

Full Length Research Paper

# Inhibitory effect of *Rhinacanthus nasutus* (Linn.) Kurz. and *Stemona tuberosa* (Lour.) extracts on herpes simplex virus infection

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**Efficacy of *Rhinacanthus nasutus* and *Stemona tuberosa* against herpes simplex viruses (HSV) was evaluated in this study. The cytotoxicities of the medicinal plant extracts were tested on Vero cell. 50% cytotoxic doses (CD<sub>50</sub>) of aqueous extracts of *R. nasutus* and *S. tuberosa* were 1268.0 and 5677.0 µg/ml, whereas ethanolic extracts of *R. nasutus* and *S. tuberosa* showed CD<sub>50</sub> of 50.4 and 1426 µg/ml, respectively. Both types of HSV were treated with the highest non-toxic concentrations of aqueous and ethanolic extracts. Inhibitory effect of these plant extracts on HSV infection was investigated by plaque reduction assay. It was found that ethanolic extract of *S. tuberosa* showed anti-HSV-1 when treated before viral attachment with therapeutic index (TI) value of  $41.30 \pm 0.25$  followed by ethanolic extract of *R. nasutus* with TI value of  $15.76 \pm 2.04$ , and aqueous extract of *S. tuberosa* showed anti-HSV-2 with TI value of  $3.64 \pm 0.01$ . During viral attachment, ethanolic extract of *S. tuberosa* showed anti-HSV-1 with TI value of  $10.75 \pm 0.13$ , whereas aqueous extract of *R. nasutus* inhibited HSV-2 with TI value of  $4.16 \pm 0.02$ . Furthermore, ethanolic extracts of *R. nasutus* and *S. tuberosa* inhibited HSV-1 after viral attachment with high TI value of  $37.78 \pm 1.4$  and  $5.79 \pm 0.14$ , whereas aqueous extract of *S. tuberosa* showed high TI values of  $5.79 \pm 0.02$  and  $7.23 \pm 0.03$  on HSV-1 and HSV-2, respectively. Moreover, viral particles were inactivated directly. Aqueous extracts of *R. nasutus* and *S. tuberosa* inhibited HSV-1 particles by  $92.23 \pm 0.98$  and  $89.12 \pm 2.14\%$ , respectively at 4 h of treatment. Ethanolic extracts of *R. nasutus* and *S. tuberosa* inhibited HSV-2 particles by  $95.12 \pm 1.43$  and  $94.11 \pm 1.43\%$ , respectively at 4 h of treatment. In addition, the highest reduction of log titer of HSV-1 and HSV-2 was observed at 30 h after treatment with ethanolic extract of *R. nasutus* by  $3.07 \pm 0.28$  and  $3.52 \pm 0.34$ , respectively. Therefore, extracts of *R. nasutus* and *S. tuberosa* showed promising anti-HSV activity.**

**Key words:** Herpes simplex virus, antiviral activity, medicinal plant, *Rhinacanthus nasutus* L., *Stemona tuberosa* L.

## INTRODUCTION

Nowadays, viral infectious diseases are important public health concern and expanded worldwide problem due to their morbidity and mortality in both developed and developing countries (Serkedjieva and Ivancheva, 1998).

One of the important viral infectious diseases is caused by herpes simplex virus (HSV). The first document about HSV has been recognized in ancient Greece. Herpes was used for descriptions of creep or crawl, which is a cutaneous spreading lesion. HSV is a member in family *Herpesviridae* and classified into subfamily *Alphaherpesvirinae*. HSV is divided into 2 types; Herpes simplex virus-1 (HSV-1) and Herpes simplex virus-2 (HSV-2). The HSV genome composes of double strand linear DNA duplex molecule, surrounded by an

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icosahedral capsid and the outer is covered with lipid envelope (Whitley et al., 1998; Roizman, 1991; Angeletti, 2006). Both types of HSV are characterized by their propensity of latency in sensory neural ganglia. After primary infection, the site of latency was established in the trigeminal ganglia for HSV-1 infection, whereas the sacral ganglia are the site in the case of HSV-2 infection. HSV-1 infection causes vesicle lesions around lips, oral cavity and facial area. Infections of cutaneous membrane, mucous membranes involved in esophageal membrane, and cerebral disease also occurred. HSV-2 is generally associated with genital infections transmitted through sexual activity. These pathologies may result from a primary infection or alternatively from reactivation of latent infection (Kott et al., 1998; Tyring, 1998; Greco et al., 2007).

HSV infection is usually managed with effective antiherpes synthetic drugs, which are continuously being developed. Nucleoside analogue, acyclovir (ACV) is successful in inhibition of virus replication by inhibition of viral DNA synthesis (Tolo et al., 2005). Moreover, other nucleoside derivatives such as penciclovir, famciclovir, valaciclovir and ganciclovir are also useful for the treatment of HSV infection worldwide (Lipipun et al., 2003). Although, the effective synthetic antiviral drugs are now available to treat the viral infection, but the drugs have some side effects and virus latency in nervous systems remains unsolved problem. Moreover, drug resistant strains may occur after receiving long-term prophylactic treatment, which can lead to many clinical problems and ineffective therapy (Kott et al., 1998; Greco et al., 2007; Coen, 2006). Thus, it is necessary to search for alternative antiviral agents, which exhibit different mechanism of action on HSV infection.

