

Full Length Research Paper

Inhibition of *Inula cappa* (Ham. ex D. Don) DC. extracts on herpes simplex virus infection *in vitro*

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The methanol and dichloromethane extracts of *Inula cappa* (Ham. ex D. Don) DC. were evaluated for inhibition of herpes simplex virus infection. Higher anti-herpes simplex virus (HSV) activity of *I. cappa* methanol extract was observed comparing with *I. cappa* dichloromethane extract since more than 50% of the virus was inhibited by *I. cappa* methanol extract and 50% effective doses of *I. cappa* methanol extract on HSV-1 and HSV-2 were 720.1±32.7 and 529.2±5.2 µg/ml respectively. However, when the virus particles were treated with *I. cappa* dichloromethane and *I. cappa* methanol extracts, both HSV-1 and HSV-2 particles were directly inactivated to negligible amount within 1 - 2 h. More than 50% interference on blocking of HSV attachment and penetration was observed after treatment the virus with *I. cappa* methanol extract. Moreover, within 30 h, the effective inhibitory activity of *I. cappa* methanol extract on HSV replication was higher than that of the *I. cappa* dichloromethane extract. Both extracts had potent activities in reducing viral deoxyribonucleic acid (DNA) synthesis since HSV DNA after treatment with the extracts were 1.3-2 fold less than HSV DNA control after determination by real time polymerase chain reaction and approximately 45 kilodalton viral proteins were clearly diminished after treatment with *I. cappa* methanol extract. Therefore, methanol extract of *I. cappa* is a potent inhibitor of herpes simplex virus infection *in vitro*.

Key words: Anti-viral activity, *Inula cappa*, plant extract, herpes simplex virus.

INTRODUCTION

Herpes simplex virus (HSV) is one of pathogens causing sexually transmitted diseases (Vermani and Garg, 2002). It has also been frequently observed among patients with acquired immunodeficiency syndromes. Both HSV-1 and HSV-2 are the members of genus *Simplexvirus*, subfamily *Alphaherpesvirinae*, family *Herpesviridae*. They have many similar characteristics but can be distinguished by clinical manifestations, biochemical and serological examinations. Effective herpes drugs such as acyclovir, ganciclovir, valaciclovir, penciclovir, famciclovir

and vidarabine are available. However, these synthetic drugs have many disadvantages such as side effects, expensive cost and drug resistant strains may emerge after long term treatment (Vermani and Garg, 2002). The utilization of medicinal plants for primary health care is widespread in both developing and developed countries. They are gaining popularity because of several advantages such as low side effects, better patient tolerance, relatively less expensive and widely acceptance due to long history of using (Hammer and Inouye, 1997).

Some Thai medicinal plants had been studied on anti-HSV activity such as *Rhus javanica* (Kurokawa et al., 1995), *Barleria lupulina*, *Clinacanthus nutans* (Yoosook et al., 1999) *Mangifera indica*, *Maclura cochinchinensis* (Yoosook et al., 2000), *Scoparia dulcis* (Galvis et al.,

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2001), *Dunbaria bella* (Akanitapichat et al., 2005), *Eugenia caryophyllus* (Tragoolpua and Jatisatienr, 2007) and *Cissus repanda* (Nikomtat et al., 2008). *Inula cappa* (Ham. ex D. Don) DC. or Naat kham (in Thai) is in Family Asteraceae. Synonyms of *I. cappa* are *I. salviodora* Sch.-Bip. and *Duhaldea cappa* (Abid and Qaiser, 2003). These plants are found at 500-2100 m. above sea-level. The plants have perennial shrub. Their stems are erect, woody in the lower parts, densely and around 1.5 m tall. The plants distributes in the North and Northeast of Thailand. Flower and fruit periods are ranging from October to February. *I. cappa* has been recommended as traditional remedies by Lanna folk. Roots of *I. cappa* is used for treatment of rash and itching whereas hill tribes in Thailand, Shan, use *I. cappa* for treating stomachache (Sukkho, 2008).

A decoction of the root is used in the treatment of fevers. The decoction is also added to bath in order to relieve body aches caused by hard physical work. A poultice made from the pounded root is applied to the forehead to relieve headaches. The juice of the bark, mixed with equal quantities of the juice from the bark of *Ficus semicordata* and *Myrica esculenta* are used in the treatment of menstrual disorders (Manandhar, 2002; Taylor et al., 1996 a,b). The active compounds in *I. cappa* were reported to be flavonoids, caffeic acid, quercetin 4-glucoside, flavone, glycosylflavones and vitexin (Abid and Qaiser, 2003). Thus, this plant possibly represents a reservoir of pharmacologically active sub-stances. Anti - HSV activities and mode of actions of *Inula cappa* (Ham. ex D. Don) DC. were investigated in this study.

