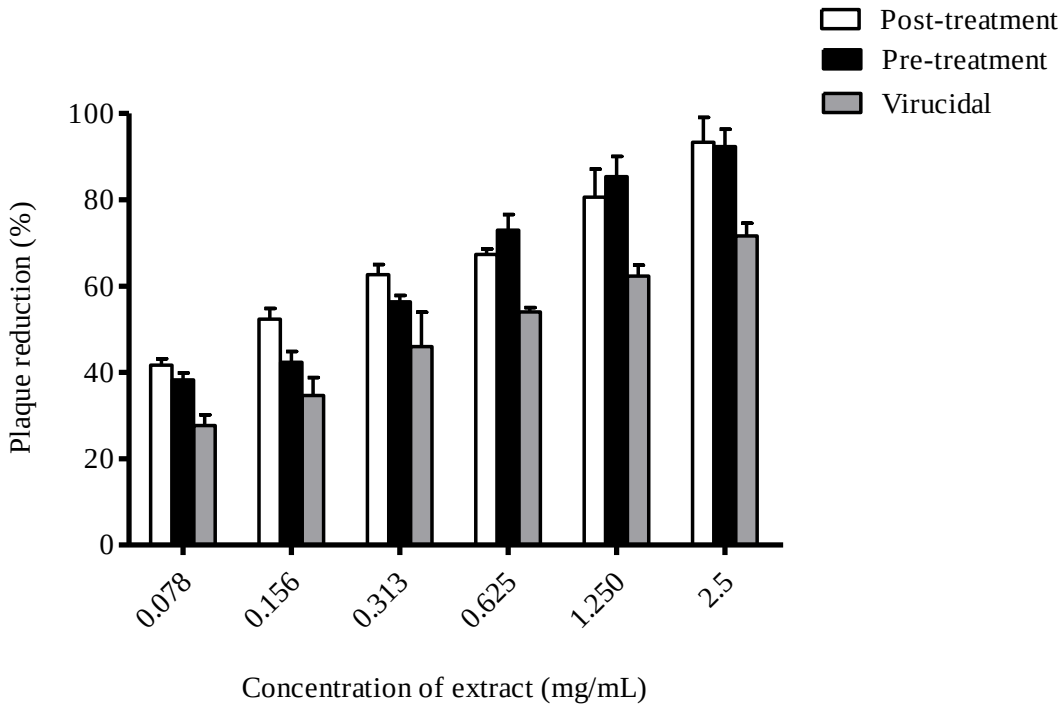

Figure 2. Antiviral activities of *C. nutans* extract against HSV-1 via post-treatment, pre-treatment, and virucidal assays.

Table 1. CC_{50}, EC_{50}, and SI values of *C. nutans* extract in post-treatment, pre-treatment, and virucidal assays.

	CC_{50} (mg/mL)	EC_{50} (mg/mL)	SI (CC_{50}/EC_{50})
Post-treatment	1.625	0.148	11
Pre-treatment	1.625	0.271	6
Virucidal	1.625	0.542	3

Anti-HSV-1 activity of *C. nutans.* Plaque reduction assays were used to evaluate the *in vitro* anti-HSV-1 activities of the *C. nutans* extract. The extract was added at different phases of viral infection: i) pre-treatment for 24 hours prior to infection for its prophylactic activity, ii) treatment for 48 hours post-adsorption, and iii) directly to cell-free virus suspension to examine its virucidal effect. Figure 2

shows the percentage of plaque reduction in post-treatment, pre-treatment, and virucidal assays. Results from the post-treatment assay showed more than 80% plaque reduction at 1.25 mg/mL. In the pre-treatment assay, more than 50% plaque reduction was achieved at a minimum concentration of 0.313 mg/mL. For the virucidal assay, more than 50% plaque reduction was observed at a minimum concentration of 0.625 mg/mL.