In Thailand, medicinal plants have been used for food ingredients. They are also used for treatment of various infectious and non infectious diseases in primary health care based on the knowledge of folk wisdom from ancient medicaments. Moreover, medicinal plants have phytochemicals as secondary metabolites of plants, which have diverse structure and bioactivities. In addition, medicinal plants are sources of many beneficial compounds against microbial infection such as carotenoids, flavonoids, diterpenes, flavonoids and stigmaterols (Murakami et al., 1995; Serkedjieva and Ivancheva, 1998; Kumar et al., 2004).

*Stemona tuberosa* (Lour.) is a member of Stemonaceae family, which is widely distributed in Southeast Asia. Beneficial constituents of this plant are from their bioactivities and phytochemicals such as alkaloids, stilbenoids and tocopherols. It is also used in Chinese medicine to treat many diseases such as pulmonary tuberculosis, cancer, microbial infections such as fungal, bacterial and virus infection. It can be used as insecticides (Brem et al., 2002; Akanitapichat et al., 2005a; Li et al., 2007; Puttarak et al., 2010; Bukke et al., 2011). *Rhinacanthus nasutus* (Linn.) Kurz. is classified

into family Acanthaceae. This plant is widely used in Thai traditional medicine for treatment of various diseases. Many beneficial bioactive components of this plant are known to be naphthoquinones such as rhinacanthins (A-D, G-Q), rhinacanthone and lignan groups (Gotoh et al., 2004; Siripong et al., 2006). Therefore, *S. tuberosa* (Lour.) and *R. nasutus* (Linn.) Kurz. medicinal plant extracts were investigated for their inhibitory activities against both types of HSV in this study.

## MATERIALS AND METHODS

### Plants and their extracts

Dried medicinal plants were purchased from Lampang Herb Conservation, Thailand. Plants were soaked in 95% ethanol for 3 days at room temperature or soaked in distilled water at 45°C for 3 h with the ratio of plant and solvent as 250 g/L. Then, the suspension of plant extracts was filtered and the solvent was evaporated by rotary evaporator and was dried with lyophilize. The dry powder of the plants was dissolved in dimethylsulfoxide (DMSO) before investigation of anti-HSV activity.

### Viruses and cells

Standard HSV type 1 strain F and type 2 strain G were used throughout this study and propagated on Vero cells, cultured as monolayer in Eagle's minimum essential medium (MEM; Hyclone, UK) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, UK) and 40 µg/ml gentamycin sulfate. The cells were cultured at 37°C in humidified 5% CO<sub>2</sub> atmosphere incubator. Virus titers were determined by plaque titration assay and expressed as plaque forming unit/milliliter (PFU/ml).

### Cell viability assay

Cytotoxicities of medicinal plant extracts were investigated on Vero cells. The extracts were serially two-fold diluted and each dilution of the extracts was added to quadruplet wells on 96-well plate. Then, Vero cells at concentration of  $1 \times 10^6$  cells/ml were added to each well and incubated for 72 h. After the medium was removed, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 min. 50% cytotoxicity dose (CD<sub>50</sub>) was expressed as the concentration of the extract that caused cell detachment from the wells by 50%. The CD<sub>50</sub> value was determined from dose-response curve and calculated according to modified protocol of Reed and Muench (1938).

### Plaque titration assay

HSV was serially ten-fold diluted in MEM and each dilution of HSV was added into the cell monolayer in 24-well plate. After viral adsorption for 1 h, the overlay medium containing growth medium and 1.5% sodium carboxymethyl cellulose was added to the cells. After incubation at 37°C in humidified 5% CO<sub>2</sub> atmosphere for 3 to 4 days, the cells were stained with 0.1% crystal violet in 1% ethanol and viral titers were expressed as plaque forming unit/ml.

### Plaque reduction assay

Vero cell monolayer was infected with 100 PFU of HSV for 1 h at

room temperature. After that, the plant extracts at non-toxic concentrations were added to the cells. After 3 to 4 days incubation at 37°C in humidified 5% CO<sub>2</sub> atmosphere, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 min. The number of plaque was counted comparing with untreated virus control and was expressed as PFU/ml. Therefore, 50% effective dose (ED<sub>50</sub>) was calculated by dose-response curves and expressed as 50% inhibition of plaque formation.

#### Effect of medicinal plant extracts on HSV before viral attachment

Various non-toxic doses of medicinal plant extracts were added into a confluence cell in 24-well plate and incubated at room temperature for 30 min. Then, the suspension was removed before being infected with 100 PFU of HSV. After 1 h, overlay medium was added and further incubated for 72 h at 37°C in 5% CO<sub>2</sub> incubator for 3 to 4 days. The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 20 min. Percentage of HSV inhibition was determined by reduction of plaque number when compared with untreated virus control. ED<sub>50</sub> value was also calculated by dose-response curves and expressed as 50% inhibition of plaque formation.

#### Effect of medicinal plant extracts on HSV during viral attachment

Medicinal plant extracts at non-toxic concentration and 100 PFU of HSV were added on 24-well tissue plate. Then, the mixture was incubated at room temperature for 1 h. After that the mixture was removed before it was overlaid with overlay medium and further incubated at 37°C in humidified 5% CO<sub>2</sub> atmosphere for 3 to 4 days. The number of plaques was counted and percentage of HSV inhibition was determined by reduction of plaque number when compared with untreated virus control. ED<sub>50</sub> value was also calculated by dose-response curves and expressed as 50% inhibition of plaque formation.

