

Inhibitory effect of *Rosmarinus officinalis* L., *Senna alata* (L.) Roxb., *Acalypha indica* L., and *Elephantopus scaber* L. against herpes simplex viruses

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Abstract

Introduction: Herpes simplex virus (HSV) is pathogenic to human. This study demonstrated anti-herpes simplex virus activity of ethanol extract of *Rosmarinus officinalis* L., *Senna alata* (L.) Roxb., *Acalypha indica* L., and *Elephantopus scaber* L. on green monkey kidney cells. They are widely used in pharmaceutical products and in traditional medicine for anti-inflammatory, anti-tumor, antimicrobial, and antiseptic activity. **Methods:** In order to determine the mode of antiviral action of these plants, viral adsorption and viral replication were performed. **Results:** These results indicated that ethanol extract of *S. alata* and *A. indica* did not affect HSV during viral adsorption and after viral adsorption. The cells were treated on HSV-1F and HSV-2G during infection by the ethanol extract of *E. scaber* with therapeutic indices (TI) of 0.35 and 0.54, respectively. Ethanol extract of *R. officinalis* demonstrated higher inhibitory effects during HSV-1F and HSV-2G adsorption with TI of 1.23 and 57.38 while TI of 9.7 and 93.67 were observed after HSV-1 and HSV-2 adsorption. **Conclusion:** This study demonstrated that the ethanol extract of *R. officinalis* and *E. scaber* showed higher inhibitory activity on HSV-2G than HSV-1F. The rosemary extract showed the highest activity when tested after HSV-2G adsorption. Therefore, this study will be useful for anti-HSV drug development from herb, *R. officinalis* and *E. scaber*.

Keywords: herpes simplex virus, *Rosmarinus officinalis* L., *Senna alata* (L.) Roxb., *Acalypha indica* L., *Elephantopus scaber* L.

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1. Introduction

Herpes simplex viruses (HSVs) are endemic in all human populations. HSV-1 and HSV-2 are members of a subfamily of the *Alphaherpesvirinae*, with common biological activities, but they are different in many aspects. The virus particle consists of a deoxyribonucleic acid genome packaged in icosahedral capsid containing 162 capsomers (Collier and Oxford, 2000). HSVs are linear double stranded DNA that consists of nucleotide composition ranged from 32 to 75% G+C, depending on the virus species. They are classified into 2 types; HSV-1 and HSV-2. HSV-1 is the primary agent of orolabial disease or fever blister whereas HSV-2 causes infection at genital area. They can cause lytic and latent infections within neurons that innervated mucosal and squamous epithelial surfaces in the oral and genital regions. The illness is more serious in patients with deteriorated cellular immunity. Since viruses are intracellular parasites and utilize organelles within host cells, so it is difficult to eliminate virus. The effective chemically synthesized antiviral drugs have been used to treat HSV infection. Acyclovir (ACV) is specifically inhibits the herpes virus DNA polymerase. However, ACV is expensive and a major problem of ACV therapy is the development of HSV resistant variants that may occur after long-term treatment (Crumpacker *et al.*, 1982). Mutations in the HSV-thymidine kinase or DNA polymerase genes of HSV may occur after long-term treatment and these mutants are particularly important as opportunistic infectious agents in immunocompromised patients (Elion, 1993). Many medicinal

plants have been used against infectious disease. Screening of plant extracts for anti-HSV activity has given interesting results for the search of new antiviral agents. The ethanol extracts of *R. officinalis* (rosemary), *S. alata*, *A. indica*, and *E. scaber* were selected for investigated of HSV during viral adsorption and HSV after adsorption. *A. indica* is widely used in tradition medicine for hepatoprotective, wound healing, anti-inflammatory and anti-bacterial activity (Jagatheeswari *et al.*, 2013). Hexane, chloroform, ethyl acetate and methanol extracts of *A. indica* against *Candida albican* and bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Govindarajan *et al.*, 2008). The extracts of *E. scaber* has been traditionally used for diverse biological functions such as anti-bacterial, anti-cancer, anti-diabetic and anti-rhinoviral activity (Wang *et al.*, 2004 and Jenny *et al.*, 2012). Organic effects of *S. alata* showed anti-microbial activity against both Gram negative and Gram positive bacteria. This plant has been used for the treatment of wound infection, gonorrhea, and other infectious skin disease (Doughari and Okafor, 2007). Rosemary is widely used as traditional herb medicines for food flavouring as well as in the cosmetics, perfumery, pharmaceutical industries, preservation of foods, treatment of various human diseases and useful for urinary aliments. Moreover, they also possess other biological activities such as insecticidal, anti-inflammatory, anti-tumor, and antimicrobials activities. (Bozinet *et al.*, 2007; Carvalho *et al.*, 2005; Flück, 1988; Ho *et al.*, 2000; Mahmoud *et al.*, 2005; Moreno *et al.*, 2006; Ody,

2000; Ouattara *et al.*, 1997; Sacchettiet *et al.*, 2005). Base on their biological activity from traditional medicine, these plants can be used for treatment of wound infection and infectious skin diseases. Therefore, in the present study, we determined mechanism of action of these herbson HSV.