MATERIALS AND METHODS

Plant material

Inula cappa (Ham. ex D. Don) DC. (Asteraceae) plants were collected from Doi Angkhang, Fang District, Chiang Mai Province, Thailand during October-December, 2005 and identified by Assoc. Prof. Dr. Chusie Trisonthi and Assist. Prof. Paritat Trisonthi, taxonomists in the Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

Voucher specimen (QSBG 22711) was 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) and 40 µg/mL gentamicin (Bio Basic Inc.), in a humidified 5% carbon dioxide (CO₂) incubator at 37°C. Herpes simplex virus types 1 (HSV-1; F strain) and 2 (HSV-2; G strain) were used throughout the study. Quantitative analysis of the virus was performed in a 24-well tissue culture plates, using a plaque titration assay. Plaques were counted and the virus titers were expressed as plaque forming units (PFU)/mL.

Plant extracts

Dried plant leaves (500 g) were milled and soaked in methanol or dichloromethane at room temperature for 3 days. The suspension was then filtered and the solvent was evaporated, using a rotary evaporator and dried in high vacuum. The dry material was dissolved in dimethyl sulfoxide (DMSO) before determination 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 assay. Briefly, GMK cells were seeded on 96-well microplates and incubated with the non toxic concentrations of plant extracts for 72 h. The 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 (CD₅₀) 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 incubated at room temperature for 1 h. Then, 200 µl of crude extract of *I. cappa* at non toxic concentrations and ACV at 50% effective dose (ED₅₀) concentration were applied in duplicate into test wells whereas the media were added into control wells. After that, 400 µl of growth media, containing 2% sodium carboxymethyl cellulose, were added to the cells. After 3 days incubation in the 5% CO₂ incubator, the cells were stained with 0.1% crystal violet in 1% ethanol. The plaques were counted and the inhibitory activities of tested extracts were calculated comparing with the control. The ED₅₀ was also determined.

Effect of plant extract on viral attachment

The GMK cell monolayers were pre-chilled at 4°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 the extracts and acyclovir. The infected cells were further incubated at 4°C for 3 h and the cells were washed with phosphate buffered saline (PBS) twice before overlaid with the growth media, containing 2% sodium carboxymethyl cellulose. After 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°C for 1 h (Cheng et al., 2002). HSVs were inoculated on cell monolayers and infected cells

were further incubated at 4°C for 3 h. The extract or acyclovir was added and then incubated at 37°C for 10 min. After that, the infected cells were treated with PBS, pH 3 for 1 min, and PBS, pH 11 was added immediately to neutralize acidic PBS. PBS was then removed and the cell monolayer was overlaid with growth media, containing 2% sodium carboxymethyl cellulose and incubated for 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 cm² flasks. The cells (6x10⁵ cells/ml) were infected with 1x10⁶ PFU/ml of HSV. Virus was allowed to adsorb to the cells for 60 min at room temperature. Unadsorbed virus was discarded and the cells were washed twice with PBS. The duplicated flasks of infected cells were maintained in the media containing crude extract of *I. cappa* at non-toxic concentration while only media or media containing acyclovir at ED₅₀ 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°C and virus titers were determined using plaque titration assay.

Viral Deoxyribonucleic acid (DNA) extraction

GMK cells (4x10⁵ cells/ml) were infected with HSVs with a multiplicity of infection (MOI) of 1 in the presence or absence of the extracts. The infected cells were harvested when 80-90% of infected cell showed cytopathic effect. The cells were lysed with lysing solution (0.25% Triton X-100, 0.5M EDTA and 1 M Tris-HCl, pH 8.0). Then, treated with 5 M NaCl, proteinase K and RNase A. The viral DNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with cold absolute ethanol. The concentration of viral DNA was determined after measuring the optical density at 260 nm. Quantitative viral DNA was then detected by real-time PCR.

Quantitative real-time Polymerase chain reaction (PCR)

Amplification and detection of viral DNA by real-time PCR was carried out using the iCycler iQ (Bio-Rad, Hercules, CA). Amplified 1350 bp region of viral DNA polymerase gene was produced. A total volume of 25 µl was obtained by adding 1 µl of viral to a PCR reaction mix. Forward primer (5'-ATGCGAAAGCAGATCCGCTCGC-3') and reverse primer (5'-GGCTCTATGCAACATTGACG-3') were used at 0.5 µM in reaction mixer. PCR products were detected using the sequence unspecific SYBR Green I dye (Maxima™ SYBR Green qPCR Master Mix (2x); Fermentas) with the melting curve analysis.

PCR conditions were as follows: activation of SYBR green I was performed at 95.0°C for 10 min followed by 40 cycles of denaturation at 95.0°C for 45 s, annealing at 60.0°C for 45 s and extension at 72.0°C for 3 min, then, melting curve programme was used at 55-95°C with increase set point temperature after cycle 2 by 0.5°C and cooling temperature down to 20°C. After cycle completed, a threshold for detection of fluorescence above background is determined by iCycler iQ software. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold, C_t. The quantity of DNA doubles every cycle during the exponential phase; therefore, relative amounts

of DNA can be calculated.

