CHAPTER 3

MATERIALS AND METHODS

1. Medicinal plants

In Thailand, tradition medicinal plants were used for primary health care to treat several illnesses and used as ingredients for medicine. The action of these selected plants was selected based on their revealed therapeutic properties to treat general disease and promote health. These plants also have the potential to providepharmacological properties such as antimicrobial infection, anti-cancer and anti-inflammatory. Furthermore, the parts of traditional plants used were considered to select based on the folklore medicaments.

Thus, this present study demonstrated anti-herpes simplex virus activity of traditionally selected medicinal plant. Dried medicinal plants that used in this study were obtained from Lampang Herb Conservation, Thailand. The selected plants were shown in Table 3.

Table 3 Medicinal plants used in this study

Medicinal plant	Thai name	Family	Part used
1. *Andrographis paniculata* (Burm. f.) Wall. ex Nees.	Fa Thalai Chon	*Acanthaceae*	Whole plant
2. *Cissus quadrangularis* L.	Phet Sang Kat	*Vitaceae*	Stem
3. *Coscinium fenestratum* (Gaertn.) Colebr.	Hamm	*Lamiaceae*	Stem
4. *Croton roxburghii* N.P.Balakr.	Plao Yai	*Euphorbiaceae*	Bark
5. *Derris scandens* (Roxb.) Benth.	Thaowanpriang	*Papilionaceae*	Leaf
6. *Eclipta prostrata* (L.) L.	Ka Meng	*Asteraceae*	Whole plant
7. *Glycyrrhiza glabra* L.	Cha Em Taet	*Papilionaceae*	Leaf
8. *Gynostemma pentaphyllum* (Thunb.) Makino.	Jiaogulan	*Cucurbitaceae*	Whole plant
9. *Houttuynia cordata* Thunb.	Khao Tong	*Saururaceae*	Whole plant
10. *Leptochloa chinensis* (L.) Nees	Ya Dokkhao	*Poaceae*	Whole plant
11. *Momordica charantia* Linn.	Mara Ki Nok	*Cucurbitaceae*	Whole plant
12. *Phyllanthus amarus* Schum.&Thonn.	LukTai Bai	*Euphorbiaceae*	Whole plant

Table 3 (continued)

Medicinal plant	Thai name	Family	Part used
13. *Pluchea indica* (Linn.) Less.	Khlu	*Asteraceae*	Leaf
14. *Pseuderatherum platiferum* (Wall.) Radlk. ex. Lindau.	Phaya Wanon	*Acanthacea*	Leaf
15. *Rhinacanthus nasutus* (Linn.) Kurz	Thong Phan Chang	*Acanthaceae*	Leaf
16. *Schefflera leucantha* R. Vig.	Hanumaa Prasaan Kaai	*Araliaceae*	Leaf
17. *Senna alata* (Linn.) Roxb.	Chum Head Ted	*Caesalpinoideae*	Leaf
18. *Sphenodesma* sp.	Hor Sar Par Kwai	*Verbenaceae*	Whole plant
19. *Stemona tuberosa* Lour.	Non Taai Yaak	*Stemmonaceae*	Rhizome
20. *Stephania venosa* (Blume) Spreng.	Sabu Liad	*Euphorbiaceae*	Corm
21. *Thunbergia laurifolia* Lindl.	Rang Chuet	*Acanthaceae*	Leaf
22. *Tinospora crispa* (Linn.) Miers ex Hook.f. & Thomson	Bora Phet	*Menispermaceae*	Stem
23. *Zingiber montanum* (Koenig) Linkex Dietr.	Phlai	*Zingiberaceae*	Rhizome

2. Extraction of medicinal plants

Dry powders of medicinal plants (250 grams) were macerated with 4 liter of 95% ethanol for 3 days at room temperature or soaked with distilled water at 45ºC in water bath for 3 hours. Then, the suspension of medicinal plant extracts was filtered through filter paper Whatman No.1 (Whatman, USA). The filtrate was concentrated by rotary evaporator to remove the solvent and dried by lyophilizer to obtain crude extracts. Next, the dried extract was reconstituted by dimethylsulfoxide (DMSO, Labscan, Ireland) and stored in amber glass bottle at 4°C before investigation of anti-HSV activity.

3. Anti-herpes simplex virus assay

3.1 Cell culture

Vero cells were used throughout this study for propagation of herpes simplex virus and investigation of anti-HSV activity. The cells were obtained from division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Vero cell were cultured as monolayer in Eagle's minimum essential medium; MEM (Gibco, UK) and supplemented with penicillin/streptomycin (PAA, Austria). Heat inactivated fetal bovine serum, 10% (Hyclone, UK) was added before used. The cells were cultured until confluence at 37ºC in humidified 5% CO_2 atmosphere incubator. Vero cells were subcultured every 2-4 days in Biosafety Cabinet Class II. The cells were washed twice with PBS (1X) and detached with trypsin-ethylenediamine tetra-acetic acid (Trypsin-EDTA, 0.05%, PAA, Austria). Trypsin-EDTA was removed and growth medium was added. The

cells were transferred into new flask with spilt ratio of 1:3 and further incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days.