#### Inactivation kinetics

HSV was treated with non-toxic concentration of medicinal plant extracts, whereas MEM was used for viral control. The mixture of virus and plant extracts was incubated for 20, 40, 60, 80, 100 and 120 min, and was kept at -80°C. Titer of residual virus was determined by plaque titration assay.

#### Effect of plant extracts on HSV replication

Vero cells were grown as monolayer on 6-well plates. After that, the cells were infected with  $1 \times 10^6$  PFU/ml of HSV for 1 h. Next, infected cells were washed twice by PBS 1X to remove unattached virus. Then, medicinal plant extracts at non toxic concentrations were added on the infected cells and incubated at 37°C in humidified 5% CO<sub>2</sub> atmosphere. Infected cells were collected at 0, 6, 12, 24 and 30 h after viral infection. After that, the cells were frozen and thawed twice, and the virus was kept at -80°C. The viral titers were determined by plaque titration assay.

## RESULTS AND DISCUSSION

Viral infectious disease is an important health problem and still a major threat to public health, especially HSV

infection. Effective antiviral drugs were produced but side effects and development of drugs resistant viral strains may occur after long-term prophylactic treatment. Thus, in this study, efficacy of aqueous and ethanolic extracts of *R. nasutus* and *S. tuberosa* were examined for inhibitory effect on HSV infection.

Yield of aqueous extracts of *R. nasutus* and *S. tuberosa* were 10.65 and 6.00%, and ethanolic extracts of *R. nasutus* and *S. tuberosa* were 15.54 and 13.20%, respectively. Then, toxicities of the plant extracts were tested as the toxicities were important for selection of the extract which should be least or not interfere with host cell activity while specific to HSV particle. Thus, the cytotoxicity dose of *R. nasutus* and *S. tuberosa* extracts were calculated and expressed as CD<sub>50</sub>, which was the concentration of the extract that caused cells death by 50%. It was found that ethanolic and aqueous extracts of *R. nasutus* showed CD<sub>50</sub> value of 50.4 and 1268.0 µg/ml, respectively. Ethanolic and aqueous extracts of *S. tuberosa* showed CD<sub>50</sub> value of 1426 and 5677.0 µg/ml, respectively. Ethanolic extracts of *R. nasutus* and *S. tuberosa* showed higher toxicity than the aqueous extracts on Vero cells.

The highest non-toxic concentrations that showed cell viability above 90% were selected to test against both types of HSV. Therefore, ethanolic extracts of *R. nasutus* and *S. tuberosa* at concentration of 3.9 and 250 µg/ml were selected to test anti-HSV in this study, while, aqueous extracts of *R. nasutus* and *S. tuberosa* at concentration of 1000 and 2000 µg/ml were selected. Moreover, ACV at ED<sub>50</sub>, which was the dose that inhibited HSV infection by 50%, was selected as positive control. Thus, ACV at concentrations of 1.5 and 3.1 µg/ml was used to inhibit HSV-1 and HSV-2 as a comparable antiviral agent.

The efficacy of *R. nasutus* and *S. tuberosa* extracts were investigated for potential inhibition of HSV infection when treating the extracts before, during and after viral attachment using plaque reduction assay. Inhibition of viral particles and viral replication were also determined. For demonstration of the efficacy of these extracts on HSV, therapeutic index (TI) value was calculated from the ratio of CD<sub>50</sub> and ED<sub>50</sub>. The high TI value reflected high therapeutic potential of the plant extracts. It was found that ethanolic extracts of *S. tuberosa* and *R. nasutus* could protect HSV-1 before viral attachment with therapeutic index (TI) of  $41.30 \pm 0.25$  and  $15.76 \pm 2.04$ , respectively. Thus, the ethanolic extract of *S. tuberosa* demonstrated higher inhibitory efficacy against HSV-1 than *R. nasutus*. Only aqueous extract of *S. tuberosa* could inhibit HSV-2 with TI value of  $3.64 \pm 0.01$  (Table 1).

Moreover, the efficacy of these plant extracts on HSV during viral attachment was also demonstrated. Ethanolic extract of *S. tuberosa* showed anti-HSV-1 with TI value of  $10.75 \pm 0.13$ , whereas the aqueous extracts of *R. nasutus* and *S. tuberosa* could inhibit HSV-2 with TI values of  $4.16 \pm 0.02$  and  $3.55 \pm 0.01$ , respectively (Table 1).

**Table 1.** Inhibitory effect of aqueous and ethanolic extracts of *R. nasutus* and *S. tuberosa* on herpes simplex virus.