Western blot analysis

GMK cells were grown as monolayers in 25 cm² flasks (4x10⁵ cells/ml). The cells were infected with HSV with MOI = 1. Virus was allowed to adsorb to the cells for 60 min at room temperature. Unadsorbed virus was discarded and the cells were washed twice with PBS. Infected cells were maintained in the media containing crude extracts of *I. cappa* at non-toxic concentration while only media were added into the control flasks. The infected cells were observed for CPE and were collected at 24 h after infection (Kuo et al, 2001). Total proteins were extracted from the infected cells using NucleoSpin[®] RNA/Protein (MACHEREY-NAGEL) and ten microliter of total protein extracts were detected by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to nitrocellulose filter and the filter was blocked by soaking overnight in 3% bovine serum albumin in saline (Towbin et al., 1979). The blotting filters were incubated overnight with horseradish peroxidase-conjugated Immunoglobulin G against HSV (AbD serotec) and washed in saline 5 times for 30 min. HSV protein bands were detected by incubating the blotting filter in 0.06% 4-chloro-1-naphthol (sigma)/ 0.01% H₂O₂ in PBS and then washed in deionized water to stop reaction.

Data analysis

The CD₅₀ and ED₅₀ are the average of two independent experiments with 5 concentrations within the inhibitory range of the extracts. The selective index (SI) is defined as CD₅₀/ED₅₀ and the program Gene Tool (Image Analysis System) was used for analyzing protein bands quantitatively.

RESULTS

Dichloromethane (ID) and methanol (IM) extracts of *I. cappa* were dissolved in DMSO for being stock extracts. The stock extracts and DMSO was diluted two-fold dilution with medium at least 10 dilution and determined for cell toxicity by MTT assay. The CD₅₀ value of *I. cappa* which was extracted with ID and IM were 87±1 and 1818±0 µg/ml, respectively. Non-toxic concentration of the ID and IM extracts at 31 and 949 µg/ml were used throughout the experiments.

The viruses were incubated with each extract at room temperature for 1 h interval up to 4 h and the titers of viruses after treatment were investigated by the plaque titration assay. It was found that all the extracts could inactivate both HSV-1 and HSV-2 directly. However, the virucidal test showed that HSV-1 was inactivated by IM more efficiently than ID extracts at 1 h after incubation. Log amount of HSV-1 and HSV-2 were drastically reduced at 1 to 2 h after incubation with the ID and IM extract (Table 1). The virucidal effect of the extracts on HSV was not due to DMSO that used to dissolve the extracts.

Only IM extract exhibited anti-HSV activity against both types of HSV using plaque reduction assay. These

Table 1. Direct inactivation of HSV-1 and HSV-2 by dichloromethane and methanol extracts of *I. cappa*.

Virus	Extracts	Concentration ($\mu\text{g/ml}$)	Log amount of viruses (PFU/ml) at one hour interval				
			0	1	2	3	4
HSV-1	IM	949	4.7	<1	<1	<1	<1
	ID	31	4.9	3.4	<1	<1	<1
	control		5.6	4.7	4.3	3.8	3.5
HSV-2	IM	949	4.7	4.1	<1	<1	<1
	ID	31	4.5	4.3	<1	<1	<1
	Control		5.6	4.7	4.6	4.5	4.1

Table 2. Anti-HSV activity and selective index of the extracts on GMK cells.

Virus	Extracts	Concentration ($\mu\text{g/ml}$)	Antiviral activity, ED ₅₀ ($\mu\text{g/ml}$)	Selective index, SI ^a
HSV-1	IM	949	720.1 \pm 32.7	2.5 \pm 0.1
	ID	31	-	-
HSV-2	IM	949	529.2 \pm 5.2	3.4 \pm 0.1
	ID	31	-	-

Values are mean \pm S.D. of two independent experiments.

^aCC₅₀/ED₅₀

experiments showed that the IM extract could inhibit HSV-1 and HSV-2 with ED₅₀ of 720.1 \pm 32.7 and 529.2 \pm 5.2 $\mu\text{g/ml}$ and the selective index (SI) values were 2.5 \pm 0.1 and 3.4 \pm 0.1 respectively. Therefore, the efficiency of IM extract on HSV was better than ID since the percentage of HSV inhibition by ID extract was less than 50% (Table 2).