2. Material and Method

Cell linesand viruses

African green monkey (GMK) cells were cultivated as monolayer in Eagle's minimum essential medium; MEM (Hyclone,UK) containing 10% heat inactivated fetal bovine calf serum (Starrate, Australia) and 40 μ g/mL gentamycin. Cells were incubated at 37°C in 5% CO_2 humidified atmosphere incubator. HSV-1 strain F and HSV-2 strain G were propagated on GMK cells. The virus stocks were prepared from supernatants of infected cells and stored at -85°C until use. Viral titers were determined by plaque titration assay and were expressed as plaque forming unit (PFU)/mL.

Plant extracts and acyclovir

Rosemary was purchased from Thai Royal project, Thailand. *S. alata* (L.) Roxb. and *E. scaber* were collected from NakhonSawan province, Thailand where as *A. indica* was collected from Mukdahan province, Thailand. Aerial part of Rosemary and leaves of *A. indica*, *E. scaber* and *S. alata* were dried at 45°C incubator. Dried plant materials were cut into small pieces, milled and soaked in ethanol at room temperature for three-day period. The extracts were filtered, concentrated and lyophilized to form dried powder

extracts and then reconstituted in dimethylsulfoxide (DMSO) before determination of anti-HSV activity.

Acyclovir (ACV) was purchased as powder from Sigma Aldrich chemical Company, USA and dissolved in sterile water before use. ACV was diluted with MEM before determination anti-HSV activity and 50% inhibition concentration (IC_{50}) of ACV was calculated.

Cytotoxicity assay

For evaluation of cytotoxicity, reconstitution of the extracts were diluted two-fold by MEM and added in quadruplicated wells of 96-well tissue culture plate. Then, GMK cells suspension containing 1×10^6 cells/mL were seeded into the culture plate. After 4 days incubation, the cells were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The cytotoxicity was expressed as the 50% cytotoxic dose (CD_{50}) and calculated according to modified protocol of Reed and Muench (1938).

Plaque titration assay

Vero cells were seeded into 24-well culture plates and incubated at 37°C in 5% CO_2 incubator. Viral stocks were serially diluted in MEM and each dilution was added into cell monolayer. After 1 hr adsorption, the infected cells were then overlaid with 1.5% carboxymethyl cellulose medium and incubated at 37°C in 5% CO_2 incubator for 4days before staining with 0.1% crystal violet in 1% ethanol for 15 minutes and then virus plaques were counted and expressed as plaque forming unit (PFU) per mL.

Effect of plant extracts on HSV during viral adsorption

Confluent cell monolayers cultivated in 24-well plate were infected with 100-200 PFU of HSV. Then, non toxic concentrations of plant extracts were added into cell monolayers and incubated for 1 hour at room temperature for virus adsorption. After viral adsorption, the inoculum was removed and infected cells were overlaid with overlay medium, and incubated at 37°C in 5% CO₂ incubator for 4 days. The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The number of plaques was counted and the 50% effective doses (ED₅₀) were determined from dose-response curves and expressed 50% inhibition of plaque numbers compared with controls.

Effect of plant extracts on HSV after viral adsorption

Confluent cell monolayers cultivated in 24-well plate were infected with 100-200 PFU of HSV and incubated for 1 hour at room temperature for virus adsorption. After viral adsorption, non toxic concentrations of the plant extracts were added onto the infected cells. The cells were overlaid with overlay medium and incubated for 4 days at 37°C in 5% CO₂ incubator. The virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 minutes and the 50% effective doses (ED₅₀) were calculated.

3. Results and Discussion

The ethanol extracts were tested for toxicity on GMK cells. It was found that 50% cytotoxicity dose of ethanol extract of *R. officinalis*, *S. alata*, *A. indica*, and *E. scaber* were 13.77, 358.2, 13.7 and 4.97mg/mL, respectively. The effects of plant extract on viral during adsorption and after viral adsorption were demonstrated as shown in Table 1 and 2. The results showed that *S. alata*, and *A. indica* extracts did not affect HSV during viral adsorption and after viral adsorption. ED₅₀ values of ethanol extract of *R. officinalis* and *E. scaber* on HSV-1F during viral adsorption onto the cells were 11.21 and 14.14 µg/mL and therapeutic index (TI; CD₅₀/ED₅₀) of 1.23 and 0.35 whereas ethanol extract of *R. officinalis* and *E. scaber* showed ED₅₀ = 0.24 and 9.12 µg/mL, respectively and TI= 57.38 and 0.54, respectively against HSV-2G. Although some extracts could inhibit HSV less than 50%, cytopathic effects of the infected cells after treating with the extract were reduced and small plaque sizes were observed comparing with control, which may resulted from reduction of viral infectivity. On the other hand, ethanol extract of rosemary was tested after HSV-1F and HSV-2G adsorption and showed TI of 9.7 and 93.67. In addition, IC₅₀ of ACV on HSV-1F and HSV-2G were 1.69 and 1.95 mg/mL respectively. These studies indicated that ethanol extract of *R. officinalis* and *E. scaber* showed inhibitory activity on HSV-2G higher than HSV-1F when using plaque titration assay. The rosemary extract also showed the highest activity when tested after viral adsorption.

