การศึกษาฤทธิ์การยับยั้งเชื้อเฮอร์ปีส์ ซิมเพล็กซ์ ไวรัส ไทป์ 1 ของสารสกัดเอทิลอะซิเตตจากสมุนไพรพญายอ

Anti-Herpes Simplex Virus Type 1 Activity of Crude Ethyl Acetate Extract of *Clinacanthus nutans*

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บทคัดย่อ

การทดสอบฤทธิ์ของสารสกัดเอทิลอะซิเตตจากพญายอในการยับยั้งเชื้อไวรัสเฮอร์ปีส์ ซิมเพล็กซ์ ไทป์ 1 (HSV-1) โดยวิธี plaque reduction assay พบว่าสารสกัดสมุนไพรความเข้มข้น ≤ 19 μg/ml ไม่เป็นพิษต่อเซลล์ โดยความเข้มข้น 76 μg/ml มีความเป็นพิษต่อเซลล์ร้อยละ 50 ซึ่งที่ระดับความเข้มข้น 7.6 μg/ml สามารถยับยั้งการติดเชื้อร้อยละ 50 โดยมีค่า selectivity index ต่อเชื้อ HSV-1 เท่ากับ 10 เมื่อทำการศึกษาว่าสารสกัดมีผลยับยั้งไวรัสในระยะก่อน (pre-infection) หรือหลังเข้าสู่เซลล์ (post-infection) พบว่าในระยะก่อนเข้าเซลล์สารสกัดสามารถลดจำนวนกลุ่มเซลล์ที่ติดเชื้อได้ร้อยละ 97 เมื่อเทียบกับกลุ่มควบคุม ซึ่งผลการยับยั้งจะเพิ่มขึ้นตามช่วงเวลาในการทดสอบ ในขณะที่การทดสอบในระยะหลังเข้าสู่เซลล์ พบว่าสารสกัดไม่สามารถยับยั้งการเพิ่มจำนวนของไวรัสได้ ซึ่งการศึกษานี้แสดงให้เห็นว่าสารสกัดเอทิลอะซิเตตจากพญายอมีผลในการยับยั้งเชื้อ HSV-1 ในระยะก่อนที่เชื้อเข้าสู่เซลล์เพื่อให้ทราบกลไกการออกฤทธิ์ของสารสกัดที่แท้จริงควรศึกษากลไกการออกฤทธิ์ในระยะดังกล่าวในระดับโมเลกุลต่อไป

ABSTRACT

Crude ethyl acetate of *Clinancanthus nutans* was investigated for its antiviral activity on herpes simplex virus type 1 (HSV-1) in vitro by using a plaque reduction assay. Results showed that the extract had no cytotoxic effect against Vero cell at concentration of 19 μg/ml or below and showed the 50% cytotoxic concentration (CC_{50}) was 76 μg/ml. While the extract exhibited anti-herpestic activity with

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inhibiting concentration 50% values or IC_{50} was 7.6 μg/ml, as determined by plaque reduction assay, indicating the selectivity index (SI) or ratio of CC_{50} to IC_{50} of 10 for HSV-1. In order to study the possible mode of action of antiviral activity of the extract, the experiment was separated into anti-HSV-1 pre-infection and anti-HSV-1 post-infection. In anti-HSV-1 pre-infection, the extract produced 97% plaque reduction with a time-dependent manner whereas it had no effect in anti-HSV-1 post-infection. This study concludes that crude ethyl acetate of *C. nutans* possesses anti-herpetic activity in inhibiting viral in the step of pre-infection. Therefore, the molecular mechanism of extract in interfering with certain step of pre-infection thus further investigates.

คำสำคัญ : พญายอ, เชื้อไวรัสเฮอร์ปีส์ ซิมเพล็กซ์ ไทป์ 1 ฤทธิ์การยับยั้งเชื้อไวรัส

Key Words : *Clinacanthus nutans* Lindau (*C. nutans* L.), Herpes simplex virus type 1, Antiviral activity

Introduction

Herpes simplex virus type 1 (HSV-1) is a human pathogen. It is an enveloped DNA virus and causes a wide range of infections, which are usually self-limiting in the immunocompetent host. Its have different degrees of severity, ranging from mild to severe manifestation including herpes labialis, genital herpes, keratitis, gingivostomatitis, keratoconjunctivitis, pharyngitis and encephalitis (Marques and Straus, 2000). HSV-1 is generally associated with primary and recurrent mucocutaneous facial, ophthalmic or genital lesions. After primary infection, HSV-1 establishes latency in sensory and autonomic neurons innervating the mucosal tissues where primary infection takes place, and reactivated by the proper stimulus to cause recurrence infection (Roizman and Sears, 1996).