The effect of crude extracts on HSV replication was determined after treating the infected cell with the extracts. The infected cells were collected at 6, 12, 24 and 30 h. Acyclovir at ED₅₀ concentration was used only for drug positive control in the test of viral growth inhibitory curve. Thus, the highest non-toxic dose of the IM extract (949 $\mu\text{g/ml}$) was used in the study for determination of the maximum inhibitory effect instead of using the ED₅₀ dose (720 $\mu\text{g/ml}$), which reflected only 50% inhibition of viral infection. Thirty hours after HSV-1 infection (Figure 1A), log amount of viruses after treatment with IM and ID extracts were 6.3 \pm 0.3 and 7.5 \pm 0.2 respectively, whereas log amount of virus control and ACV treated virus were 8.3 \pm 0.02 and 4.8 \pm 0.2 respectively. Moreover, HSV-2 replication was completely inhibited after treatment with IM extract 24 hours after infection. However, 30 hours after HSV-2 infection, log amount of viruses after treatment with IM and ID extracts were 2.5 \pm 0.5 and 5.6 \pm 0.6 respectively, while log amount of virus control and ACV treated virus were 8.5 \pm 0.4 and 5.6 \pm 0.9 respectively (Figure 1B). Therefore, effective inhibitory

activity of IM on HSV replication was higher than that of ID extract.

The attachment of HSV-1 on GMK cells was blocked after treatment with IM and ID extracts. The percentage of inhibition was 60.4 \pm 7.5 and 13.5 \pm 9.6 respectively. Inhibition of HSV-2 attachment was also observed as the virus was blocked by IM extract with percentage of 93.2 \pm 2.3 (Figure 2). Additionally, the extracts also inhibited HSV penetration. The effective inhibition of IM extract on HSV-1 penetration was similar to the inhibition of HSV attachment. IM extract showed percentage of HSV inhibition more than ID extract. Penetration of HSV-1 was inhibited with the percentage of 51.1 \pm 5 and 18.9 \pm 0.9 respectively while HSV-2 penetration was inhibited by 53.3 \pm 0.5 and 24.3 \pm 15.0% respectively (Figure 2).

To determine HSV DNA replication in the presence or absence of the extract by real time PCR using primer specific to HSV DNA polymerase gene, PCR products were detected at 1350 bp. It was showed that the cycle threshold, C_t of IM, ID extracts and HSV-1 control were 23.8 \pm 3.1, 15.7 \pm 2.4 and 12.0 \pm 3.6 respectively whereas average C_t of HSV-2 DNA after treatment with IM, ID extracts and HSV-2 control were 22.6 \pm 1.9, 16.6 \pm 2.9 and 12.1 \pm 1.9 respectively. It indicated that high C_t reflected many cycles of PCR to generate DNA product. Thus, HSV DNA after treatment with the extracts was less than HSV DNA control approximately 1.3 to

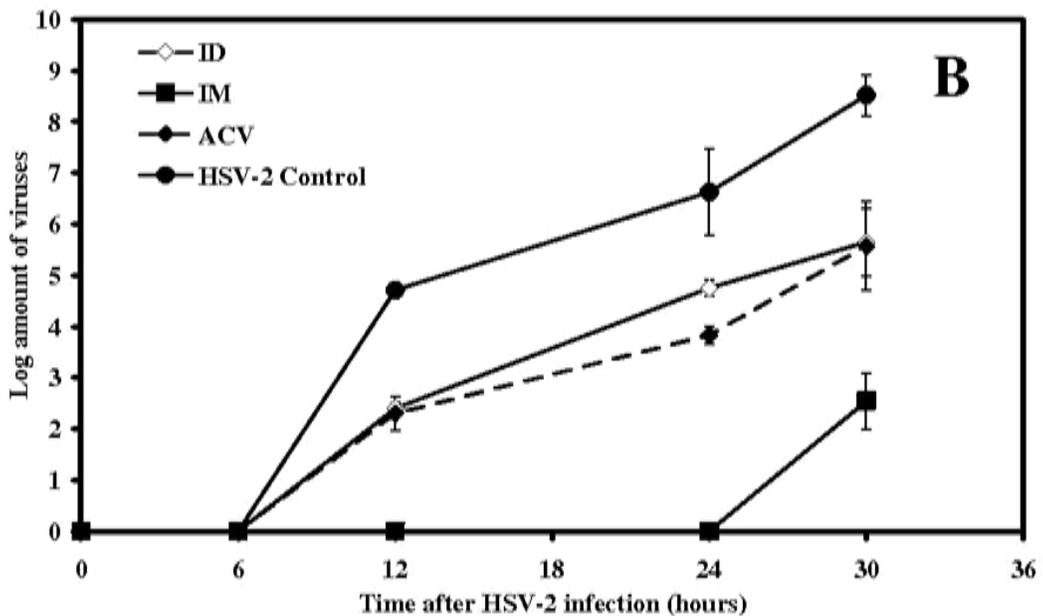
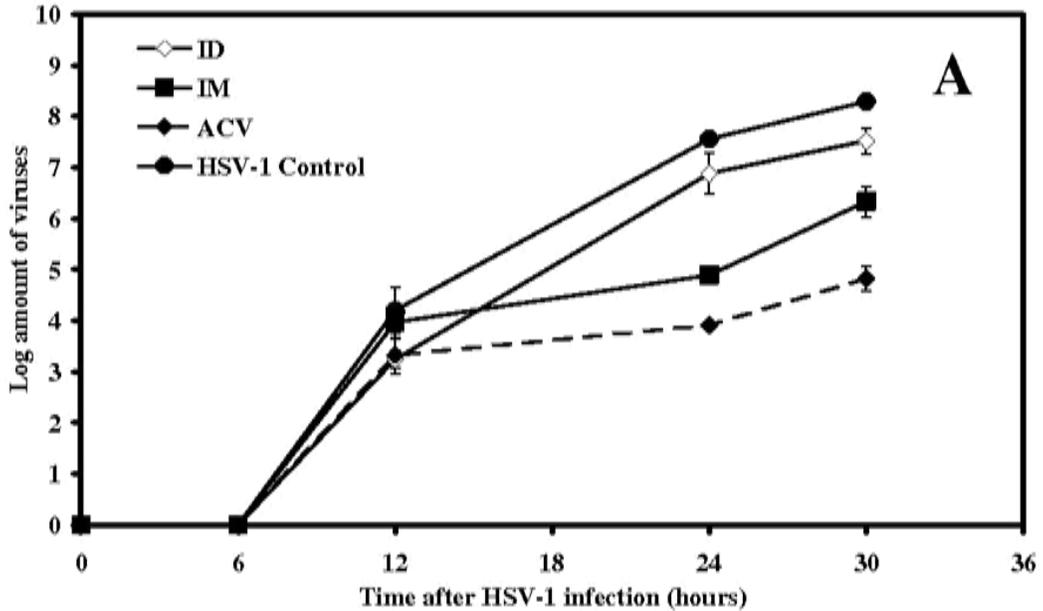


