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## Anti-Viral Activity of *Cissus repanda* Vahl. Plant Extract on Herpes Simplex Virus

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**Abstract:** The effect of *Cissus repanda* Vahl. plant extract on herpes simplex virus type 1 and type 2 was investigated in this study. The cytotoxicity of dichloromethane and methanol extracts of *C. repanda* on GMK cells was determined by MTT assay. Non toxic concentrations were used in the study. Methanol extracts of *C. repanda* showed higher anti-HSV efficacy than dichloromethane extracts, by plaque reduction assay, although, HSV particles were directly inhibited by both extracts. Inhibition of HSV attachment, penetration and replication were also observed, after treatment of HSV with *C. repanda* extracts. The most affected stage was the attachment period. Moreover, *C. repanda* extracts inhibited replication of HSV-2 more than HSV-1.

**Key words:** Anti-viral activity, *Cissus repanda*, plant extract, herpes simplex virus

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### INTRODUCTION

Herpes simplex virus (HSV) is a common human pathogen, widespread in the human population, with two types, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). HSV-1 infects orofacial mucosal surfaces whilst HSV-2 infects genital mucosal surfaces, resulting in clinical symptoms such as herpes labialis, encephalitis and genital herpes. After primary infection, HSV tends to persist in ganglia (Yang *et al.*, 2005). The virus can be reactivated, causing frequent recurrent infections in some patients, while most people experience few recurrences (Tyring, 1998). Moreover, infections in immunocompromised patients are usually more severe than in immunocompetent hosts (Schuhmacher *et al.*, 2003; Logu *et al.*, 2000). Although, acyclovir (ACV), valaciclovir and famciclovir have been used to treat herpes simplex virus infections, the efficacy of these drugs is limited by side effects and increasing resistance of the viruses at present. Antiviral-resistant HSV strains can occur following mutation in either HSV thymidine kinase (TK) or DNA polymerase. Moreover, several patients with frequent attacks may not be able to afford the cost of long-term treatment, particularly immunocompromised patients (Hammer and Inouye, 1997). Therefore, new antiviral agents from natural products are demanded for prevention and treatment of HSV infection.

Anti-HSV activity has been studied on medicinal plants such as *Camellia sinensis* Ktze. (Fukuchi *et al.*, 1989; Sydiskia *et al.*, 1991), *Nicotiana tabacum* Linn. (Larsson *et al.*, 1992), *Glycyrrhiza glabra* Linn. (Ryu *et al.*, 1993), *Rhus javanica* Linn. (Kurokawa *et al.*, 1995), *Barleria lupulina* Lindl., *Clinacanthus nutans* (Burm.f.) Lindau (Yoosook *et al.*, 1999), *Mangifera indica* Linn., *Maclura cochinchinensis* Cornor (Yoosook *et al.*, 2000), *Scoparia dulcis* Linn. (Galvis *et al.*, 2001), *Dunbaria bella* Prain (Akanitapichat *et al.*, 2006) and *Eugenia caryophyllus* (Spreng.) Bullock

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and S.G. Harrison (Tragoolpua and Jatisatienr, 2007). In this study, we aimed to investigate the anti-HSV activity of *Cissus repanda* Vahl. (Vitaceae). This medicinal plant can be found in northern Thailand. The plant can be used for treating rashes and detoxication (Chattawanith, 1997).

## MATERIALS AND METHODS

### Plant Material

*Cissus repanda* Vahl. (Vitaceae) plants were collected from Queen Sirikit Botanic Garden during July-September, 2005 and were identified by Associate Professor Dr. Chusie Trisonthi and Assistant Professor Paritat Trisonthi, Department of Biology, Faculty of Science, Chiangmai University, Chiangmai, Thailand.

Voucher specimens (QSBG 12006, QSBG 12053) were deposited in the herbarium at Queen Sirikit Botanic Garden, Chiang Mai Province, Thailand.

### Cell Line and Viruses

Green monkey kidney cells (GMK) were grown in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated bovine calf serum (Hyclone), 40  $\mu\text{g mL}^{-1}$  gentamicin (BIO BASIC INC.), in a humidified 5%  $\text{CO}_2$  incubator at 37°C.