3.2 Herpes simplex viruses

Standard strains of herpes simplex virus (HSV) type 1 strain F and type 2 strain G (HSV-1F and HSV-2G) were used throughout this study. HSV-1F and HSV-2G were obtained from Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. The virus at multiplicity of infection (MOI) of 0.1-1 was propagated on monolayer of Vero Cells in 25 cm^2 flask after adsorption at room temperature for 1 hour. After adsorption period, the inoculum was removed before washing twice with PBS (1X). Then, maintenance medium supplemented with 2% heat inactivated fetal bovine serum was added. The infected cells were cultured at 37ºC in humidified 5% CO_2 atmosphere incubator until 80-90% of cytopathic effect (CPE) of the cells was observed. The infected cells were collected and virus stock was prepared by freezing and thawing of infected cells twice. Quantitation of the virus titers was performed in 24-well tissue culture plates by plaque titration assay and expressed as plaque forming unit per milliliters (PFU/ml).

3.3 Acyclovir

Acyclovir (ACV, Sigma Aldrich), 0.10 g was dissolved in 1 ml of sterile deionized water and stored in -20°C before use. ACV was diluted in MEM and tested against both types of HSV. ACV at 50% effective dose (ED_{50}), which was the dose

that inhibited HSV infection by 50% was determined and selected as positive control for investigation of anti-HSV activity.

3.4 Plaque titration assay

HSV was serially ten-fold diluted in MEM and each dilution of HSV was added into the confluence of Vero cells in 24-well tissue culture plate. After viral adsorption for 1 hour, the overlay media containing growth medium and 1.5% sodium carboxymethyl cellulose were added to each well. After incubation at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes for observed toxicity on Vero cell. Then, plaques were counted and viral titers were expressed as plaque forming unit/ml (PFU/ml).

3.5 Cell cytotoxicity

Cytotoxicity of medicinal plant extracts was investigated on Vero cells. The extracts were serially two-fold diluted with MEM and each concentration of the extracts was added to quadruplet wells onto 96-well tissue culture plate. Then, Vero cells at concentration of 1×10^5 cells/ml was added to each well and incubated for 72 hours at 37ºC in humidified 5% CO_2 atmosphere incubator. Media was discarded and the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes to evaluate toxicity of medicinal plant extracts on Vero cells. The concentrations of 50% cytotoxicity dose (CD_{50}) were expressed as a concentration of the extract that caused cell detachment from the wells by 50%. Moreover, the CD_{50} value was

determined from dose-response curve and calculated according to modified protocol of Reed and Muench (1938).

3.6 Plaque reduction assay

Vero cells were grown as monolayer in 24-well tissue culture plates. Each well was infected with 50-100 PFU of HSV and incubated on rocking platform for 1 hour at room temperature. Then, medicinal plant extracts or fractions at non-toxic concentrations and ACV at 50% effective dose (ED_{50}) were added to the cells and overlaid with overlay medium. After incubation for 3-4 days at 37ºC in humidified 5% CO_2 atmosphere incubator, the extracts were removed and washed once by PBS (1X). Then, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. The number of plaques was counted comparing with untreated virus control and expressed as plaque forming unit per ml (PFU/ml). 50% effective dose (ED_{50}) was expressed as 50% inhibition of plaque formation and calculated by dose-response curves.

3.7 Mechanism of medicinal plant extracts on HSV inhibition

3.7.1 Inactivation kinetic

Inactivation kinetic of the medicinal plant extracts on HSV particles was investigated. HSV was treated with the highest non-toxic concentration of medicinal plant extracts. The mixture of virus and aqueous or ethanolic plant extracts was incubated at room temperature for 20, 40, 60, 80, 100, 120, 180 and 240 minutes. In addition, the extract that exerted the strong efficacy to inactivate HSV particle was used at lower concentrations and incubated with the virus for 5, 10 and 15 minutes.

The mixture was collected at the end of each incubation period and kept at -80ºC. Titers of residual virus after treatment with medicinal plant extracts were determined by plaque titration assay and the percentage of viral particles inhibition by plant extracts was calculated and compared to untreated virus control.

3.7.2 Effect of medicinal plant extracts on HSV before viral attachment

Effect of medicinal plant extracts on viral attachment to cellular receptor was evaluated. Various non-toxic concentrations of medicinal plant extracts were added into a confluence of cell monolayer in 24-well tissue culture plate and incubated at room temperature for 1 hour. After that, the extracts were removed and the cells were washed with PBS (1X) once. Then, 50-100 PFU of virus was added and overlaid with overlay media. The inoculum was incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days. After incubation, the overlay media were discarded and plaques were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. Percentage of HSV inhibition was determined by calculation of plaque number after treatment with plant extracts comparing with antiviral agent, ACV and virus control. ED_{50} values of plant extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

3.7.3 Effect of medicinal plant extracts on HSV during viral attachment

Effect of medicinal plant extracts on viral attachment to cell culture was investigated. The medicinal plant extracts at various non-toxic concentrations and 100 plaques/0.1 ml of HSV were added into monolayer of Vero cell in 24-well tissue

culture plates. Then, the virus was adsorbed at room temperature for 1 hour on rocking platform. After that, the inoculum was removed before overlaid with overlay medium and incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days. After incubation period, the numbers of plaques were counted after stained with 0.1% crystal violet in 1% ethanol for 20 minutes and percentage of inhibition was determined by calculation of plaque number compared to untreated viral infected cells control. ED_{50} values of plant extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