Figure 1. Kinetic of inhibition of HSV-1 (A) and HSV-2 (B) replication by the ID and IM extracts.

2fold (Figure 3).

Moreover, the effects of dichloromethane and methanol extracts of *I. cappa* were determined on the synthesis of HSV proteins in the presence and absence of the extracts. Ten microliter of total protein was loaded into 10% SDS-PAGE, then transfer to membrane. The viral

proteins were specified by direct detection using horseradish peroxidase-conjugated IgG against HSV and protein from uninfected cell was included in this assay. Approximately 45 kDa viral proteins were remarkably reduced after treatment with the extracts compared with the protein of viral control and protein marker. The

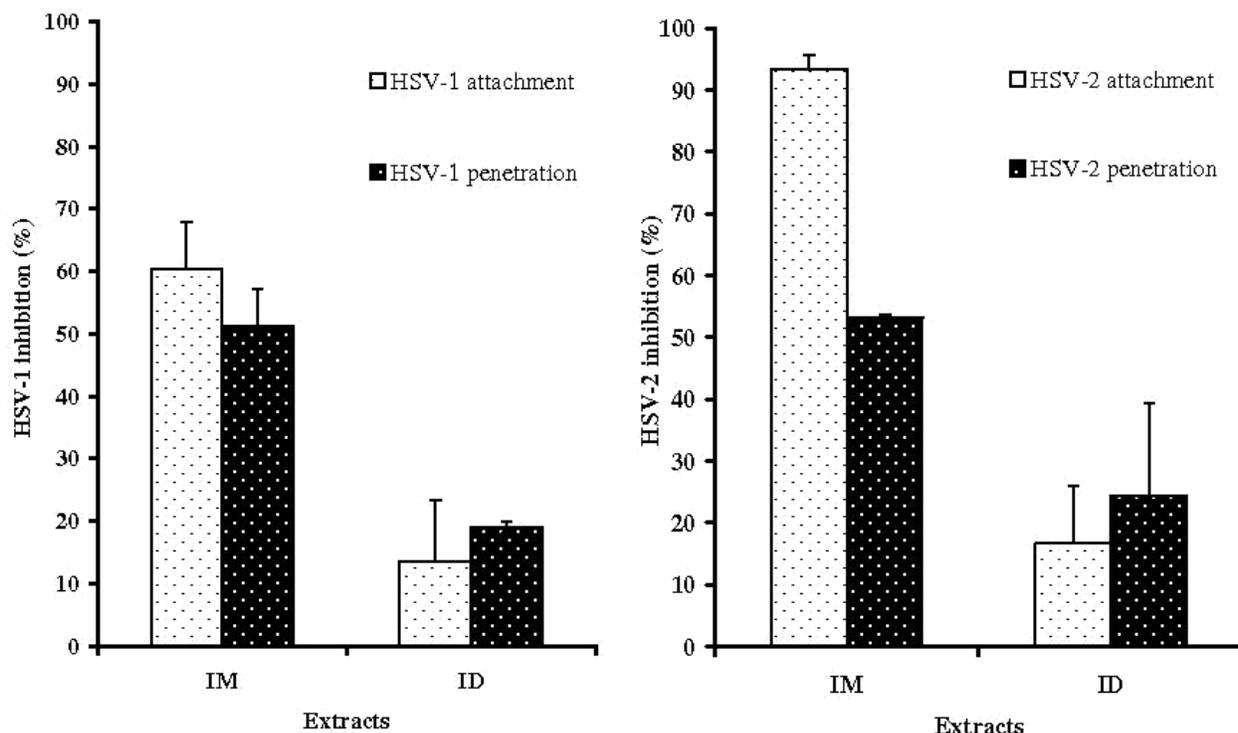


Figure 2. Effect of IM and ID extracts on HSV attachment and penetration to GMK cells. The percentage of inhibition of the extracts was evaluated by plaque assay. Each bar represents the mean±S.D. for two independent experiments.