Herpes simplex virus types 1 (HSV-1; F strain) and 2 (HSV-2; G strain) were used throughout the study. Quantitation of the virus was performed in 24-well tissue culture plates, using a plaque titration assay. Plaques were counted and the virus titers were expressed as plaque forming units (PFU)  $\text{mL}^{-1}$ .

### Plant Extracts

Dried plant leaves and climbers (500 g) were milled and soaked with dichloromethane or methanol at room temperature for 3 days. Then, the suspension was filtered and the solvent was evaporated, using a rotary evaporator and dried with high vacuum. The dry material was dissolved in dimethyl sulfoxide (DMSO) before investigation of anti-HSV activity.

### Cytotoxicity Test by MTT Assay

Cytotoxicity tests were performed following previously published procedures (Yu *et al.*, 2004). Cell toxicity was quantified using a MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide assay). GMK cells were seeded on 96-well microplates and incubated with the tested plant extracts for 72 h. Media were removed before being incubated with MTT solution for 4 h. Then, the blue formazan product was eluted from cells by adding DMSO. The absorbance at 570 nm was determined using a microplate reader. The concentration of the 50% cytotoxicity dose ( $\text{CD}_{50}$ ) was calculated.

### Inactivation Kinetics

HSVs were treated with non-toxic concentrations of the extracts at room temperature. Aliquots of the virus-extract mixture were taken and diluted for residual virus assay at 1 h intervals for up to 4 h, using the plaque titration assay. The residual virus titers were determined from two independent experiments

### Plaque Reduction Assay

The GMK cells were grown in 24-well tissue culture plates as a monolayer. Approximately 100 PFU of HSV were added to the cells per well and were incubated at room temperature for 1 h. Then, 200  $\mu\text{L}$  of media, dichloromethane or methanol extract of *C. repanda* at non toxic concentrations and ACV at  $\text{ED}_{50}$  concentration, were applied into duplicate test wells whereas, media were added into

control wells. After that, 400  $\mu\text{L}$  of growth media, containing 2% sodium carboxymethyl cellulose, was added to the cells. After 2-3 days incubation in the  $\text{CO}_2$  incubator, the cells were stained with 0.1% crystal violet in 1% ethanol. The plaques were counted and inhibitory activities of tested extracts were calculated comparing to control. The 50% effective dose ( $\text{ED}_{50}$ ) was also calculated.

#### **Effect of Plant Extract on Viral Attachment**

The GMK cell monolayers were pre-chilled at  $4^\circ\text{C}$  for 1 h (Cheng *et al.*, 2002). The media were aspirated and the cell monolayers were infected with HSV in the absence or presence of extracts and acyclovir. Then, the infected cells were further incubation at  $4^\circ\text{C}$  for 3 h and the cells were washed with PBS twice before overlaid with growth media, containing 2% sodium carboxymethyl cellulose. After 2-3 days incubation, the cells were stained with 0.1% crystal violet in 1% ethanol. The percentage of inhibition for HSV attachment to GMK monolayer was calculated.

#### **Effect of Plant Extract on Viral Penetration**

GMK cell monolayers were pre-chilled at  $4^\circ\text{C}$  for 1 h (Cheng *et al.*, 2002). The media were aspirated and cell monolayers were infected by HSV. Infected cells were further incubated at  $4^\circ\text{C}$  for 3 h and the extract or acyclovir was added. After further incubation at  $37^\circ\text{C}$  for 10 min, the infected cells were treated with PBS, pH 3 for 1 min and PBS, pH 11 were added immediately to neutralize acidic PBS. PBS was then removed and cell monolayer was overlaid with growth media, containing 2% sodium carboxymethyl cellulose and incubation for 2-3 days. The cells were stained with 0.1% crystal violet in 1% ethanol and the percentage of inhibition for HSV penetration was calculated.

#### **Effect of Plant Extract on HSV Replication**

GMK cells were grown as monolayers in  $25\text{ cm}^2$  flasks. The cells were infected with  $1 \times 10^6$  PFU  $\text{mL}^{-1}$  of HSV. Virus was allowed to adsorb to the cells for 60 min at room temperature. Then, unadsorbed virus was discarded and the cells were washed twice with PBS. The duplicated flasks of infected cells were maintained in media containing crude extract of *C. repanda* at non-toxic concentration while only media or media containing acyclovir at  $\text{ED}_{50}$  concentration were added into control flasks. Infected cells were observed for cytopathic effects (CPE) and were collected at 6, 12, 24 and 30 h after viral infection. The cells were frozen and thawed twice. The supernatants containing viruses were kept at  $-80^\circ\text{C}$  and virus titers were determined using plaque titration assay.

