

Phytochemistry, cytotoxicity and antiviral activity of *Catharanthus roseus*

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ABSTRACT

Background and Objectives: *Catharanthus roseus* is generally used to treat many diseases in folklore remedies. The present study is aimed at determining phytochemical constituents, cytotoxicity and antiviral activities for crude extract of the plant.

Materials and Methods: The whole plant of *C. roseus* was extracted using methanol extraction method. Phytochemical qualitative screening was carried out for *C. roseus* extract according to standard procedures used to test for the presence of alkaloid, saponin, terpenoid and steroid. 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. roseus* extract against herpes simplex virus type 1 (HSV-1). These include post-treatment, pre-treatment and virucidal assays.

Results: *C. roseus* extract contain secondary metabolites such as alkaloid, saponin and terpenoid but does not contain steroid. Cytotoxicity screening against Vero cells using MTT assay showed that the CC₅₀ values for crude extract of *C. roseus* was 0.5 mg/mL. The extract prepared from *C. roseus* possesses phytochemical compound that was non-cytotoxic to the cell with potential antiviral activity. Plaque reduction assays against herpes simplex virus type 1 (HSV-1) showed that the selective indices (SI = CC₅₀ / EC₅₀) of *C. roseus* extract in post-treatment, pre-treatment and virucidal assays were 36, 20 and 4.7 respectively. The results revealed that the extract prepared from *C. roseus* possesses phytochemical compound that was non-cytotoxic to the cell with potential antiviral activity.

Conclusion: This study showed that *C. roseus* extract has promising potential to be explored as anti-HSV-1 agent regardless of the mode of treatment.

Keywords: *Catharanthus roseus*; Herpes simplex virus type-1; Plaque assay

INTRODUCTION

Catharanthus roseus is a perennial tropical medicinal plant belongs to the Family Apocynaceae in which the leaf extracted from *C. roseus* showed significantly higher activity, suggesting that its bio-active compounds can be potentially exploited as antibacterial agents (1). Out of 36 alkaloids of *C. roseus*

evaluated, nine showed a degree of antiviral has been reported (2). Pericalline, perivine, periformylone, leurosivine, leurocristine, vincalokoblastine, perividine, vindolinine and carosine all showed antiviral activity against the vaccinia and polio type III viruses. Extract of *C. roseus* has been reported to exhibit antiviral activities against dengue virus type 2 (3). *C. roseus* also exhibited broad-spectrum antibacterial activity against *Salmonella* Typhi and *Shigella boydii* (4). In addition, *C. roseus* extract also showed antifungal properties against *Rigidoporus microporus*, *Ganoderma philippii* and *Phellinus noxius* (5).

Herpes Simplex Virus type-1 (HSV-1) is a common pathogen that causes cold sores or common cold and orolabial infection. Normal sites of infection are

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mucosal epithelium including keratitis labial herpes, gingivostomatitis and genital herpes (6). However, these common symptoms can develop into serious illness when infection disseminates from mucosal epithelium to other tissues with slow healing. The effect has more detrimental outcomes in immunocompromised individuals (7) which includes newborn babies, transplant patients or HIV patients who are readily struggling with immature immunity, immune-suppressive drugs regimen and prolonged toxicity and prophylaxis, respectively (8). HSV infection can be successfully treated with Acyclovir and related nucleoside analogue but the emergence of drug resistance to acyclovir has created a barrier for the treatment especially in immunocompromised patients (9). Acyclovir resistance has been increasingly described and is caused by mutations in either the thymidine kinase or the DNA polymerase genes (10). Therefore, alternative therapies for patients with documented resistance are required to reduce the clinical impact of drug-resistant herpes viruses (11). So, in order to combat this resistant HSV-1 strain, new antiviral agents with a different mode of actions are indeed important.