quantitative analysis of an image analysis system showed that the intensity of the HSV-1 protein bands after treatment with ID and IM extracts were reduced by $33.1 \pm 3.28\%$ and $75.5 \pm 2.0\%$ (Lanes 1 and 2) comparing with HSV-1 protein control band (Lane 3) and uninfected cells (Lane 4), whereas the intensities of the HSV-2 protein bands after treatment with ID and IM extracts were reduced by $74.4 \pm 0.2\%$ and $83.0 \pm 2.0\%$ (Lanes 5 and 6) comparing with HSV-2 protein control bands (Lane 7) and uninfected cells (Lane 4) as shown in Figure 4.

Thus, IM extract could reduce the viral protein synthesis better than ID extract and HSV-2 protein was inhibited more than HSV-1 protein.

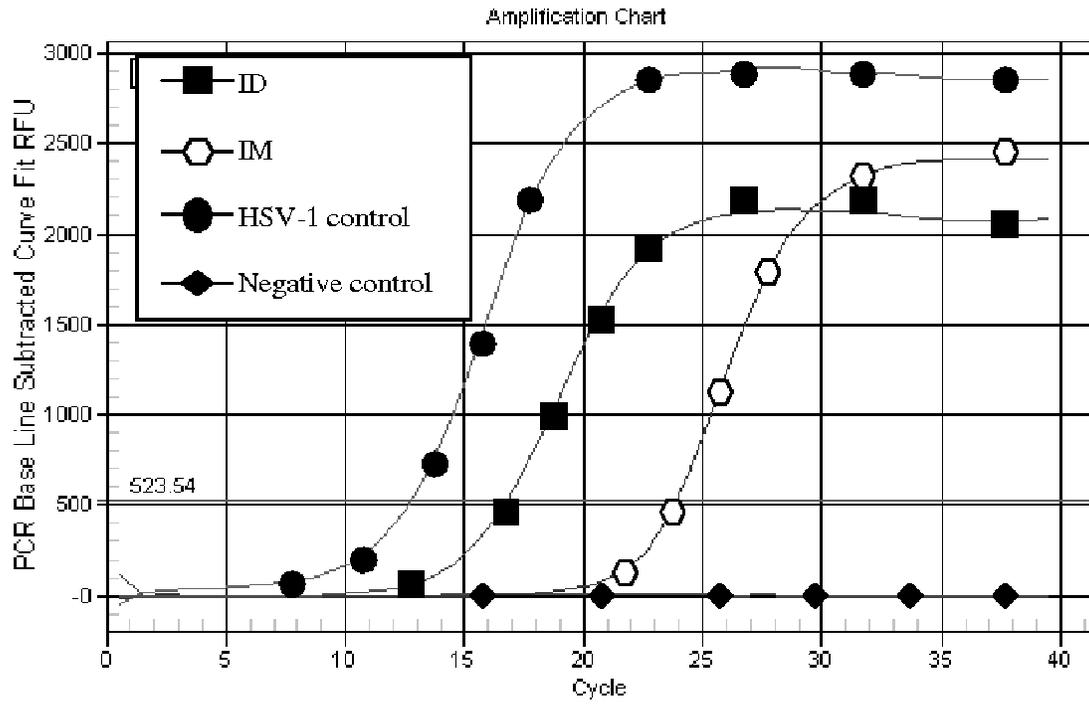
DISCUSSION

The dry material, 10 g of crude ID or IM extracts from leaves of *I. cappa* was obtained after evaporation of the solvent after extraction and drying in high vacuum. The ID and IM extracts were found to inhibit herpes simplex virus. Cytotoxicity test indicated that ID extract was more toxic to the cells than IM extract as observed from the CD_{50} values since the CD_{50} value of ID extract was lower than that of IM extract. Higher anti-HSV activity of IM extract was observed comparing with ID extract because