Many therapeutic agents have been developed and used for treatment of HSV disease, most of these are nucleoside analogs with selective antiviral activity (Darby *et al*, 1994). Among those acyclovir is widely used for the systemic treatment of HSV infections. It is a highly selective antiviral agent because it is specifically phosphorylated by viral thymidine kinase (tk) in infected cells (Furman *et al*., 1979; Elion *et al*, 1997). However, it has recently been observed that the acyclovir-resistant HSV infection has come from immunocompromised patients such as transplant patients and patients with AIDS (Coen *et al*, 1996; Kimberlin and Whitley, 1996). Therefore, it is of interest to develop new anti-HSV agents from natural source that herbal medicine may be employed as an alternative therapy for HSV infection in the future.

Thai herbs are potential sources of useful edible and medicinal plants. *Clinacanthus nutans* Lindau (*C.nutans* L.) is a Thai medicinal plant, the fresh leaves of which have long been used domestically for treatment of herpes simplex skin infection and shingles and for the relief of pain from of insect bites (Thawarananth *et al*, 1992; Tuntiwachwuttikul *et al*., 2004). There are controversies about the study of effects on HSV. Since 1992, the investigation about anti-HSV activity both *in vitro* and *in vivo* studies of *C. nutans* crude extracts have been noted. For *in vitro*

studied, the leaves extract of C. nutans was tested the virucidal activity on HSV-2 by plaque reduction assay, this extract had inhibited HSV-2 plaque formation in baby hamster kidney cell line (BHK cell) (Jayavasu *et al.*, 1992a). However, the methanolic extract of *C. nutans* and *Barleria lupulina (B. lupulina)* were investigated the antiviral activity against HSV-2 strain G and five clinical HSV-2 isolates. The result showed that *B. lupulina* extract exhibited activity against all five isolates but not effect to HSV-2 strain G, whereas *C. nutans* did not shown any activity against these viruses as determined by plaque inhibition assay. These results suggest that there are a therapeutic potential of *B. lupulina* but not *C. nutans* against HSV-2 infection (Yoosook *et al.*, 1999). For investigated the clinical trials by using cream base, developed from the crude extract of *C. nutans* in the treatment of genital herpes patients, the result indicated that the extract of *C. nutans* had good efficacy in shortening the duration of infection, reduced severity and no side effect was observed in patients who were treated with C. nutans when compared with control group (Jayavasu *et al.*, 1992b).

Therefore, this study attempted to evaluate the antiviral activities of crude ethyl acetate extract of *C. nutans* against HSV-1 strain F by plaque reduction assay.

Materials and methods

Virus and cell culture preparation

HSV-1 (F strain) stocks were prepared in Vero cells. For standard viral preparation, the cells were infected by HSV-1 at 0.01 and subsequently 0.1 multiplicity of infection (m.o.i.) and the infected cells were harvested at 72 h post-infection (p.i.) and centrifuged at 2000 rpm, at 4°C for 15 min. The supernatant was collected and the virus titres were determined by a standard plaque assay. The infectious titres of the stock solutions were 10^6 PFU/ml and virus stock was stored at -70°C until use.

Vero cells (African green monkey kidney cell line) were used for propagation of HSV-1 and investigation of anti-HSV activity. They were cultured as a monolayer and maintained in Opti-modified eagle's medium(Opti-MEM; Gibco-BRL, Gaithersburg, MD) supplemented with 2% fetal bovine serum (FBS; Seromed, Berlin, Germany) 100 units/ml of penicillin, 100 micrograms/ml of streptomycin, 40 micrograms/ml of gentamicin, and 2.5 micrograms/ml of amphotericin B. As the cell became confluent, they were subcultured following dispersingwith 0.25% trypsin-ethylenediaminetetraacetate (EDTA). The cells were incubated at 37°C in a 5% CO_2 incubator.