MATERIALS AND METHODS

Plant Material. The fresh plant of *C. roseus* was collected from the state of Terengganu, Malaysia. The plants authentication was performed by competent botanist from Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin. The plants were cleaned with tap water to remove dirt and oven-dried at 60°C. The dried whole plants consisting of leaf, stem and root were cut into smaller pieces and ground into powder using a grinder. The powder (200 g) was soaked in 500 mL methanol for three days. The extracts were filtered and solvent was evaporated under reduced pressure using rotary vacuum evaporator (Eyela).

Cells and virus. The 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. Vero cells were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 5% Fetal Bovine Serum (Nacalai Tesque), penicillin/streptomycin (100 U/L) and non-essential

amino acid. The cell culture was maintained in an incubator at 37°C and humidified 5% CO₂ atmosphere. HSV-1 was propagated in Vero cells and incubated until cytopathic effects developed. The titer of the virus was estimated and stored at -80°C until used.

Phytochemical screening. Phytochemical qualitative screening was carried out for *C. roseus* extract according to standard procedures used to test for the presence of alkaloids, saponins, terpenoid and steroids (12).

Determination of alkaloid. 10 mg/mL of extract was diluted in 2 mL of 25% ammonia for several minutes. After that, 5 mL of chloroform was added and shaken gently to extract the alkaloidal base. Mayer's reagent was added. The formation of a cream with Mayer's reagent was observed indicating the presence of alkaloids.

Determination of saponin. 2 mL of distilled water was added to extract suspended in ethanol and was shaken vigorously. The formation of copious foam layer indicates the presence of saponins.

Determination of terpenoid. 2 mL of chloroform was added to 10 mg of the extract. After that, 3 mL of concentrated sulphuric acid (H₂SO₄) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

Determination of steroid. 2 mL of chloroform and 2 mL of concentrated H₂SO₄ was added to 10 mg/mL of extract and shaken well. Chloroform layer appeared red. This confirm the presence of sterols steroid.

Cytotoxicity test. Cytotoxicity test was performed to determine the maximum non toxic dose of the plant extracts. Confluent Vero cells (2.0 × 10⁴ cell/ well) grown in 96-well microtitre plate were treated with different concentrations of extract. After 48 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added into each well and was further incubated for 3 h. Excess MTT was removed and 100 μL of dimethylsulfoxide (DMSO) was added. The absorbance was measured at 540 nm by using microplate reader (Tecan Infinite 200 Pro). The percentage of cell viability was calculated and the CC₅₀ value was obtained directly from graph of cell population versus extract concentration.

Antiviral assay. Antiviral assays were determined by plaque assay. Antiviral assays composed of post-treatment, pre-treatment and virucidal assays.

Post-treatment assay. Vero cells (1.0×10^5 cell/well) were seeded into 24 well plate and incubated overnight. Upon confluency, cells were inoculated with 200 μ L of virus at ~50 PFU (plaque forming unit). Cells were incubated for 2 h to allow virus adsorption. After adsorption period, 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 mixed with DMEM+methyl cellulose. Cells were then incubated for 48 h. After incubation, cells were stained using crystal violet and plaques were counted.

Pre-treatment assay. Vero cells (1.0×10^5 cell/well) were seeded in 24 well plate and incubated overnight. Cells were pre-treated with six concentrations of extracts for 24 h before being removed and infected with 200 μ L of virus at ~50 PFU. After adsorption period, DMEM+methylcellulose was added and incubated for 48 h. After incubation, cells were stained using crystal violet and plaques were counted.

Virucidal assay. Direct virucidal effect of the extract was investigated by incubating virus with extract for 1 hour before it was inoculated on the cells. After adsorption period, DMEM+methylcellulose was added and incubated for 48 h. After incubation, cells were stained using crystal violet and plaques were counted.

RESULTS

Phytochemical analysis. Phytochemical analysis shown in Table 1 indicates the methanolic extract of *C. roseus* contained a few secondary metabolites such as alkaloid, saponin and terpenoid. Phytochemical analysis also indicates the *C. roseus* extract does not contain steroid.