3.7.4 Effect of medicinal plant extracts on HSV after viral attachment

Vero cells were cultured as monolayer at 37ºC in humidified 5% CO_2 atmosphere incubator in 24-well tissue culture plates. Then, 100 plaques/0.1 ml of HSV were added for 1 hour at room temperature on rocking platform. After that the cells were treated with various concentrations of medicinal plant extracts and overlaid with overlay medium before incubation for 3-4 days at 37ºC in humidified 5% CO_2 atmosphere incubator. Then, media was discarded and infected cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. Percentage of inhibition was determined by reduction of plaque number comparing to untreated virus control. ED_{50} values of plant extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

3.7.5 Effect of medicinal plant extracts on HSV multiplication

Effect of medicinal plant extracts on viral multiplication was determined. Vero cells were grown as monolayer in 6 well tissue culture plates. The cells were infected with $1x10^6$ PFU/ml of HSV for 1 hour at room temperature on rocking platform. After that, the inoculum was removed and unattached virus was washed twice by PBS (1X). Then, the highest nontoxic concentrations of medicinal plant extracts were added to each well and incubated at 37ºC in 5% humidified CO_2 atmosphere incubator. ACV at ED_{50} concentration and MEM were used as positive and negative control, respectively. Infected cells were collected at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after viral infection. Moreover, the extracts that showed the strong inhibition of HSV multiplication were selected and used at lower concentration to compare inhibitory activity against HSV multiplication cycle. After that the cells were frozen and thawed twice and virus in supernatant was kept at -80ºC before determination of virus titers by plaque titration assay.

3.7.6 Effect of medicinal plant extracts on HSV DNA

Inhibitory effect of medicinal plant extracts against HSV DNA replication involved 2 steps of viral DNA extraction and detection of viral DNA.

3.7.6.1 Viral DNA extraction

Vero cells were grown as monolayer in 75 cm^2 flask and were infected with HSV-1F and HSV-2G with MOI of 1. After incubation at room temperature on rocking platform for 1 hour, unabsorbed virus was removed and washed twice with PBS (1X). After that, infected cells were treated with the highest non-toxic concentration of each medicinal plant extract and incubated at 37ºC in humidified 5%

CO_2 atmosphere incubator. MEM was used as a negative control. The infected cells were harvested when cytopathic effect was observed by 80-90%. The inoculum was removed and the cells were washed twice by 5 ml of cold PBS (1X). The infected cells were collected by scraping with cell scrapers (Nunc, England). Then, the cell suspension was pooled and transferred into 50 ml microcentrifuge tube, which was kept in the ice box all the time. After centrifugation of cell suspension at 4ºC 1,200 rpm for 5 minutes, PBS (1X) was discarded and the cell pellet was resuspended by 1 ml of cold PBS (1X). The cell suspension was transferred to 1.5 ml microcentrifuge tube and centrifuged at 1,200 rpm 4ºC for 5 minutes. After centrifugation, the supernatant was discarded and cell pellet was lysed by adding lysing solution 1 ml and mixed thoroughly by a vortex mixer. After incubation at 4ºC for 5 minutes, 40 µl of sodium chloride (5M) was added and the mixture was centrifuged at 10,000 rpm at 4ºC for 10 minutes. Then, the supernatant was transferred to a new microcentrifuge tube. After that, 55 µl of 10% SDS, 20 µl of proteinase K (Amresco, USA) and 10 µl of 10 mg/ml RNase A were added, gently mixed by inversion, and incubated at 37ºC in water bath for 1.5 hours. Phenol : chloroform : isoamyl alcohol (50:50:1) was added at the same volume of the mixture, mixed thoroughly by vortex mixer and centrifuged at 4ºC 10,000 rpm for 5 minutes. Then, supernatant was carefully collected and transferred into new microcentrifuge tube. Chloroform : isoamyl alcohol (50:1) was added at the same volume of supernatant and mixed by vortex mixer. After centrifugation at 4ºC 10,000 rpm for 5 minutes, the supernatant was transferred into new microcentrifuge tube and the ice cold sodium acetate (3M) and cold absolute ethanol were added. Then, mixture was kept at -20ºC at least 24 hours in order to precipitate viral DNA. After that, the mixture was centrifuged at 4ºC

10,000 rpm for 15 minutes, the solution was removed and the viral DNA was washed by 250 µl of cool 70% ethanol after centrifugation at 10,000 rpm 4ºC for 5 minutes. Finally, ethanol residual was removed, viral DNA was air dried and resuspended in 20 µl of sterile distilled water and kept at -20ºC. Viral DNA after treatment with plant extracts was determined by 0.8% agarose gel electrophoresis (Research organics, Inc, USA) compared to untreated viral control (modified from Deethae, 2008).