Test	TI value (ED <sub>50</sub> ± SD)					
	Before viral attachment		During viral attachment		After viral attachment	
	HSV-1	HSV-2	HSV-1	HSV-2	HSV-1	HSV-2
<b>Aqueous extract</b>						
<i>R. nasutus</i> (1000 µg/ml)	- (-) <sup>a</sup>	- (-) <sup>a</sup>	- (-) <sup>a</sup>	4.16±0.02 (305.17±1.32) <sup>a</sup>	- (-) <sup>a</sup>	1.82±0.02 (697.19±11.50) <sup>a</sup>
<i>S. tuberosa</i> (2000 µg/ml)	- (-) <sup>b</sup>	3.64±0.01 (1559.18±5.12) <sup>b</sup>	- (-) <sup>b</sup>	3.55±0.01 (1600.85±5.49) <sup>b</sup>	5.79±0.02 (981.14±3.54) <sup>b</sup>	7.23±0.03 (785.56±3.07) <sup>b</sup>
<b>EtOH extract</b>						
<i>R. nasutus</i> (3.9 µg/ml)	15.76±2.04 (3.23±0.42) <sup>a</sup>	- (-) <sup>a</sup>	- (-) <sup>a</sup>	- (-) <sup>a</sup>	37.78±1.4 (1.34±0.05) <sup>a</sup>	- (-) <sup>a</sup>
<i>S. tuberosa</i> (250 µg/ml)	41.30±0.25 (34.53±0.21) <sup>a</sup>	- (-) <sup>a</sup>	10.75±0.13 (132.68±1.58) <sup>a</sup>	- (-) <sup>a</sup>	5.79±0.14 (246.29±6.09) <sup>a</sup>	- (-) <sup>a</sup>

TI = Therapeutic index (CD<sub>50</sub>/ED<sub>50</sub>); Data were presented as mean ± standard deviation (SD) of duplicate experiments. Statistical comparisons between groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b statistical tests were shown. Values with the different alphabets within each column were significantly different (P < 0.05).

In addition, medicinal plant extracts were also determined for inhibitory effect on HSV infection after viral attachment to the cell. The result showed that HSV-1 was inhibited by ethanolic extract of *R. nasutus* and *S. tuberosa* with TI value of 37.78 ± 1.4 and 5.79 ± 0.14, whereas aqueous extract of *S. tuberosa* inhibited HSV-1 with TI value of 5.79 ± 0.02. Moreover, aqueous extracts of *S. tuberosa* and *R. nasutus* inhibited HSV-2 with TI values of 7.23 ± 0.03 and 1.82 ± 0.02, respectively (Table 1).

Therefore, aqueous extract of *S. tuberosa* exerted highest anti-HSV-1 and 2 activities when treated after viral attachment. Ethanolic extract of *S. tuberosa* also demonstrated anti-HSV-1 when adding the extract before viral attachment, followed by during and after viral attachment. Thus, the extract might inactivate viral particles and also interfered with viral attachment process. Ethanolic extract of *R. nasutus* showed the highest anti-HSV-1 activity when treated after viral attachment to the cells, so that the extract might interfere with viral multiplication cycle. Moreover, plaques size of HSV were also smaller than the

plaque size of viral control after treatment with the extracts, although, some of these plant extracts inhibited HSV less than 50%. Thus, it was possible that plant extract was able to reduce viral infectivity to neighboring cells (Yoosook et al., 1999).

Effect of these plant extracts on virus particles was also investigated. Aqueous extracts of *R. nasutus* and *S. tuberosa* showed the highest inhibition on HSV-1 particles at 240 min by 92.23 ± 0.98 and 89.12 ± 2.14%, respectively. Moreover, HSV-2 particles were also inhibited by aqueous extracts of *R. nasutus* and *S. tuberosa* by 83.15 ± 1.56 and 88.94 ± 1.53%, respectively (Table 2).

HSV-2 particles were inhibited directly after treatment with ethanolic extracts of *R. nasutus* and *S. tuberosa* by 95.12 ± 1.43 and 94.11 ± 1.43%, respectively, whereas ethanolic extracts of *R. nasutus* and *S. tuberosa* inhibited HSV-1 by 73.43 ± 0.12 and 92.83 ± 0.13%, respectively (Table 3). The ability of these medicinal plant extracts to inhibit HSV-1 and 2 infections was significantly increased by time with the highest anti-viral activity observed at 240 min of

incubation.

Furthermore, inhibitory effect of medicinal plant extracts on viral replication at 0, 6, 12, 24 and 30 h was investigated. The results showed that HSV titers decreased upon the time when treated with these medicinal plant extracts. HSV-1 and HSV-2 yields were reduced after treatment with aqueous extract of *R. nasutus* by 0.98 ± 0.01 and 1.13 ± 0.21 log PFU/ml, respectively, while yields of HSV-1 and HSV-2 were reduced after treatment with ethanolic extract of *R. nasutus* by 3.07 ± 0.28 and 3.52 ± 0.34 log PFU/ml, respectively (Table 4, Figures 1 and 3). Reductions of HSV-1 and HSV-2 yields were 1.71 ± 0.17 and 0.47 ± 0.01 log PFU/ml after treatment with aqueous extracts of *S. tuberosa*. Yield reductions of HSV-1 and HSV-2 were 1.23 ± 0.00 and 1.10 ± 0.13 log PFU/ml after treatment with ethanolic extracts of *S. tuberosa* (Table 4, Figures 2 and 4). Ethanolic extract of *R. nasutus* showed significantly the highest ability to inhibit viral replication against both types of HSV. Further study should be performed to clarify the effect of the extracts on other stages of multiplication cycle, such as viral DNA and

**Table 2.** Percentage of inhibition on HSV-1 and HSV-2 viral particles by aqueous extracts of *R. nasutus* and *S. tuberosa*.