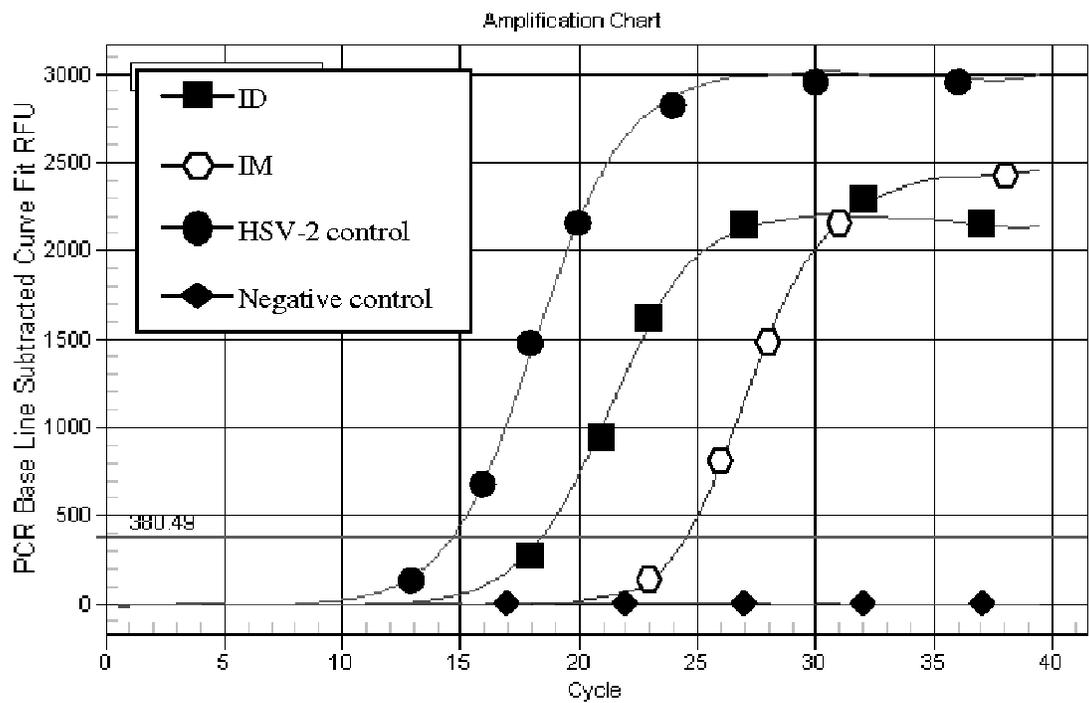
the viruses were inhibited by IM extract more than 50% and ED_{50} values of IM extract on HSV-1 and HSV-2 were 720.1 ± 32.7 and 529.2 ± 5.2 $\mu\text{g/ml}$. Although the ED_{50} of the IM extract was quite high, the extract showed low toxicity to the cells tested. Thus, the selective index, which reflects the therapeutic effect of the extract, was demonstrated by 2.5 and 3.4 when treated with HSV-1 and HSV-2 respectively.

However, when the virus particles were treated with ID and IM extract, both HSV-1 and HSV-2 particles were directly inactivated to negligible amount within 1-2 h. Moreover, within 30 h, the effective inhibitory activity of IM extract on HSV replication was higher than ID extract as observed by the inhibition of the amount of HSV-1 by 24.1 and 9.6%, while the inhibition of amount of HSV-2 were 70.6 and 34.1% respectively. Thus, the inhibition of HSV-2 replication was higher than HSV-1.

Mode of inhibition of the extracts on viral attachment, viral penetration, viral DNA replication and viral protein synthesis were determined. The attachment and penetration of HSV-1 and HSV-2 into cells were blocked more than 50% by IM extract. Therefore, the interference may occur during HSV attachment, which mediated by viral glycoprotein C interaction with cellular heparan sulfate (Cheng et al., 2002) and interference with the stability of attachment between viral glycoprotein D to the cell



(a)



(b)

Figure 3. Fluorescence curve from SYBR Green I detection of HSV-1 DNA (a) and HSV-2 DNA (b) after treatment with ID and IM extracts compared with the control.