Cytotoxicity of *C. roseus* extracts. Cytotoxicity of *C. roseus* extracts on Vero cells were evaluated using the MTT assay. To determine the nontoxic dose, Vero cells were exposed to two-fold serially diluted

C. roseus methanol extracts at concentrations ranging from 0.039 to 1.25 mg/mL. The related CC_{50} was then calculated using Graph Pad Prism for Windows, version 5 (Fig. 1). In this assay, the CC_{50} value of *C. roseus* extract was determined at 0.5 mg/mL.

Anti-HSV-1 activity of *C. roseus*. Plaque reduction assays was used to evaluate the *in vitro* anti-HSV-1 activities of *C. roseus* extract. *C. roseus* extract was added at the 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 direct virucidal effect. Fig. 2 shows the percentage of plaque reduction in post-treatment, pre-treatment and virucidal assays, respectively. The results from post-treatment assay showed more than 75% plaque reduction was observed at 0.250 mg/mL. In pre-treatment assay, 100% plaque reduction was achieved at a minimum concentration of 0.125 mg/mL. For virucidal assay, more than 50% plaque reduction was observed at a minimum concentration of 0.016 mg/mL.

The effectiveness of the *C. roseus* extract as an

Table 1. Phytochemical analysis of *C. roseus* methanolic extract

Phytochemical substances	+: Present; -: Absent
Alkaloid	+
Saponin	+
Terpenoid	+
Steroid	-

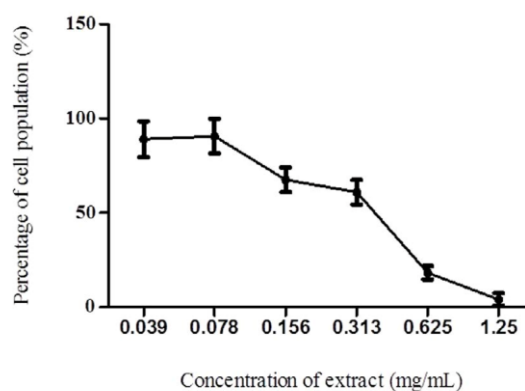


Fig. 1. Effect of different concentration of *C. roseus* extract towards the population of Vero cells.

antiviral compound expressed as selectivity index (SI). In post adsorption assay, *C. roseus* extract exhibited potent antiviral activity against HSV-1 with $EC_{50} = 0.014$ mg/mL and with SI value of 36 (Table 2). Pre-treatment of Vero cells with *C. roseus* extract exhibited the prophylactic activity of extract against HSV-1 infection with $EC_{50} = 0.025$ mg/mL and with SI value of 20 (Table 2). *C. roseus* extract when added simultaneously with the virus showed anti-adsorption activity against HSV-1 with $EC_{50} = 0.106$ mg/mL and with SI value of 4.7 (Table 2). Results from direct virucidal activity assessment of *C. roseus* extract showed that the extract exhibited a weak extracellular anti-HSV-1 activity. Result revealed that *C. roseus* extract had greater SI value in post-treatment followed by pre-treatment and virucidal assays. Any antimicrobial compound that has SI values higher than 10 ($SI > 10$) ensures the potential to be developed as an agent of antiviral drug (13). Selectivity index of *C. roseus* extract against HSV-1 was more than 10 indicating potential as antiviral agent.

DISCUSSION

Many natural products are known with their differ-

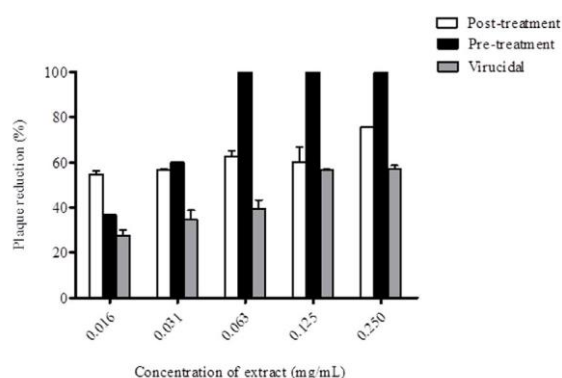


Fig. 2. Antiviral activities of *C. roseus* extract against HSV-1 via post-treatment, pre-treatment and virucidal assays.