## **RESULTS**

The  $\text{CD}_{50}$  values of *C. repanda*, extracted with dichloromethane (CD) and methanol (CM) were  $737 \pm 26$  and  $1876 \pm 0$   $\mu\text{g mL}^{-1}$ , respectively. Thus, non-toxic concentrations of *C. repanda* extract were used to determine anti-HSV activity.

After incubation of viruses with the dichloromethane or methanol extract of *C. repanda* at room temperature for 1 h intervals up to 4 h, both HSV-1 and HSV-2 were directly inactivated (Table 1). After incubation of HSV-1 and HSV-2 with CD extract for 2 h, the viruses were directly inactivated to negligible amounts. Similar results were observed when HSV-2 was incubated with CM extract, whereas HSV-1 was inactivated after incubation with CM extract for only 1 h.

*C. repanda* extracts demonstrated anti-HSV activity against both types of HSVs using plaque reduction assay. HSV-1 was inhibited by CM and CD extracts with  $\text{ED}_{50}$  values of  $310.6 \pm 50.0$  and  $249.2 \pm 40.7$   $\mu\text{g mL}^{-1}$  and selective index (SI) values of  $6 \pm 1.2$  and  $2.9 \pm 0.4$ , respectively (Table 2). HSV-2 was also inhibited by CM and CD extracts with  $\text{ED}_{50}$  values of  $295.4 \pm 56.4$  and  $253.4 \pm 15.9$   $\mu\text{g mL}^{-1}$  and SI values of  $6.3 \pm 1.6$  and  $2.9 \pm 0.2$ , respectively. Therefore, the SI of CM

Table 1: Direct inactivation of HSV-1 and HSV-2 by methanol (CM) and dichloromethane (CD) extracts of *C. repanda*

Time of inactivation (h)	Log titer of virus <sup>a</sup>					
	HSV-1(F)			HSV-2(G)		
	CM	CD	Control	CM	CD	Control
0	4	4.8	5.6	4.7	5.4	5.7
1	<1	2.6	4.7	3.6	4.2	4.7
2	<1	<1	4.3	<1	<1	4.6
3	<1	<1	3.8	<1	<1	4.5
4	<1	<1	3.5	<1	<1	4.1

<sup>a</sup>Values are means of two independent experiments

Table 2: Anti-HSV activity of methanol (CM) and dichloromethane (CD) extracts of *C. repanda* on GMK cells

Viruses	Extracts	ED <sub>50</sub> (µg mL <sup>-1</sup> ) <sup>b</sup>	Selectivity index <sup>c</sup>
HSV-1	CM	310.6±50.0	6.0±1.2
	CD	249.2±40.7	2.9±0.4
HSV-2	CM	295.4±56.4	6.3±1.6
	CD	253.4±15.9	2.9±0.2

<sup>a,b</sup>Values are means±SD of two independent experiments <sup>c</sup>CD<sub>50</sub> ED<sub>50</sub><sup>-1</sup>

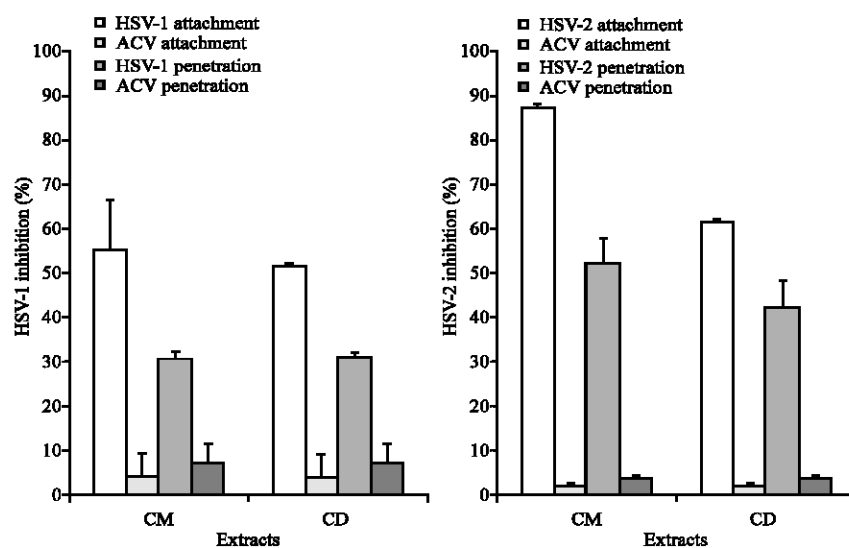


Fig. 1: Effects of extracts and acyclovir on HSV attachment and penetration to GMK cells