Medicinal plant extract	Inhibition effect of medicinal plant extracts on HSV at 60-240 min $\pm$ SD*					
	60	80	100	120	180	240
<b>HSV-1</b>						
<i>R. nasutus</i> (1000 $\mu$ g/ml)	43.23 $\pm$ 1.82 <sup>b</sup>	54.50 $\pm$ 3.36 <sup>b</sup>	63.44 $\pm$ 2.21 <sup>b</sup>	83.67 $\pm$ 0.47 <sup>b</sup>	89.53 $\pm$ 1.13 <sup>b</sup>	92.23 $\pm$ 0.98 <sup>b</sup>
<i>S. tuberosa</i> (2000 $\mu$ g/ml)	57.4 $\pm$ 1.41 <sup>b</sup>	60.55 $\pm$ 2.44 <sup>b</sup>	75.36 $\pm$ 2.24 <sup>b</sup>	79.80 $\pm$ 6.23 <sup>b</sup>	82.13 $\pm$ 3.21 <sup>b</sup>	89.12 $\pm$ 2.14 <sup>b</sup>
<b>HSV-2</b>						
<i>R. nasutus</i> (1000 $\mu$ g/ml)	7.26 $\pm$ 2.69 <sup>a</sup>	26.47 $\pm$ 1.16 <sup>a</sup>	42.86 $\pm$ 2.52 <sup>a</sup>	71.21 $\pm$ 0.00 <sup>a</sup>	78.31 $\pm$ 2.12 <sup>a</sup>	83.15 $\pm$ 1.56 <sup>a</sup>
<i>S. tuberosa</i> (2000 $\mu$ g/ml)	41.43 $\pm$ 2.02 <sup>b</sup>	55.51 $\pm$ 1.18 <sup>b</sup>	66.16 $\pm$ 1.83 <sup>b</sup>	78.43 $\pm$ 0.74 <sup>b</sup>	83.13 $\pm$ 3.17 <sup>b</sup>	88.94 $\pm$ 1.53 <sup>b</sup>

\*Data were presented as mean  $\pm$  standard deviation (SD) of duplicate experiments. Statistical comparisons between groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b tests were shown, and different alphabets showed significantly different value (P < 0.05).

**Table 3.** Percentage of inhibition on HSV-1 and HSV-2 viral particles by ethanolic extracts of *R. nasutus* and *S. tuberosa*.

Medicinal plant extract	Inhibition effect of medicinal plant extracts on HSV (min) $\pm$ SD*					
	60	80	100	120	180	240
<b>HSV-1</b>						
<i>R. nasutus</i> (3.9 $\mu$ g/ml)	0.00 $\pm$ 0.00 <sup>a</sup>	31.36 $\pm$ 3.99 <sup>a</sup>	46.00 $\pm$ 0.00 <sup>a</sup>	59.75 $\pm$ 0.89 <sup>a</sup>	69.31 $\pm$ 2.12 <sup>a</sup>	73.43 $\pm$ 0.12 <sup>a</sup>
<i>S. tuberosa</i> (250 $\mu$ g/ml)	43.80 $\pm$ 2.05 <sup>b</sup>	51.49 $\pm$ 2.47 <sup>b</sup>	66.26 $\pm$ 2.78 <sup>b</sup>	76.59 $\pm$ 3.54 <sup>b</sup>	84.44 $\pm$ 1.53 <sup>b</sup>	92.83 $\pm$ 0.13 <sup>b</sup>
<b>HSV-2</b>						
<i>R. nasutus</i> (3.9 $\mu$ g/ml)	54.93 $\pm$ 0.00 <sup>b</sup>	61.54 $\pm$ 2.63 <sup>b</sup>	75.42 $\pm$ 1.77 <sup>b</sup>	88.49 $\pm$ 3.22 <sup>b</sup>	92.21 $\pm$ 3.11 <sup>b</sup>	95.12 $\pm$ 1.43 <sup>b</sup>
<i>S. tuberosa</i> (250 $\mu$ g/ml)	63.75 $\pm$ 4.16 <sup>b</sup>	69.67 $\pm$ 4.66 <sup>b</sup>	82.34 $\pm$ 2.59 <sup>b</sup>	87.36 $\pm$ 5.33 <sup>b</sup>	93.11 $\pm$ 1.45 <sup>b</sup>	94.11 $\pm$ 1.43 <sup>b</sup>

\*Data were presented as mean standard  $\pm$  deviation (SD) of duplicate experiments. Statistical comparisons between groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b tests were shown, and different alphabets showed significantly different value (P < 0.05).