3.7.6.2 Detection of viral DNA by agarose gel electrophoresis

Viral DNA was detected by 0.8% agarose gel electrophoresis. Agarose gel was prepared by adding 0.32 g of agarose powder into 40 ml of TAE buffer, 1X and heated on hot plate until agarose was completely dissolved. Then, the molten agarose was cool down to 55-60ºC, poured the solution on to a casting tray and a comb was placed. After that, the comb was carefully removed and the casting tray were placed in an electrophoresis chamber and filled with TAE buffer to cover the surface of the gel. DNA samples was mixed with bromophenol blue loading buffer (Merck, Germany) and then the mixtures were carefully loaded into well. Electricity at 90V was applied to the gel for 1 hour. After the electrophoresis was completed, the gel was stained with ethidium bromide (Bio Basic Inc., Canada) for 15 minutes. Finally, the gel was soaked in water for 5 minutes before observation of viral DNA band under UV transluminator. The quantitative of viral DNA was determined by program Gene Tools Match (LAB Focus, Co. ltd) and percentage of inhibition of viral DNA synthesis was calculated.

3.7.7 Effect of medicinal plant extracts on HSV protein

Inhibitory efficacy of medicinal plant extracts were examined against HSV protein synthesis by Western blotting. Viral proteins from whole cell lysates were detected by specific antibody. The procedure can be divided into several steps as follows

3.7.7.1 Preparation of HSV infected cell

A monolayer of Vero cells in the flask 25 cm^2 was infected with HSV-1F and HSV-2G with MOI of 1 at room temperature for 1 hour. After infection, unabsorbed virus was removed and cells were washed with 5 ml of PBS (1X). Cells were treated with medicinal plant extracts and incubated at 37ºC in 5% CO_2 incubator. Cytopathic effect of infected cells was observed comparing to untreated cell control under inverted microscope before being harvested at 24 hours. The culture media were discarded and the infected cells were collected by scraping with sterile cell scrapers and washed twice with 5 ml of ice-cold PBS (1X). The cell suspension was completely transferred into 15 ml microcentrifuge tube before centrifugation at 4ºC, 3,000 rpm for 5 minutes. Then, supernatant was discarded, 1 ml of ice-cold PBS (1X) was added to cell pellets and cell suspension was transferred to new microcentrifuge tube. After spinning at 4ºC 3,000 rpm for 5 minutes, PBS (1X) was removed and cell pellets were kept at -80ºC (modified from Nikomtat, 2010 and Yucharoen, 2011).

3.7.7.2 Extraction of HSV protein

Cell pellets were suspended in 400 µl of cold NP-40 lysis buffer (Calbiochem, UK) containing protease inhibitors cocktail No.3 (1:200) (Calbiochem, UK), gently mixed and then incubated on ice box for 20 minutes. After incubation,

the mixture was spinned at 4ºC 14,000 rpm for 10 minutes. Then, supernatant was carefully collected and transferred to a sterile microcentrifuge tube. Total viral proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.7.7.3 Determination of HSV protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The inhibitory effect of medicinal plant extracts on viral protein synthesis was detected by separation of treated viral protein on SDS gel. Bio-Rad Mini-PROTEIN Tetra Handset's equipment was cleaned with 70% ethanol prior to casting the gel. Then, separating gel was poured into gel casting, 70% ethanol was overlaid and the gel was allowed to completely polymerize about 30-45 minutes. Ethanol was then removed and stacking gel was added. After that, comb was inserted and allowed the gel to completely polymerize about 20-30 minutes. The gel was placed into clamping frame and electrode was assembled into gel tank. Running buffer was added and the comb was removed. Protein samples from infected cells after treatment with medicinal plant extracts, viral control protein and cell control protein were denatured for 5 minutes at 95°C in water bath. Then, these proteins were centrifuged at 13,000 rpm at 4°C for 1 minute before loading into SDS-polyacrylamide gel comparing to pre-stained dye molecular weight marker (Page Ruler, Fermentus, Canada). Gel electrophoresis was performed at 95 V for 1 hour and 40 minutes.

3.7.7.4 Detection of HSV protein by Western blot analysis

After separation each protein by SDS-PAGE, proteins on polyacrylamide gel were electrotransferred to nitrocellulose membrane using semi-dry blotting.

Nitrocellulose membrane and filter papers were soaked at 4°C for 30-45 minutes in towbin transfer buffer, pH 8.3 using flat-blade forceps or gloves to manipulate the membrane. Then, the apparatus of semi-dry botting was assembled as shown in Figure 40. Three pre-wet filter papers, nitrocellulose membrane (PROTRAN®; Schleicher&Schuell Inc., U.S.A), polyacrylamide gel and three pre-wet filter papers were placed from anode to cathode following manufacturer's instructions for semi-dry blotting.