extract on HSV was higher than CD extract. Moreover, ED<sub>50</sub> values of ACV on HSV-1 and HSV-2 were 2.31 and 14.92 µg mL<sup>-1</sup>, respectively.

The effects of *C. repanda* extracts on HSV attachment are shown in (Fig. 1). CM and CD extracts blocked HSV-1 attachment with percentage of 55.3±11.1 and 51.5±0.8, respectively. Similar results were observed when CM extract was applied to determine the blocking of HSV-2 attachment. The efficacy of CM extract was better than CD extract with percentages of 87.5±0.7 and 61.5±0.4, respectively.

Furthermore, CM and CD extracts inhibited HSV-1 penetration with percentages of 30.6±1 and 31.0±0.8, respectively, while HSV-2 penetration was blocked by CM and CD extracts with percentages of 51.8±5.9 and 42.1±6.4, respectively (Fig. 1).

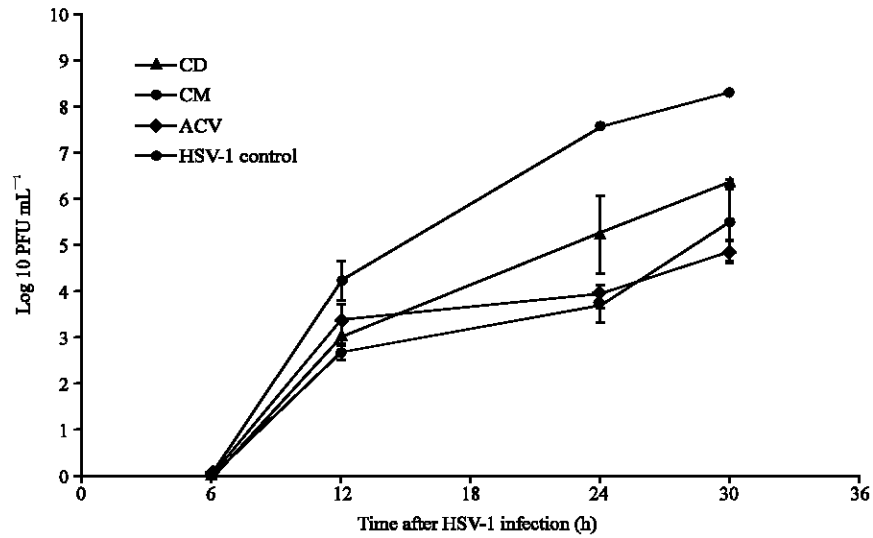


Fig. 2: Inhibition of HSV-1 replication by dichloromethane (CD) and methanol (CM) of *C. repanda* extract compared with ACV and control