The effectiveness of the *C. nutans* extract as an antiviral compound is expressed as selectivity index (SI). In the post adsorption assay, the extract exhibited potent antiviral activities against HSV-1 with EC_{50} = 0.148 mg/mL and SI value of 11 (Table 1). Pre-treatment of Vero cells with the extract exhibited the prophylactic activity of the extract against HSV-1 infection with EC_{50} = 0.271 mg/mL and SI value of 6 (Table 1). The extract when added simultaneously with the virus showed anti-adsorption activity against HSV-1 with EC_{50} = 0.542 mg/mL and SI value of 3 (Table 1). Results from direct virucidal activity assessment of the extract exhibited a weak extracellular anti-HSV-1 activity. Results revealed that the *C. nutans* extract had greater SI value in the post-treatment assay, followed by the pre-treatment and virucidal assays. Any antimicrobial compounds that have SI values higher than 10 (SI>10) ensure the potential to be developed as antiviral drugs [14]. Selectivity index of the *C. nutans* extract against HSV-1 was more than 10, indicating a potential as an antiviral agent.

DISCUSSION

Many natural products are known for their different secondary metabolites, and part of them has already been used for the treatment of various chronic human diseases [15]. Interestingly, different secondary plant metabolites such as flavonoids, saponins, lignans, tannins, alkaloids and thiophenes, and phenolic acids were found to have significant antiviral activities against a variety of viruses [16-19]. In this study, the *C. nutans* extract was shown to be non-cytotoxic with CC_{50} of 1.625 mg/mL. The high SI values identified with the *C. nutans* extract might be due do the presence of unique phytochemical constituents. Based on phytochemical analyses, *C. nutans* has been proven to be rich in secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, steroids, terpenoids, phenols, and tannins [20]. This plant contains high contents of flavonoid and phenolic, which are the active compounds of antivirus agents that will inhibit viral replication [21]. Flavonoid groups have been reported by several researchers to exhibit a wide range of biological activities, such as antimicrobial, antioxidant, anti-analgesic, anti-inflammatory, anti-cancer, and anti-allergic [22]. There have been reports on flavonoids from plants that inhibited dengue virus type -2 (DENV-2) [23], severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [24], and Enterovirus A71 (EV-A71) [25]. Flavonoids from many of plant extracts were found to cause maximum antimicrobial activity towards Gram-positive and Gram-negative bacteria [26-28]. Thus, the richness of alkaloids and flavonoids in *C. nutans* may contribute to anti-HSV-1 properties.

The calculated selective index (SI) value of more than 10 of the *C. nutans* extract in the post-treatment assay demonstrates its worth to be further studied as an antiviral agent. The *C. nutans* extract could affect viral infection on post-treated cells. The pre-treatment assay was conducted to investigate the effect of the extract on cells before being infected with HSV-1. During 24 h of the cell-extract incubation period, the extract had enough time to be absorbed and affect the cells. The activity of the extract in the pre-treated cells towards HSV-1 infection was concentration dependent, as shown in Figure 2. This could happen because the extract was able to bind to the Vero cells and interfere with the glycoprotein receptors on the cell membrane or block HSV-1 from binding to the cell surface [29]. Meanwhile, the post-treatment assay was conducted to investigate the activity of the extract in inhibiting HSV-1 viral replication cycle, such as during the transcription of viral genes, envelopment process or during egression [30]. Extracellular anti-HSV-1 activity of the *C. nutans* extract was investigated by the virucidal assay. Results from direct virucidal activity assessment of the *C. nutans* extract showed that the extract exhibited a weak extracellular anti-HSV-1 activity. Based on the calculated SI values, the *C. nutans* extract showed the ability to decrease viral replication more in the post-treated cells compared to the pre-treated cells. This result indicates that the *C. nutans* extract was capable in controlling viral infection after 2 h, which is the initial phase in virus replication cycle involving virus attachment process [31]. This result proposes that the *C. nutans* extract was effective in inhibiting early viral entry including attachment, penetration phases and viral replication, but incapable of inactivating free virus particles.

CONCLUSION

As a conclusion, our findings suggest that *C. nutans* extract exhibits potent anti-herpes activities *in vitro* and contains antiviral active compounds and could be a potential antiviral agent. Results from this study can help further *in vivo* anti-viral studies as part of the developmental process for development of *C. nutans* extract as a potential anti-HSV-1 therapeutic. Future studies are warranted for further identification of bioactive compounds in *C. nutans* with antiviral activities.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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