Preparation of extracts

The crude ethyl acetate extract of *C. nutans* was dissolved in dimethylsulfoxide (DMSO) and prepared in Opti-MEM to a concentration of 152 μg/ml. The stock solution was stored at 4°C until use.

Cytotoxicity study

Subtoxic concentration of the crude ethyl acetate extract of *C. nutans* was determined before the study of anti-HSV-1 activity. Vero cells were seeded in a 96-well tissue culture plate (Costar, Cambridge, MA, USA) and then incubated at 37°C, 5% CO_2 overnight. Then the monolayer cells were incubated in Opti-MEM with or without serial two-fold dilutions of the extract and 40% DMSO in Opti-MEM at 37°C, 5% CO_2 for 72 h. The cells were washed with cold PBS twice. After

staining the cell with 3% crystal violet solution 50 µl per well for 15 min, the cells were washed with water and air-dried at room temperature overnight. Crystal violet precipitates were dissolved in DMSO and OD_{620} of the solution were measured.

The cell viability was evaluated by the percentage of the mean value of the optical density resulting from the cell control that set 100%. The subtoxic concentration was the maximal concentration that has %cell viability not less than 90% of cell control. The 50% cytotoxic concentration (CC_{50}) of the extract was calculated from the mean dose-response of three independent assays.

Anti-HSV-1 activity

The anti-HSV-1 activity of crude ethyl acetate extract of *C. nutans* was determined by plaque reduction assay. The subtoxic concentration of the extract was incubated with 500 PFU/ml of HSV-1 at 37°C for 60 min. Controls were mixtures of HSV-1 + medium (virus control) and HSV-1 + 5 µg/ml of acyclovir (inhibition of HSV DNA replication control). After 60 min incubation, all of the mixtures were added 50 µl per wells and adsorbed on confluent Vero cell monolayer in a 96-well culture plate at 37°C for 1 h under a humidified 5% CO_2 atmosphere. After virus adsorption, the mixtures were aspirated, the cells were washed twice with PBS (pH 7.5) and once with Opti-MEM and then replaced with 0.8% of CMC in Opti-MEM with subtoxic concentration of the extract. The controls were replaced with 0.8% of CMC in Opti-MEM without or with 5 µg/ml of acyclovir, respectively.

After incubation at 37°C under a humidified 5% CO_2 atmosphere for 72 h, the cell monolayer was fixed in 10% formalin and stained with 3% crystal violet. Plaques were counted and compared with control group. The inhibitory activities of test extracts and acyclovir on HSV-1 replication were calculated as follows:

$$\text{Inhibitory activity (\%)} = \frac{\text{Number of plaques (Control-Experiment)}}{\text{Number of plaques (Control)}} \times 100$$

If the extract was exhibited anti-HSV-1 activity more than 80% of virus control, it was further investigated for anti-viral activity in pre-infection and post-infection. 50% inhibitory concentration (IC_{50}) was determined by the same experiment with a serial two-fold dilution of the subtoxic concentration extract. The IC_{50} was analyzed by regression analysis of the dose-response curves.

Anti-viral activity in pre-infection

The subtoxic concentration of the extract was incubated with 500 pfu/ml of HSV-1 at 37°C for 10, 30 and 60 min. Controls were mixtures of HSV-1+medium (virus control) and HSV-1 + 2 µg/ml of dextran sulfate (inhibition of HSV entry control).After each of incubation times, all of the mixtures were added 50 µl per well and adsorbed on confluent Vero cell monolayer in a 96-well tissue culture plate at 37°C for 1 h under a humidified 5% CO_2 atmosphere. After virus adsorption, the mixtures were aspirated, the cells were washed twice with PBS (pH 7.5) and once with Opti-MEM and replaced with 0.8% of CMC in Opti-MEM.

After incubation at 37oC under a humidified 5% CO_2 atmosphere for 72 h, the cell monolayer was fixed in 10% formalin and stained with 3% crystal violet. Plaques were counted and compared with control group. The inhibitory activities of the extract and dextran sulfate on HSV-1 activity in pre-infection and IC_{50} were calculated as described previously.