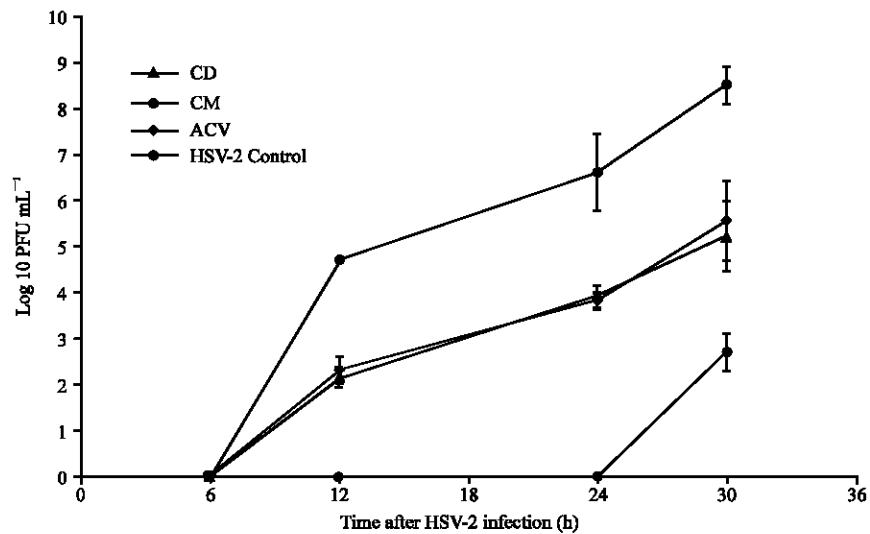


Fig. 3: Inhibition of HSV-2 replication by dichloromethane (CD) and methanol (CM) of *C. repanda* extracts compared with ACV and control

To determine the effects of *C. repanda* extracts on HSV yield, infected cells were maintained in the extract and were collected at 6, 12, 24 and 30 h. At 30 h after HSV-1 infection, log<sub>10</sub> PFU mL<sup>-1</sup> of virus control and ACV treated virus were 8.3±0.01 and 4.8±0.2, respectively. An inhibition of HSV-1 replication was observed, since the log<sub>10</sub> PFU mL<sup>-1</sup> of virus after treatment with CM and CD extracts were 5.5±0.8 and 6.3±0.1, respectively (Fig. 2). HSV-2 replication was also inhibited after treatment with the extracts. At 30 h after infection the log<sub>10</sub> PFU mL<sup>-1</sup> of HSV-2 were 2.7±0.4 and 5.2±0.8 after treatment with CM and CD extracts compared with 8.5±0.4 of control virus and 5.6±0.9 of ACV treated virus (Fig. 3). Therefore, the inhibitory activity of CM extract on HSV replication was higher than CD extract.

## DISCUSSION

In this study, we investigated anti-HSV activity of *C. repanda*, which is a medicinal plant species found in northern Thailand, used in traditional remedies (Chattawanith, 1997). The toxicity on GMK cells of dichloromethane extract was higher than methanol extract of *C. repanda*. Anti-HSV activity was observed after treatment of HSV with non-toxic concentrations of the extracts using the plaque reduction assay. ED<sub>50</sub> values of the CD extract were slightly lower than those obtained with the CM extract, when HSV-1 and HSV-2 were tested with the extracts. However, SI values (CD<sub>50</sub> ED<sub>50</sub><sup>-1</sup>) of CM extracts were about 2-fold higher than treatment with CD extracts. Thus, CM extracts showed higher anti-HSV efficacy than CD extracts. Moreover, the effects of the extracts on various stages of HSV multiplication cycles were determined to elucidate modes of action of the extracts. Both CM and CD extracts had an ability to inhibit HSV particles. HSV-1 was directly inactivated by CM and CD extracts to negligible amounts within 1 and 2 h, respectively, whereas the inactivation of HSV-2 particles was observed after incubation with CM and CD extracts for 2 h. Therefore, the efficiency of the CM extracts on inactivation of HSV-1 particle was higher than that of the CD extracts. Inhibition of HSV attachment and penetration was also observed. CM and CD extracts inhibited HSV-1 attachment 1.8 and 1.7 fold higher than penetration respectively. Similar results were obtained after treatment of HSV-2 with the extracts, with inhibition of HSV-2 attachment being 1.7 and 1.5 fold higher than viral penetration, after treatment with CM and CD extracts respectively.

Thirty hours after HSV-1 replication, the amounts of the virus were reduced by 33.7 and 24.1%, compared with the control after treatment with CM and CD extracts, respectively. Higher percentage inhibition of viral replication was observed on HSV-2 after treatment with CM and CD extracts with the reduction of viral amounts of 68.2 and 38.8%, respectively.

These results indicate that dichloromethane and methanol extracts of *C. repanda* inhibit HSV at various stages in the viral multiplication cycle i.e. attachment, penetration and replication. The most affected stage was the attachment period. The highest anti-HSV activity was obtained with methanolic extracts from *C. repanda*. Moreover, *C. repanda* extracts inhibited replication of HSV-2 more than HSV-1. This is the first report of anti-HSV activity of *C. repanda*, besides the activity on HSV-1 by crude extract of endophytic fungi isolated from *C. repanda* (Wiyakrutta *et al.*, 2004). However, biologically active compounds from *C. repanda* and their modes of action should be further studied.

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