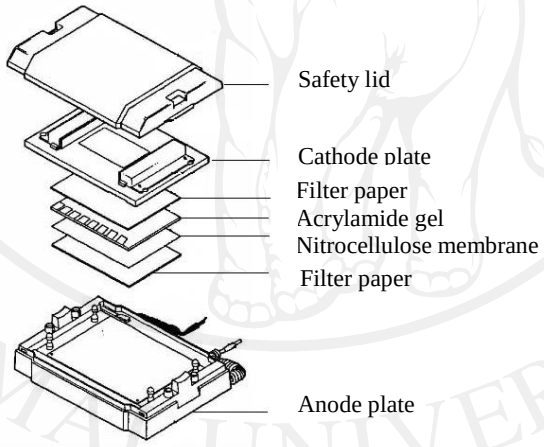


Figure 40 Semi-dry botting apparatus (modified from Garfin, 2003)

Air bubbles between layers were removed using rolling clean glass pipette and carefully cover with cathode plate apparatus. Protein transferring was performed at 15 V for 4.3 hours at 25°C. After transferring, each filter paper and gel was removed carefully by forceps. Next, the membrane was wash twice with saline on rocking platform at room temperature and bovine serum albumin (BSA, 3%)

(Hyclone, UK) in saline was added to membrane, and incubated overnight in dark box at 4°C to block non-specific binding on the membrane. After incubation period, the membrane was washed twice by saline before incubation with horseradish peroxidase-conjugated IgG against HSV (AbD serotec, Bio-Rad) in dark box at 4°C overnight with gentle rocking. Antibody was used at ratio of 1:100 by dilution of 50 µl of antibody in 5 mL of BSA. After that, the blotting filter membrane were washed in saline twice for 5 minutes and HRP antibodies were detected by incubating the blotting filter in 0.06% 4-chloro-1-naphthol (Sigma-Aldrich, USA)/ 0.01% H_2O_2 in PBS. Viral proteins on the membrane were shown in blue-black color. The membrane was washed with deionized water to stop reaction, air dried and viral protein band intensity was analyzed by Gene Tools Match (LAB Focus, Co.Itd.)

4. Separation and partial purification of medicinal plant extracts

The medicinal plant extracts that showed highest anti-HSV activity were selected and separated by partition technique and subsequently further purified using column chromatography to identify the major secondary metabolites. The procedure of separation was as follows.

4.1 Separation of medicinal plant extracts by partition technique

Crude extracts of medicinal plant extract, which showed the highest anti HSV activity were separated by partition technique. Different solvent based on polarity was used. Distilled water (56 ml) was initially added to 25.0 g of dried powder of extract before it was partitioned with 50 ml of hexane in separatory funnel four times to give the hexane fraction. The residual of extracts was further partitioned by gradually enrichment with 50 ml of ethyl acetate (EtOAc, Merck, Germany) four

times to give the EtOAc fraction. Then, the supernatant were selected and further partitioned with 50 ml of n-butanol to give n-butanol fraction and aqueous fraction. Finally, each partition fractions were dried in high vacuum to obtain dry yield of four fractions and the fractions of extracts were further dissolved in DMSO before testing for their anti-HSV activity by plaque reduction assay.

4.2 Column chromatography

4.2.1 Isolation of *E. prostrata*

The ethanolic extract of *E. prostrata* (100 g) was fractionated by column chromatography using celite (Fluka, Switzerland) as stationary phase. Celite was mixed with plant extract and packed in column and elution was started with eluted solvent; hexane, hexane:EtOAc, EtOAc and 95% EtOH, consecutively. Each 25 ml fraction was collected and combined after visualization with TLC using ultraviolet light (254 and 366 nm) to give 12 fractions, K101 to K112. The isolation of *E. prostrata* was performed in Figure 41.

After that, each fraction was dried in high vacuum and dissolved in DMSO before study the efficacy against HSV activity by plaque reduction assay.

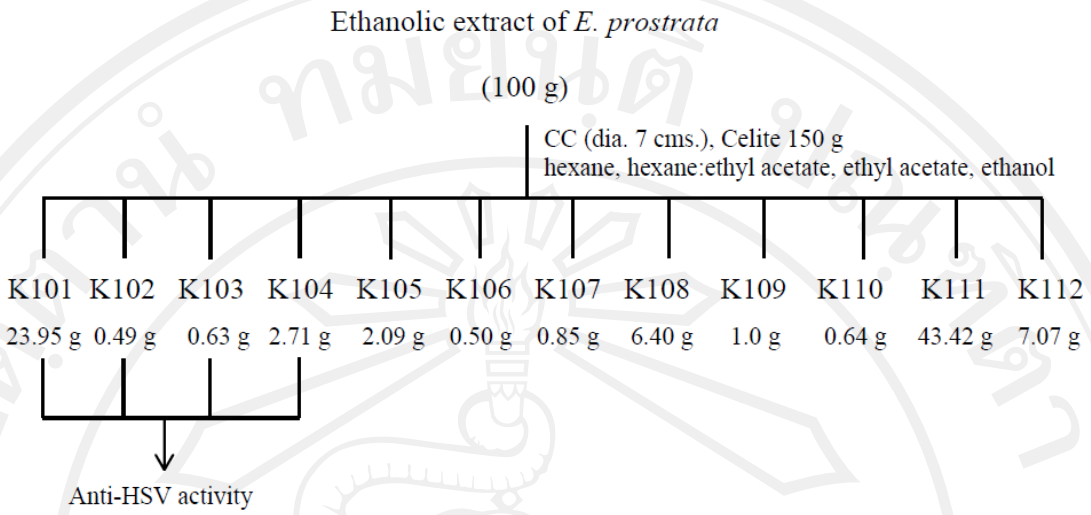


Figure 41 Isolation scheme of ethanolic extract of *E. prostrata*

4.2.2 Isolation of *Sphenodesme* sp.

The ethanolic extract of *Sphenodesme* sp. (100 g) was fractionated by column chromatography using celite as stationary phase, and hexane: EtOAc, EtOAc, EtOAc: EtOH and 95% EtOH were used consecutively as mobile phases. Each 25 ml fraction was collected and combined according to their TLC pattern to give 10 fractions, P101 to P110 (Figure 42).