Table 2. CC_{50} , EC_{50} and SI values of *C. roseus* extracts in post-treatment, pre-treatment and virucidal assay.

	CC_{50} (mg/mL)	EC_{50} (mg/mL)	SI (CC_{50}/EC_{50})
Post-treatment	0.5	0.014	36
Pre-treatment	0.5	0.025	20
Virucidal	0.5	0.106	4.7

ent secondary metabolites and part of them has been already used for the treatment of various chronic diseases in human (14). Interestingly, different secondary plant metabolites such as flavonoids, saponins, lignans, tannins, alkaloids and thiophenes, phenolic acids were found to have significant antiviral activity against variety of viruses (15, 16). Based on phytochemical analyses, *C. roseus* extract has been proven to be rich in secondary metabolites such as alkaloids, saponin and terpenoid. According to Moradi et al. (17), 43 alkaloids extracted from plants, fungi and bacteria showed inhibitory effect on influenza virus replication and inhibition of inflammation of the lungs. Lee et al. (18) demonstrated that hepatitis C virus replication was inhibited by saponin. Similarly, Ikeda et al. (19) demonstrated that some triterpenoidal saponins exhibit anti-HSV-1 activity *in vitro*. Wen et al. (20) reported the elucidation of the mechanism of the anti-severe acute respiratory syndrome associated coronavirus (SARS-CoV) activity of plant terpenoids. In this study, we investigated whether *C. roseus* extract could confer antiviral activities against HSV-1 infection. This antiviral analysis was performed on Vero cells as a model of infection in mammalian cells.

The cytotoxicity evaluation assay was performed to determine the cytotoxic concentration that kills 50% cell population which is known as CC_{50} value. The CC_{50} value of *C. roseus* extract was determined at 0.5 mg/mL. The results of the greater SI value was identified with *C. roseus* extract might be due to the presence of the unique phytochemical constituents. The anti-HSV-1 activity can be related to the presence of active compounds in *C. roseus* methanol extract such as alkaloids (21), saponins (22) and terpenes (23). The calculated selective index (SI) value of more than 10 for *C. roseus* extract in post-treatment and pre-treatment which its worth to be further studied as antiviral agent. *C. roseus* extract can affect virus infection to pre-treated and post-treated cells. The pre-treatment assay was conducted to investigate the effect of the extract on the cells before being infected with HSV-1. During the 24 h of cell-extract incubation period, the extract can have 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 Fig. 2. This may happened because of the extract was able to bind to Vero cells and interfere with the glycoprotein receptor on the cell membrane

or block the HSV-1 from binding to the cell surface (24). Meanwhile, the post-treatment assay was conducted to investigate whether HSV-1 viral replication cycle and assembly in infected cells, could be affected (25). Extracellular anti-HSV-1 activity of *C. roseus* extract was investigated by virucidal assay. Results from direct virucidal activity assessment of *C. roseus* extract showed that the extract exhibited a weak extracellular anti-HSV-1 activity. According to calculated SI value, *C. roseus* extract showed the ability to decrease viral replication more in the post-treated cell compared to the pre-treated cell. This result indicated that *C. roseus* extract was capable in controlling viral infection after 2 h which is the initial phase in virus replication cycle involving virus attachment process (26). This result proposed that *C. roseus* extract was effective in inhibiting early viral entry including attachment, penetration phases and viral replication but incapable inactivate free virus particles.

In conclusion, *C. roseus* extract exhibited potent anti-dengue activity *in vitro* and contains antiviral active compounds and could be 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. roseus* extract as potential anti-HSV-1 therapeutic.

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