Anti-viral activity in post-infection

Confluent Vero cell monolayer in a 96-well tissue culture plate was adsorbed with 500

PFU/ml (25 PFU/well) of HSV-1 at 37°C for 1 h under a humidified 5% CO_2 atmosphere. After adsorption, the mixtures were aspirated then the cells were washed twice with PBS (pH 7.5) and once with Opti-MEM and replaced with 0.8% of CMC in Opti-MEM with the extract at subtoxic concentration. The controls were replaced with 0.8% of CMC in Opti-MEM without or with 5 μg/ml of acyclovir. After incubation at 37°C under a humidified 5% CO_2 atmosphere for 72 h, the cell monolayer were fixed in 10% formalin and stained with 3% crystal violet. Plaques were counted and compared with the control. The inhibitory activities of the extract and acyclovir on HSV-1 activity in post-infection and IC_{50} were calculated as described previously.

Data analysis

Data were presented as means±standard deviation, and the differences between groups were assessed with Student's *t*-test. Each experiment was performed at least three times.

Results

Effect of crude ethyl acetate extract of C. nutans on cell viability

The cytotoxicity of crude ethyl acetate extract of C. nutans was examined on Vero cells using crystal violet uptake assay. We examined the viability of Vero cells after they had been treated with the extract for 72 h. At concentrations more than 19 μg/ml, the cell viability decreased especially at 152 μg/ml of the extract whereas at concentration of 19 μg/ml and below

the cells were not cytotoxic (Figure 1 and 2). The DMSO at the concentration used in all dilutions did not affect the cell viability (Figure 1). The 50% cytotoxic concentration (CC50) of the extract was 76 μg/ml. Results indicated that the extract had no effect on the viability of cells, even up to concentrations at 19 μg/ml. Therefore, we assumed that this concentration of extract was the subtoxic concentration of extract and used in further study of anti-HSV-1 activity.

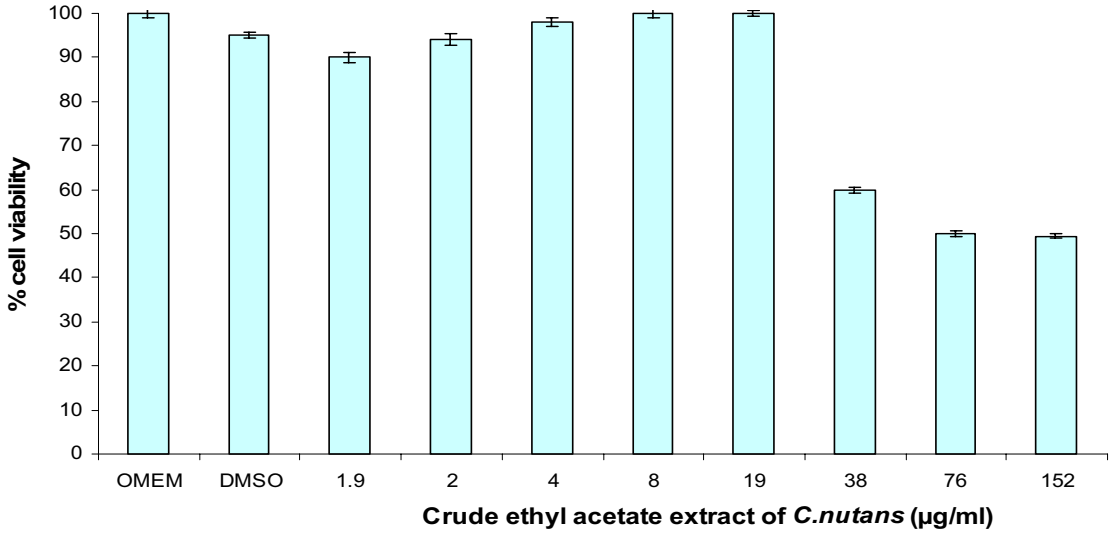


Figure 1 Subtoxic concentration of crude ethyl acetate extract of *C. nutans* on Vero cells determined by crystal violet uptake assay.