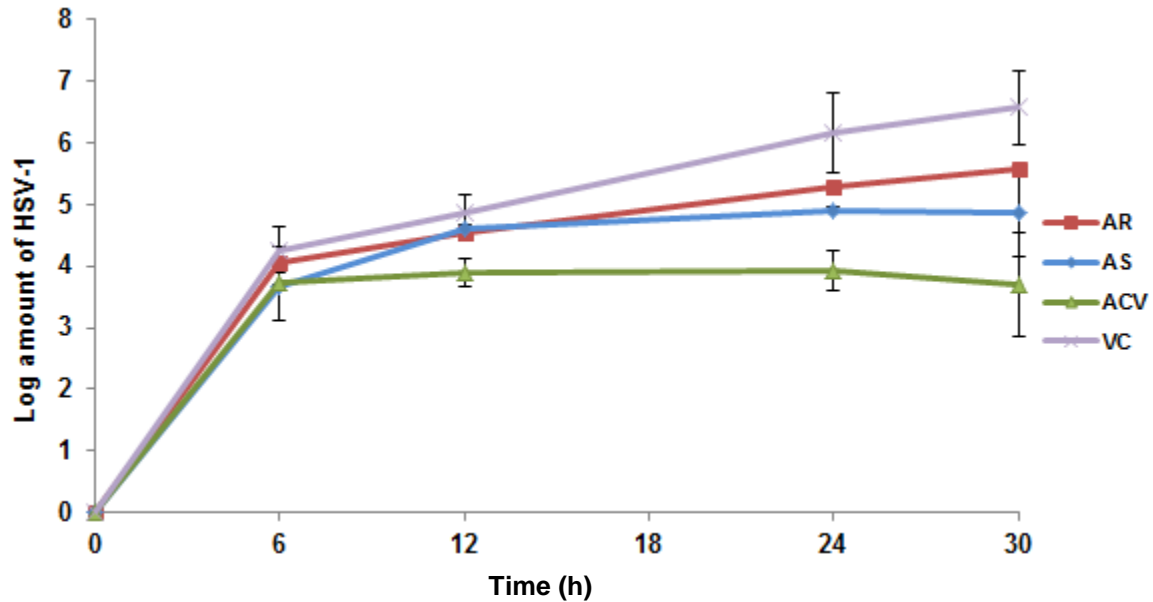
**Table 4.** Yield reduction of log HSV-1 and HSV-2 titers after treatment with aqueous and ethanolic extracts of *R. nasutus* and *S. tuberosa* at 30 h after viral infection.

Medicinal plant extract	Concentration ( $\mu$ g/ml)	Yield reduction*	
		Log PFU/ml of HSV-1	Log PFU/ml of HSV-2
<b>Aqueous extract</b>			
<i>R. nasutus</i>	1000	0.98 $\pm$ 0.01 <sup>a</sup>	1.13 $\pm$ 0.21 <sup>a</sup>
<i>S. tuberosa</i>	2000	1.71 $\pm$ 0.17 <sup>a</sup>	0.47 $\pm$ 0.01 <sup>a</sup>
<b>EtOH extract</b>			
<i>R. nasutus</i>	3.9	3.07 $\pm$ 0.28 <sup>b</sup>	3.52 $\pm$ 0.34 <sup>b</sup>
<i>S. tuberosa</i>	250	1.23 $\pm$ 0.00 <sup>a</sup>	1.10 $\pm$ 0.13 <sup>a</sup>

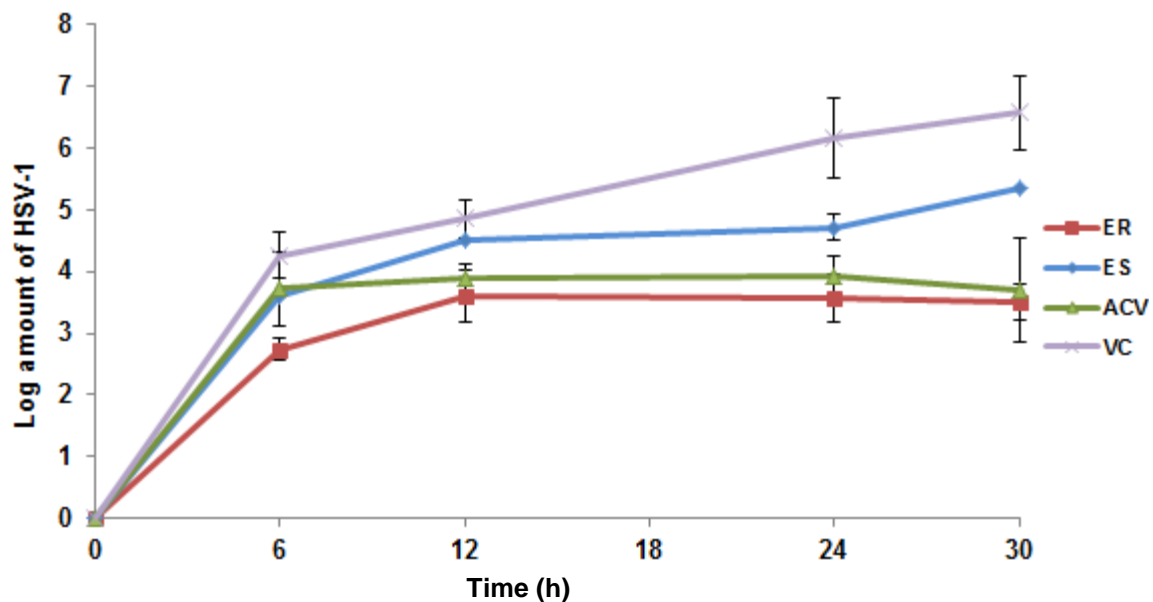
\*Data were presented as mean  $\pm$  standard deviation (SD) of duplicate experiments. Statistical comparisons between groups in each column using randomized complete blocks (RCB) statistical test were shown. Values with the different alphabets within each column were significantly different (P < 0.05).

protein synthesis, and possibly, synergistic effect of the combination of these extracts. Moreover, active compounds of the extracts that exert anti-viral activity against HSV-1 and HSV-2 should be elucidated.