After determination of anti HSV activity, P107 active fraction (0.20 g) was further separated using column chromatography on Sephadex LH 20 column chromatography. 95% EtOH, 75% EtOH: 25% H_2O, 50% EtOH: 50% H_2O, 25% EtOH: 75% H_2O, 25% acetone: 75% H_2O, 50% acetone: 50% H_2O, 75% acetone: 25% H_2O and acetone were used consecutively as eluting solvent and collected fractions of each extract were combined based on their TLC patterns to obtain 6 fractions (Figure 42). Anti-HSV activities of each selected fraction were investigated by plaque reduction assay.

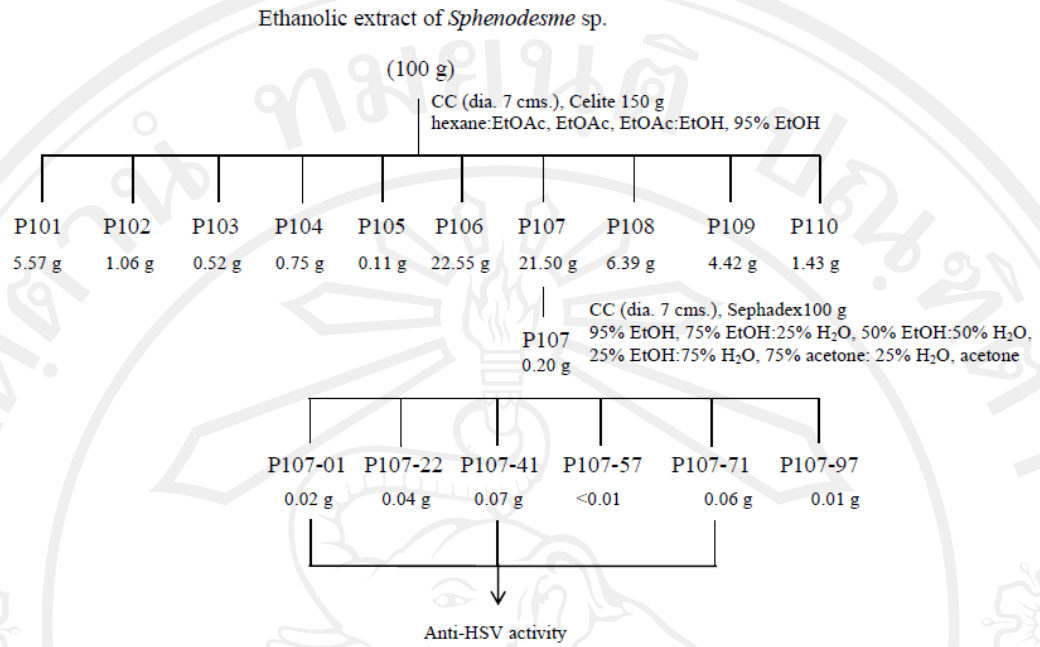


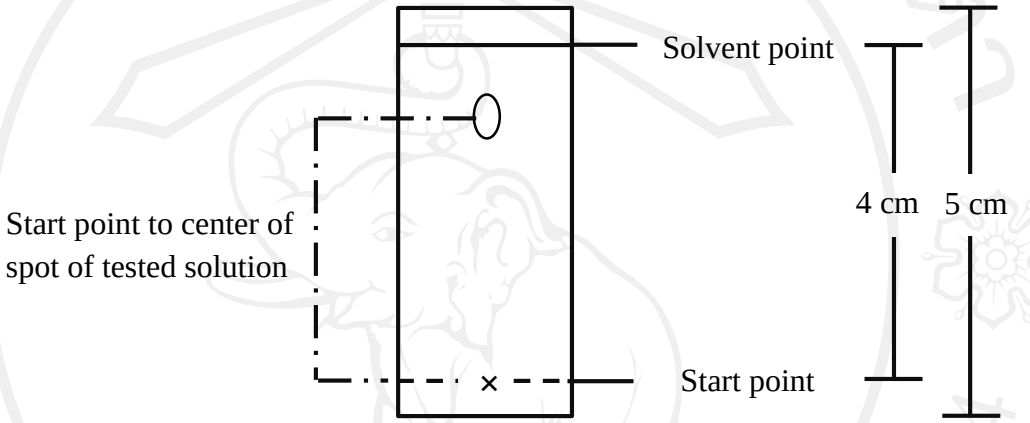
Figure 42 Isolation scheme of ethanolic extract of *Sphenodesme* sp.