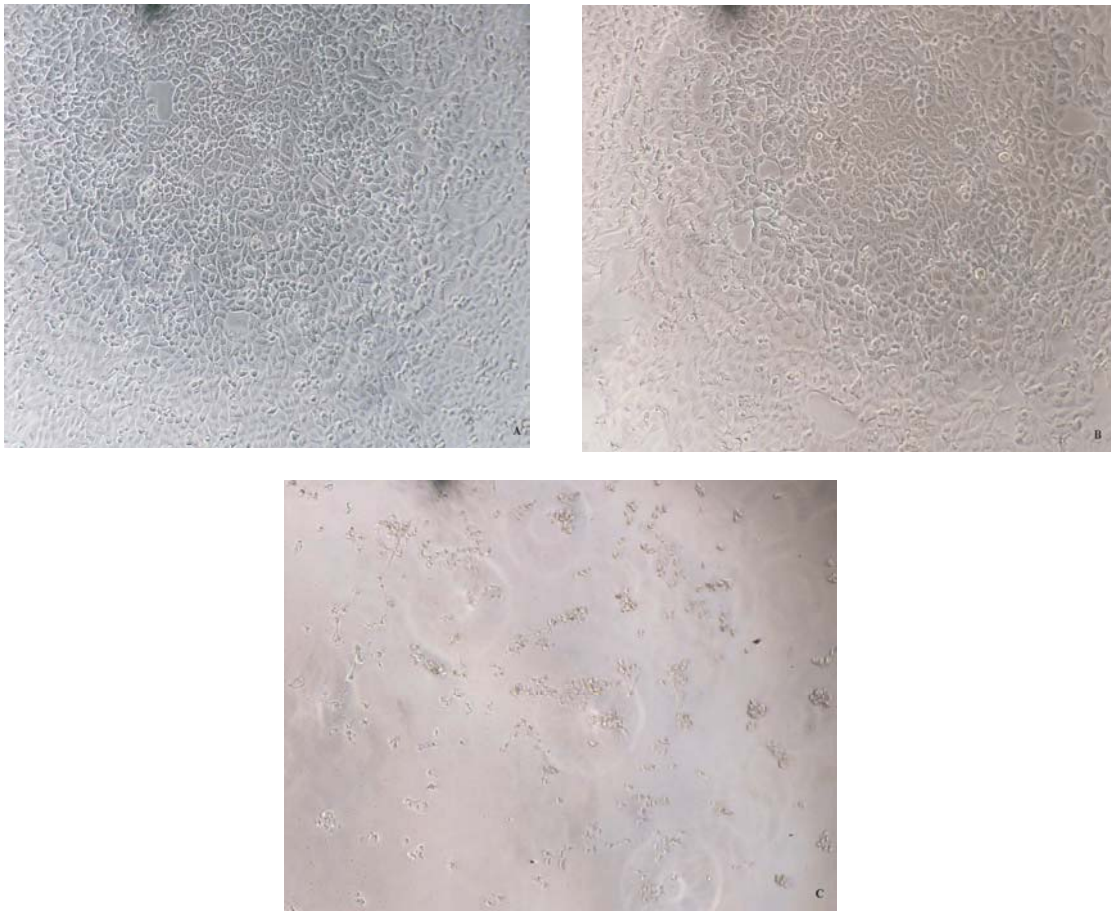


Figure 2 Vero cells viability and cell cytotoxicity affected crude ethyl acetate extract of *C. nutans* was observed under an inverted light microscope. The extract (at serial concentrations) was added to Vero cells and incubated for 72 h. The cell monolayers were photographed at about 48 h after incubated. (A) Untreated Vero cells; (B) Vero cell viability after treated with extract at 19 μg/ml; (C) Vero cell cytotoxicity treated with the extract at 152 μg/ml. (Magnification of micrographs : X100).

Effect of crude ethyl acetate extract of C. nutans on viral infectivity

In the preliminary screening test for anti-HSV-1 activity by plaque reduction assay, the extract at 19 μg/ml was incubated with HSV-1 500 PFU /ml at 37°C before adsorption and then the infected cells were incubated in the extract at 19 μg/ml. Five μg/ml of acyclovir (ACV) was used as a positive control. The extract inhibited plaque formation by 100% of control (Table 1) whereas the HSV-1 plaque formation in Vero cells was not affected by DMSO treatment. The IC_{50} value of the extract was approximately 7.6 μg/ml and the ratio of CC_{50}/ IC_{50} or selectivity index (SI) of extract was 10.

Table 1 Anti-HSV-1 activities of crude ethyl acetate extract of *C. nutans* in Vero cells.