Other studies on anti-viral activities of *Rhinacanthus* and *Stemona* were demonstrated. Kernan et al. (1997) reported that two new lignans; rhinacanthin-E and rhinacanthin-F, which were extracted from aerial part of



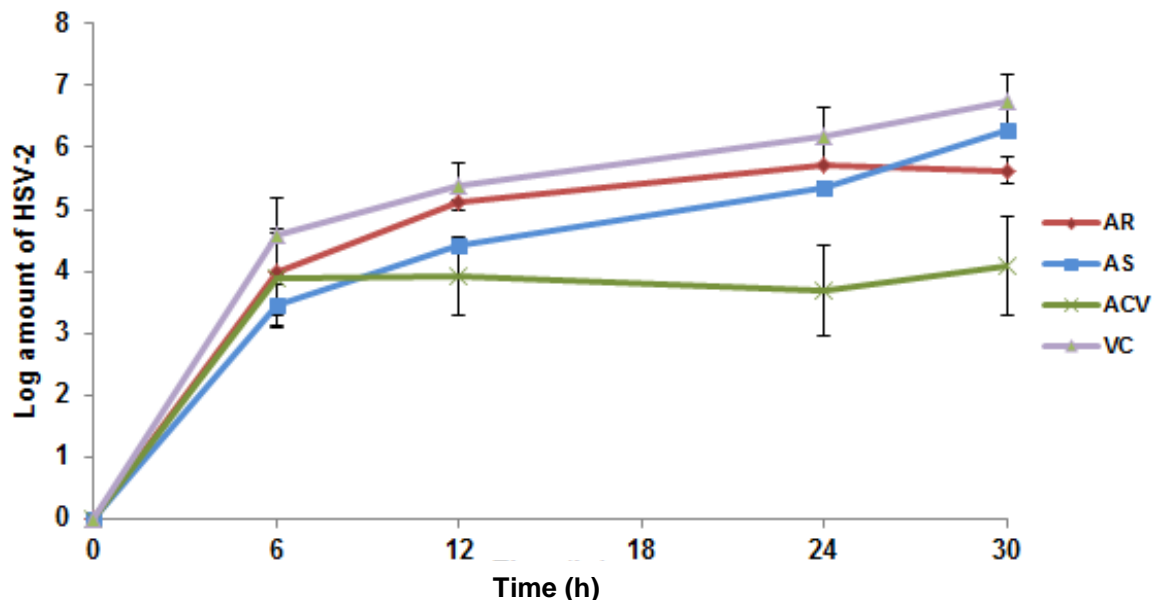
**Figure 1.** Yield of HSV-1 at 0, 6, 12, 24 and 30 h after treatment with aqueous extracts of *R. nasutus* (1000  $\mu\text{g/ml}$ , AR) and *S. tuberosa* (2000  $\mu\text{g/ml}$ , AS) compared with antiviral agent, acyclovir (ACV) at 1.5  $\mu\text{g/ml}$  and virus control (VC).



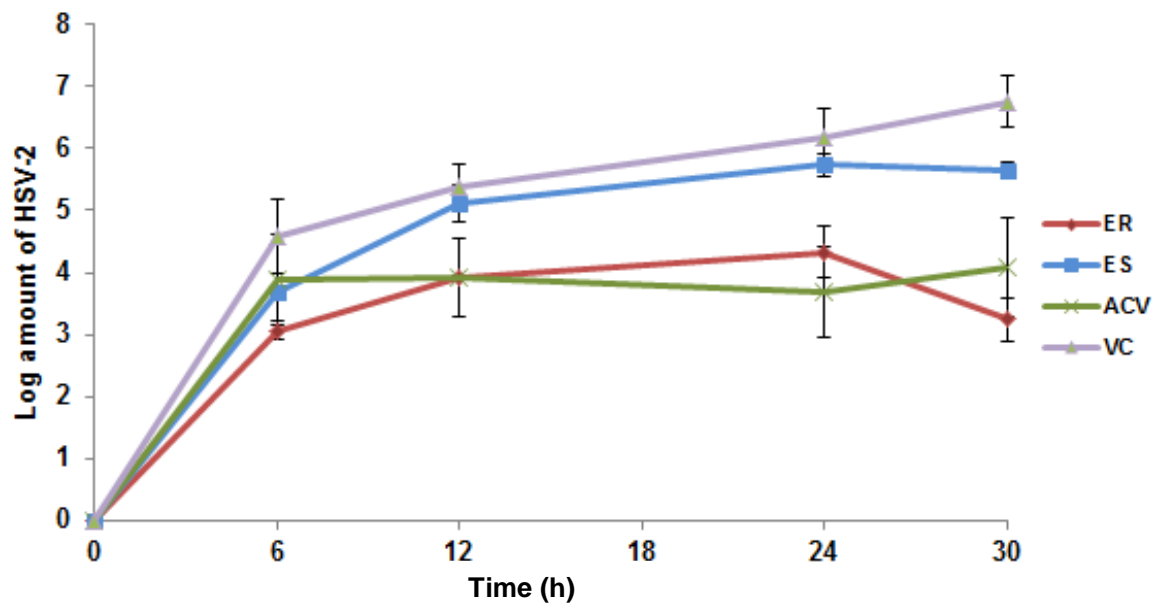
**Figure 2.** Yield of HSV-1 at 0, 6, 12, 24 and 30 h after treatment with ethanolic extracts of *R. nasutus* (3.9  $\mu\text{g/ml}$ , ER) and *S. tuberosa* (250  $\mu\text{g/ml}$ , ES) compared with antiviral agent, acyclovir (ACV) at 1.5  $\mu\text{g/ml}$  and virus control (VC).