Table 1: Anti-HSV-1F activities of ethanol extract of *R.officinalis*, *S.alata*, *E.scaber*, and *A.indica*

Treatment stage	<i>R.officinalis</i>		<i>S.alata</i>		<i>E.scaber</i>		<i>A.indica</i>	
	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI
During viral adsorption	11.21±0.25	1.23 ± 0.57	-	-	14.14 ±1.21	0.35 ±0.87	-	-
After adsorption	1.42±0.44	9.7 ±0.45	-	-	-	-	-	-

Data in table are given as mean ± standard deviation (SD) from triplicate experiments.

(-) = HSVs were inhibited less than 50% and ED₅₀ was not calculated.

Table 2: Anti-HSV-2G activities of ethanol extract of *R.officinalis*, *S.alata*, *E.scaber*, and *A.indica*

Treatment stage	<i>R. officinalis</i>		<i>S. alata</i>		<i>E. scaber</i>		<i>A.indica</i>	
	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI	ED ₅₀ (µg/mL)	TI
During viral adsorption	0.24± 0.51	57.38 ± 0.67	-	-	9.12 ±1.24	0.54 ± 2.31	-	-
After adsorption	0.147± 0.95	93.67 ±0.75	-	-	-	-	-	-

Data in table are given as mean ± standard deviation (SD) from triplicate experiments.

(-) = HSVs were inhibited less than 50% and ED₅₀ was not calculated.

These results suggested that the ethanol extract of *R. officinalis* and *E. scaber* might block the binding of HSV-1F and HSV-2G particles to cellular receptor. Moreover, interference of process of fusion of virion envelope to cell surface receptor might be occurred. Surprisingly, HSV-1F and HSV-2G were not inhibited during adsorption when treatment with ethanol extracts of *S.alata* and *A.indica* since these plants was demonstrated many biological activities such as antibacterial activities (Adedayoet al., 2001). However, viral infectivity was reduced from the observation of smaller plaque size after treatment with the plant extracts (Chaliewchaladet al., 2013; Swapnilet al., 2013).

Many plants had been reported to affect the initial stages of viral infection, for example, the essential oil of *Mentha piperita*, *Matricaria recutita*, chamomile oil, *Melissa officinalis* (lemon balm), *Hyssopus officinalis* (hypssop), *Thymus vulgaris* (thyme) and *Zingiber officinalis* (ginger) and the extracts of lemon balm, peppermint, sage, thyme, prunella and *Phyllanthus urinaria* (Koch et al., 2008; Nolkemperet al., 2006; Schnitzler et al., 2008; Schuhmacheret al., 2003).

Moreover, PB233'OG isolated from the bark of *Myricarubra* exhibited anti-HSV-2 activity (Cheng et al., 2003). The extracts of *Pterocaryas tenoptera*, *Pelargonium sidoides* and *Terminali arjuna* inhibited HSV from attachment and

penetration into cells. Besides these plants, Lactoferrin was shown to inhibit HSV-1 adsorption to verocells (Marchettiet al., 1996).

From these results, the highest anti-HSV activity obtained from ethanol extract of rosemary was more pronounced when applied after viral adsorption. These implied that these extracts may interfere with viral multiplication cycle. Therefore, the effect of these extracts after viral infection was due to inhibition of viral production and prevention of immediately early (α)-genes transcription and possibly repressing function VP16 tegument protein (Kianiet al., 2008). The similar results were observed from *Peganum harmala* extract that HSV-1 was inhibited after adsorption. A'lvarez and coworker (2009) found that *Phyllanthus orbicularis* extract was active on early stages of HSV-1 and HSV-2 replication. Wang and coworker (2009) reported that the titer of HSV-1 was reduced about 10% when the *Pheloden dron amurense* extract was added after adsorption period.

4. Conclusions

Our findings demonstrated that the ethanol extract of *R. officinalis* and *E. scaber* showed inhibitory activity on HSV-2G higher than HSV-1F. The ethanol extract of rosemary showed the highest anti-HSV-2G activity after viral adsorption. Thus, the interfering after HSV-2G adsorption was more effective than during adsorption stage. Therefore, the knowledge of biological activity of these extracts obtained from this study will be useful to exploit these plants as food and food supplement in order to promote hygiene and

health care formulation. Moreover, therapeutic potential agent from these plants should be developed as anti-herpetic agents.

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