4.3 Thin layer chromatography (TLC)

Identification of phytochemical constituents was further carried out by TLC using pre-coated silica gel 60F 264 (Merk, Germany) as an adsorbent with layer thickness of 0.2 mm and 5 cm distance. First, a small spot of each sample solution was applied to a silica gel plate at starting point about 1 cm from the base and the plate was placed in to saturated solvent chamber system. Different compounds in the sample mixture could move at different rates due to differences in solubility in the solvent and stationary phase. Then, TLC plate was removed after the solvent moved to the solvent point and air-dried at room temperature. After that, the numbers of spots on the developed plates were observed under UV light at 254 nm and 366 nm. Spots were visualized by spraying with 2% anisaldehyde-sulfuric acid reagents to determine the different compounds following by heating in oven at 110ºC for 5-10 minutes. Colorful spots of positive control after spraying this reagent were varied

depending on the compound observed. In addition, Rf values of each spot were calculated and measured as follows.

$$Rf = \frac{\text{Distance of the tested solution}}{\text{Distance of the solvent}}$$



5. Determination of phytochemical compounds in the medicinal plant extracts

Phytochemical compound in crude extracts and fractions of the medicinal plants were investigated in this study. The major compounds of the medicinal plant extracts were identified for secondary metabolite constituents such as alkaloids, antraquinon glycoside, cardiac glycosides, cumarin, flavonoid, phenolics, saponins and tannin using standard phytochemical test as follows

5.1 Detection of alkaloids

Alkaloids were determined by dissolving 0.3 g of the powdered extracts in 15 ml of 2N hydrochloric acid (HCl) and incubated in water bath for 10 minutes. Then, the mixture was cooled and filtered using Whatman No. 1 filter paper. After

that, 2 ml of filtrate was divided into 4 tubes and each individual tube was treated carefully with various alkaloid reagents.

A few drops of Mayer's reagent (Potassium Mercuric Iodine) were added to the filtrate by the side of the first test tube. A turbid or white color of precipitate indicated the presence of alkaloids in the filtrate. An orange or reddish color of precipitate indicated the presence of alkaloids, was observe after adding a few drops of Dragendroff's reagent (Solution of Potassium Bismuth Iodide) to the filtrate by the side of the second test tube. A few drops of Wagner's reagent (Iodine in Potassium Iodide) were added to the filtrate by the side of the test tube. A brown or reddish color of precipitate indicated the presence of alkaloids. A few drops of Hager's reagent (Saturated picric acid solution) were added to the filtrate by the side of the fourth test tube. A yellow color of precipitate indicated the presence of alkaloid

5.2 Detection of antraquinon glycoside

Borntrager's test was used to identify antraquinon glycoside. Briefly, 0.3 g of the extract was mixed with 20 ml of HCl in the test tube and incubated for a few minutes on water bath for 5 minutes. The mixture was cooled, filtered and extracted twice with 10 ml of chloroform in separating funnel. Then, chloroform layer was collected and 1N NaOH was added to the chloroform layer. Formation of rose-pink, red or violet color of alkaline layer indicated the presence of antraquinon glycoside.

5.3 Detection of cardiac glycoside

The extract, 0.3 g was dissolved in 10% ethanol and filtrated through Whatman No.1. The filtrate was partitioned with chloroform 3 times and chloroform

part was evaporated in hot plate before separated into 2 tubes and the present of cardiac glycoside was tested as follows

1. Detection of steroidal nucleus by Liebermann - Burchard test

Three drops of acetic anhydride and concentrated sulphuric acid (H_2SO_4) were gently added along the side of the test tube into the sample. The presence of color changes into pink, red, purple, blue and green was observed within 5, 15 and 30 minutes at the interface of two layers indicated positive result.

2. Detection of deoxysugar by Keller - kiliani test

The evaporated extract was dissolved with a few drops of chloroform. Then, 3 ml of 10% ferric chloride in acetic acid and concentrated H_2SO_4 was slowly added. The present of brown ring at the interface layer indicated the presence of deoxysugar.

5.4 Detection of coumarin

Small amount of water was added into tube containing 0.3 g of medicinal plant extract. Then, a piece of filter paper was immersed with 1N NaOH and placed upper in water bath for 3-5 minutes. After that, the filter paper was cooled, dried and visualized under UV light 366 nm. The blue-green fluorescence indicated the presence of coumarin.

5.5 Detection of flavonoids

Flavonoids were tested by Shinoda's test. The medicinal plants extracts, 0.3 g was extracted twice with 10 ml of petroleum ether to remove lipid. The residue was dissolved in 10 ml of 50% ethanol and heated. Magnesium metal strip was added and

formation of orange to reddish color was observed in 3 minutes after adding 5-6 drops of concentrated HCl, indicated the presence of flavonoids.

5.6 Detection of phenolic compound

Medicinal plant extract (0.3 g) was mixed with 6 ml of 70% ethanol in a test tube and heated in water bath for 2 minutes. After cooling, the whole solution was filtered through Whatman No.1 filter paper. Then, 3-4 drops of 5% iron (III) chloride solution was added. A blue green color was produced if phenolic substances were present.