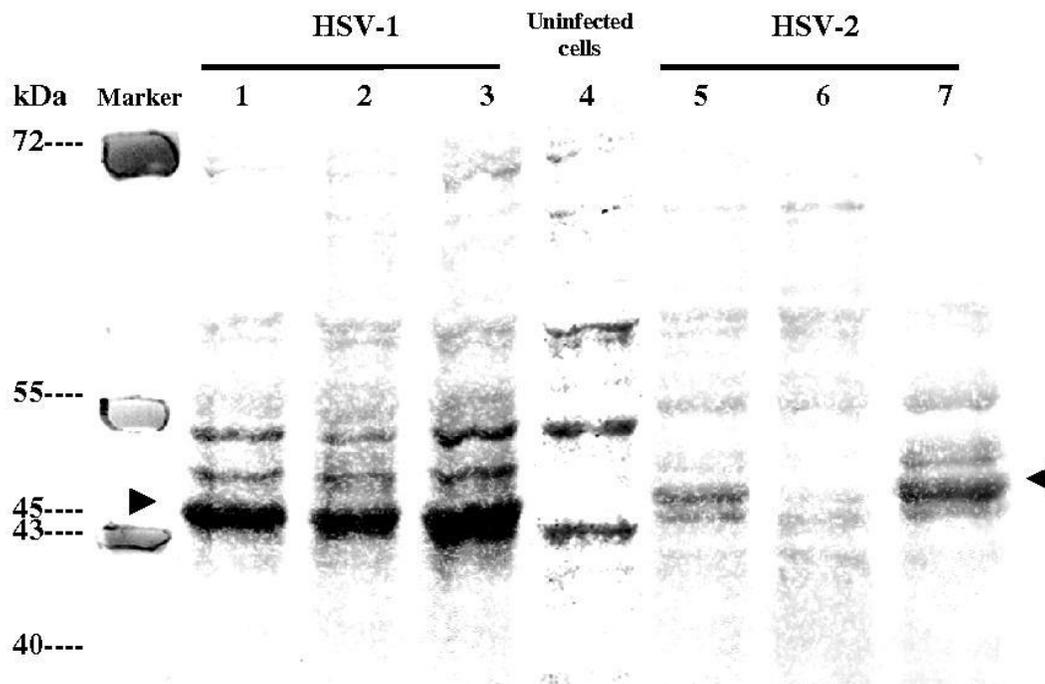


Figure 4. Effect of the ID and IM extracts on viral protein synthesis compared with the control. Western Blots of HSV-1 protein in the presence of ID extract (lane 1), IM extract (lane 2), absence of the extract (lane 3) and uninfected cells (lane 4), and HSV-2 protein in the presence of ID extract (lane 5), IM extract (lane 6) and absence of the extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV compared with protein marker. Arrow heads indicate the reduction of viral proteins after treatment with the extracts.

cell (Rajcáni and Vojvodová, 1998) and/or inhibition of membrane fusion between virion envelope glycoproteins D, B, H, L and plasma membrane of the target cell (Spear, 1993).

In addition, Quantitative RT-PCR was performed to detect HSV DNA replication. The viral DNA synthesis was affected after treatment with IM extract more than ID extract as observed by crossing point values, that were calculated directly as the coordinates of points in which the threshold line actually crossed (Larionov et al., 2005). It indicated that HSV DNA after treatment with the extracts was 1.3 to 2 fold less than HSV DNA control. Thus, it was suggested that the extract may interfere with HSV DNA synthesis by affecting HSV DNA replication directly or compounds in the extract may inactivate essential enzymes for viral DNA synthesis e.g. origin-binding protein, single-stranded binding protein, DNA polymerase or helicase-primase. These findings also reflected inhibition of viral protein expression by the extract. Approximately 45 kDa HSV-2 proteins were clearly diminished after treatment with IM extract. From the previous studies, molecular weight of VP21 (*UL26* gene product) was around 40-47 kDa (Person et al., 1993) whereas the molecular weight of different forms of

HSV-1 VP22a (*UL26.5* gene product) from purified virion and infected cells on Western blots ranged from 52 to 36 kDa (Yang et al., 2000). However, VP22a, the scaffold of the HSV-1 B capsid is the most abundant protein found in the B capsid, approximately 1,100 copies of this protein being used to form the inner core while VP21 are less abundant and present at approximately 150 molecules per capsid (Person et al., 1993; Newcomb and Brown, 1991; Baines and Duffy, 2006). From the result, high amount of approximately 45 kDa viral proteins were observed on virus control lanes (Lanes 3 and 7, Figure 4). Therefore, the viral protein was likely to be VP22a. The extracts could reduce the amount of this protein, which is required as scaffold for assembly of viral capsid. In the absence of the scaffolding function, the proteins that form the outer shell assemble into aberrant structures (Dasai et al., 1994; Tatman et al., 1994; Thomsen et al., 1994; 1995).

Although, the ability of viral infectivity after treatment with ID extract determined by number of plaques on infected cells was less than 50%, both IM and ID extracts affect HSV particles directly. Moreover, many steps in multiplication cycles of HSV such as attachment, penetration, viral DNA replication and viral protein

synthesis was found to inhibit by both extracts. However, the activities of ID extract on herpes simplex viruses were less than IM extract as shown in Figures 1 to 4.

Thus, the activities of the extracts are suggested to be sequential event. However, the activities of the extract on direct inactivation of viral particle and the interference on viral attachment stage may act at the same time when the virus particles exposed to the extract during the attachment of viruses to the cells.

Besides the anti-HSV activity of *I. cappa* leave extract, the extract from roots of *I. cappa* was also found to inhibit human poliovirus (Taylor et al., 1996a). From this study, methanol extract of *I. cappa* showed higher anti-HSV activity than dichloromethane extract. Therefore, the biological active compound on HSV may reside in methanol extract. Some compounds in the extract of *I. cappa* were reported for anti-HSV activity. Flavonoids were found to inhibit HSV-2 multiplication (Allahverdiyev et al., 2004). Quercetin could inactivate HSV directly (Khan et al., 2005) and it was effective on HSV-1 at the early stage of multiplication (Chiang et al., 2003).

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