Incubated solution	Plaque numbers (% inhibition)
Opti-MEM	29.3±1.5 (0)
DMSO	26.3±1.5(0)
ACV (5 μg/ml)	0 (100)
C.nutans crude extract (19 μg/ml)	0 (100)

Effect of crude ethyl acetate extract of C. nutans on viral infectivity in pre-infection step

The extract was further investigated for anti-HSV-1 activity in pre-infection. Nineteen microgram per milliliter of the extract was incubated with HSV-1 500 PFU/ml at a various times (10, 30 and 60 min) before adsorption. 2 μg/ml of dextran sulfate was used as positive control. The extract and dextran sulfate were reduced plaques number depending on times of exposure as showed in Figure 3. The degree of inhibition of this extract was 97% of control at 60 min of incubation (Data not shown) whereas DMSO did not inhibit HSV-1 infection. This result demonstrated that crude ethyl acetate extract of *C. nutans* exhibited a time-dependent manner for anti-HSV-1 activity in pre-infectin.

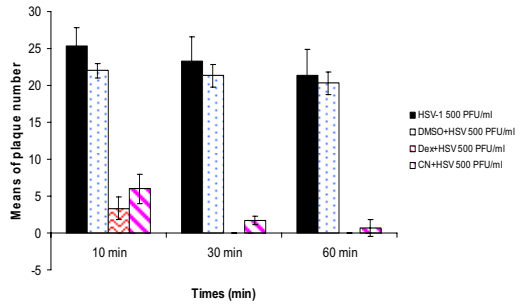


Figure 3 Inhibition of HSV-1 activity in pre-infection by crude ethyl acetate extract of *C. nutans* on Vero cells.

Effect of crude ethyl acetate extract of C. nutans on viral infectivity in post-infection step

The anti-HSV-1 activity in post-infection of the extract was investigated by adding the extract after adsorption with HSV-1 500 PFU /ml. Five μg/ml of acyclovir was used as a positive control. The extract did not reduce plaque as well as DMSO. The percentage of inhibition of the extract was shown in Table 2. The result concluded that crude ethyl acetate extract of *C. nutans* had no anti-HSV-1 activity in post-infection.

Table 2 Anti-HSV-1 activities in post-infection of crude ethyl acetate extract of *C. nutans*.

Incubated solution	Plaque numbers (% inhibition)
Opti-MEM	22±4.3 (0)
DMSO	20.3±3.0 (0)
ACV (5 μg/ml)	0 (100)
C.nutans crude extract (19 μg/ml)	22.6±2.3 (0)

Discussion and Conclusion

In the present study, we investigated antiviral activity of crude ethyl acetate extract of *C. nutans* against HSV-1 by plaque reduction assay, it was observed that crude ethyl acetate extract of *C. nutans* had an inhibitory effect on HSV-1. Based on the host-dependent life cycle of viruses, a mode of action of crude ethyl acetate extract of *C. nutans* on HSV-1 was studied preliminarily using treatments at two different stages: namely, pre-infection and post-infection activities. Anti-HSV-1 activity in pre-infection of the extract resulted in a time-dependent reduction of

plaques and also dependent on the amount of virus input (data not shown), suggesting that the extract may interfere with virion envelope structures or be masking viral compounds which are necessary for adsorption or entry into host cells. When crude ethyl acetate extract of *C. nutans* was added after virus infection (post-infection), the amount of plaques of HSV-1 was not reduced. The result similar as mentioned by A. Schuhmacher (Schuhmacher et al., 2003) shown the virucidal effect of peppermint oil effect on virus before adsorption of HSV-1 and -2 to RC-37 cells and Langeland N. (Langeland *et al.*, 1988) showed that the inhibitory effect of polylysines and polyarginine blocked the binding of HSV-1 to receptors by interfering with the cellular receptor function.

The result demonstrated that the mechanism of interaction between crude ethyl acetate extract of *C. nutans* and ACV with HSV is different. In cell-treated with crude ethyl acetate extract of *C. nutans*, free virus is varying sensitive to the virucidal effect of extract. The inhibitory effect of crude ethyl acetate extract of *C. nutans* probably inactivates HSV before it enters the cells or may be due to binding of the extract to viral glycoproteins involved in host cell adsorption and penetration, possible their envelopes, thereby impairing their ability to infect host cells, whereas the meachanism of ACV that inhibits virus replication by interference with the DNA polymerase inside the infected cells (Roizman and Sears, 1996). Therefore, in further study ethyl acetate extract of *C. nutans* will be further separated for the active compound and investigated the molecular mechanism of extract against HSV and these extract may be used in a synergistic treatment of HSV infection in the future.

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