5.7 Detection of saponins

Saponin was detected in the medicinal plant extract by Froth's test. Briefly, plant extract (0.3 g) was mixed with 10 mL of water and boiled in water bath for 5 minutes. After cooling, the solution of extract was filtered through Whatman No 1. Then, 2 ml of filtrate was shaken vigorously for 1-2 minutes. The presence of honeycomb froth (creamy miss of small bubbles) was produced 1 cm above the surface of liquid and persisted more than 30 minutes indicated saponin in the sample.

5.8 Detection of tannin

Tannin was tested by mixing with 0.3 g of medicinal plant extract with 20 ml of distilled water and boiled in water bath. After cooling, the solution was filtered. Then, 2 ml of the filtrate was divided into 7 test tubes for tannin detection as follows.

1. Negative control.

2. Gelatin test

Few drops of 1% gelatin (Fluka Biochemika) were added. The white precipitate indicated the present of hydrolysable tannin.

3. Ferric chloride test

To confirm the presence of tannin, 2-3 drops of $FeCl_3$ (Ferric chloride, Ajax Finechem, australia) was added and brownish green or a blue-black color were observed when condense tannin or hydrolysable tannin was detected.

4. Lime test

Lime water (1 ml) was added to extract. The dark-grey and blue precipitate indicated that hydrolysable tannin was presented.

5. Lead acetates test

Lead acetate (PbAcO, 10%) 2 ml was added to the solution of plant extract. If blue precipitate occurred within 15 minutes indicated that hydrolysable tannin was present.

6. Formaldehyde-HCl test

Three drops of formaldehyde (40%) and 6 drops of 10% HCl were added and then boiled in water bath for 2 minutes. If pink or red precipitate was occurred, condense tannin was presented.

7. Vanillin-HCl test.

Vallinin (Merck, Germany) and 1 ml of HCl was added in the filtrate. Tannin was present if the condensed pink-red was observed.

6. Chromatographic fingerprint of plant extract

Medicinal plant extracts with the highest anti HSV activity were selected to determine the bioactive plant compounds in the extract.

6.1 Gas chromatography/Mass spectrometry assay (GC/MS)

Volatile composition of plant extract, which showed the highest anti-HSV activity was send to Central Laboratory, Thailand.co.Lid in order to analyze the compound. The crude extract (0.2 g) was chromatographed by capillary GC and filtrate with MS using selectivity for bases (SB) column (150 x 4.6 mm), formate buffer 10mM, pH 4: acetonitrile (60:40). The sample was compared with library database (Wiley version 7) at minimum 80% matched as reference.

6.2 High preformance liquid chromatography (HPLC)

Medicinal plant extract, which showed the highest anti-HSV activity was sent to Faculty of Pharmacy, Chiang Mai University to analyze and evaluate the plant composition using High performance liquid chromatography (HPLC)**.** The extract (0.5 mg) was injected to C18 column on temperature at 35ºC. The mobile phase composed of 0.05% (v/v) trifluoroacetic acid (TFA) and 100% acetonitrile at a flow rate of 1.0 ml/min. The elute compounds were detected with UV detector at wavelength 254, 263 and 366 nm.

7. Development of herbal gel product for treatment of HSV infection

The medicinal plant extract, which showed the highest anti-HSV activity was selected and used to prepare herbal gel product. Plant extract at highest non-toxic concentration was added to gel base (Sangtongdee *et al*., 2006), which contained carbopol, methyl paraben, propylene glycol, tween 80, triethanolamine and distilled water. The contents were mix thoroughly by homoinizer. After that, the herbal gel was tested against HSV-1F and HSV-2G using plaque reducing assay.

7.1 Stability test of herbal gel product (modified from Boonmachai, 2010; Sarnjai, 2010)

Herbal gel containing medicinal plant extract was produced. Then, the herbal gel was stored at different temperatures which were 4, 25 and 45ºC for 210 days. Moreover, the stability of herbal gel and efficacy against HSV infection were determined every month for 7 months.

1. Physical properties determination

The stability of gel and physical properties were observed such as the separation of layer, homogenization, color, smell, pH and intensity.

2. Toxicity of herbal gel product

The toxicity of the herbal gel product on Vero cell was performed on Vero cell.

3. The efficiency of the herbal gel product against HSV-1F and HSV-2G infection was investigated by plaque reduction assay.

4. Stability of the herbal gel product

Heating-cooling cycle of herbal gel was performed. Herbal gel was kept at 4ºC for 48 hours and then kept at 45ºC for 48 hours for 6 cycles and anti HSV activity were tested by plaque reduction assay. Physical properties were also determined such as pH, viscosity, color and odor.

5. Irritation test of herbal gel product on volunteers skin

Irritation test of herbal gel product to the skin was tested in 10 volunteers, five males and five females with age ranging from 20-28 years. Herbal gel was applied on a surface area approximately was 1×1 cm^2 of lateral arms compared with deionized water. The gel was further washed to remove any remaining gel residue

after 24 hours of the test. The tested skin was observed for the irritation and irritation index was recorded.

8. Statistical analysis

All values were given as mean ± standard deviation (SD) from triplicate samples of three independent experiments for antiviral assay. Data values of cell viability assay and Western blot analysis was expressed as means ± SD from duplicate experiment. Differences alphabets were considered statistically significant when $p < 0.05$.