*R. nasutus* showed strong inhibitory efficacy on influenza virus, whereas inactive against HSV-2G. Lipophilic crude extracts from the leaves and roots of *Stemona collinsae* and *S. tuberosa* could inhibit *Spodoptera littoralis* (Brem et al., 2002). Moreover, the extracts of *R. nasutus* and rhinacanthin C showed antiproliferative activity on human

prostate (PC-3) and bladder carcinoma cell lines (T24) *in vitro* (Gotoh et al., 2004). Dichloromethane-methanol extract of *S. collinsae* inhibited HSV-1 and HSV-2 while aqueous and ethanolic extracts inhibited HSV with low activity, and anti-cancer activity of the extract of *S. collinsae* against KB and MCF-7 cancer cells were also



**Figure 3.** Yield of HSV-2 at 0, 6, 12, 24 and 30 h after treatment with aqueous extracts of *R. nasutus* (1000 µg/ml, AR) and *S. tuberosa* (2000 µg/ml, AS) compared with antiviral agent, acyclovir (ACV) at 3.1 µg/ml and virus control (VC).



**Figure 4.** Yield of HSV-2 at 0, 6, 12, 24 and 30 h after treatment with ethanolic extracts of *R. nasutus* (3.9 µg/ml, ER) and *S. tuberosa* (250 µg/ml, ES) compared with antiviral agent, acyclovir (ACV) at 3.1 µg/ml and virus control (VC).

shown (Akanitapichat et al., 2005a). In addition, Li et al. (2007) reported that dichloromethane fraction of *S. tuberosa* could inhibit tumor cell growth and also induced apoptosis of human medullary thyroid carcinoma cells.

Beside this study, various kinds of medicinal plants had been investigated on anti-HSV activity. Anti-HSV type

1 activity of the crude aqueous extracts from shoots of *Helichrysum aurenitens* was demonstrated (Meyer et al., 1996). Anti-HSV activities of *Geranium sanguineum* Linn (Serkedjieva and Ivancheva, 1998) and *Rhus javanica* were determined (Nakano et al., 1998). Ethanolic extract of *Annona muricata* and aqueous extract of *Petunia*

*nyctaginiflora* were found to inhibit HSV-1 (Padma et al., 1998). Extracts of *Holoptelea integrifolia* and *Nerium indicum* exhibited considerable antiviral activity against HSV (Rajbhandari et al., 2000). Methanolic extracts of *Barleria lupulina* and *Clinacanthus nutans* were active against HSV-2 (Yoosook et al., 1999). Inhibitory effect of HSV-1 and 2 was also shown from a partially purified fraction derived from a dichloromethane-methanol extract of *Dunbaria bella* Prain (Akanitapichat et al., 2005b) and crude aqueous extract of *Carissa edulis* (Tolo et al., 2005). Compounds hyperbrasilol B, amentoflavone, and luteoforol isolated from methanolic extract of *Hypericum connatum* Lam had activity on HSV-1 (Fritz et al., 2007). Methanol extract of *Inula cappa* was potent inhibitor of HSV infection *in vitro* (Nikomtat et al., 2011). Aromatic herbs, chamomile, sage, lavender and peppermint extracts were demonstrated to possess anti-HSV activities at various stages of the viral multiplication cycle (Yucharoen et al., 2011; Yucharoen et al., 2012).

## Conclusion

Inhibitory effect of the aqueous and ethanolic extracts of *R. nasutus* and *S. tuberosa* against HSV-1 and HSV-2 infection on Vero cells was demonstrated on various stages of HSV multiplication cycle. Aqueous extract of *S. tuberosa* exerted the highest anti-HSV-1 and 2 activities when treatment after viral attachment. Ethanolic extract of *S. tuberosa* demonstrated the highest anti-HSV-1 when adding the extract before viral attachment. Ethanolic extract of *R. nasutus* showed the highest anti-HSV-1 activity when treated after viral attachment to the cells. Furthermore, the efficacy of medicinal plant extracts on inhibition of HSV directly was also determined. It was found that aqueous and ethanolic extracts of *R. nasutus* and *S. tuberosa* showed significant inhibition of HSV-1 and HSV-2 viral particles. Viral replication at 30 h after treatment was also reduced after treatment with extracts of *R. nasutus* and *S. tuberosa*. The ethanolic extract of *R. nasutus* showed the highest activity on HSV replication. Therefore, *R. nasutus* and *S. tuberosa* should be used as potential medicinal plants for treatment of HSV infection.

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