CHAPTER III

MATERIALS AND METHODS

1. Cell culture

A continuous cell line of African green monkey (*Cercopithecus aethiops*) kidney, called Vero cell, was kindly provided from Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. The cells were propagated in a growth medium.

The cells were washed once with phosphate buffered saline solution (PBS) and 1 ml of trypsin-EDTA mixture was added. When the cells were detached, trypsin-EDTA was discarded, and 2 ml of growth medium was added. The cells were tapped lightly and mixed thoroughly by a pipette. The viable cells were counted by trypan blue staining. The cell suspension was diluted in growth medium to an appropriate concentration and distributed into a new 25 cm^2 tissue culture flask. Then, the cells were incubated in a 5% CO_2 incubator at 37°C until the cell monolayers were confluent.

2. Herpes simplex virus

HSV-1, strain KOS, HSV-2, strain Baylor 186 was kindly provided from Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University. A virus stock was prepared from Vero cell monolayers infected with the virus at multiplication of infection (MOI) of about 0.1 plaque forming unit per cell (PFU/cell). After one hour of viral adsorption, unadsorbed viruses were washed away with PBS and replaced with a maintenance medium. The infected Vero cells were incubated in a 5% CO_2 incubator at 37°C until the cell population showed complete CPE. Then, they were disrupted by being repeatedly frozen at –70°C and thawed at room temperature for three times. The disrupted cell suspension was pelleted by centrifugation at 3,000 rpm for 10 minutes. The supernatant was distributed in small aliquots into microtubes and stored at –70°C.

3. Extracts of medicinal plants

Table 3 Plant materials used in this study

No.	Plants [Thai name]	Family	Part of use	Collection date
1	*Glycosmis pentaphylla* (Retz.) DC.[เขยตาย] [1]	Rutaceae	Leaves	2/04/02
2	*Ipomoea maxima* (Linn.f.)[สะอึก] [1]	Convolvulaceae	Leaves	2/04/02
3	*Willughbeia edulis* Roxb. [ลุย] [2]	Apocynaceae	Leaves	2/05/02

[1] From , Salaya Nakornpathom province.

[2] From Chantaburi province.

4. Acyclovir

Acyclovir powder (Sigma, USA) was prepared into a stock solution in concentration of 1,000 μg/ml in PBS. The ACV stock solution was distributed into small aliquots and stored at –20°C until used.

5. Plant extraction

The leaves from each plant were chopped into small pieces and extracted with 95% ethanol for 3-7 days. Then, the extract was filtered and the filtrate was evaporated under reduced pressure at 40°C. In this study, this ethanol extract is refered to fraction 1 (F1).

A volume of F1 was partitioned with an equal volume of chloroform. If the mixture was not completely separated into two layers, the water was added until they were completely separated. The chloroform extract was evaporated under reduced pressure at 40°C to give fraction 2 (F2). The chloroform-water extract was evaporated on the water bath until dried, to give an aqueous extract (fraction 5, F5).

A volume of F2 was solubilized in methanol and hexane partitioned with an equal volume of hexane. A quantity of hexane was added until the 2 layers were completely separated. The methanol layer was evaporated under reduced pressure at 40°C to give

the methanol extract called fraction 4 (F4). The methanol fraction was also evaporated to give fraction 3 (F3). Each fraction (F1, F2, F3, F4 and F5) was placed in an evaporating dish and all solvents were then removed by evaporating on the water bath before use. The extraction scheme was shown in Figure 4.

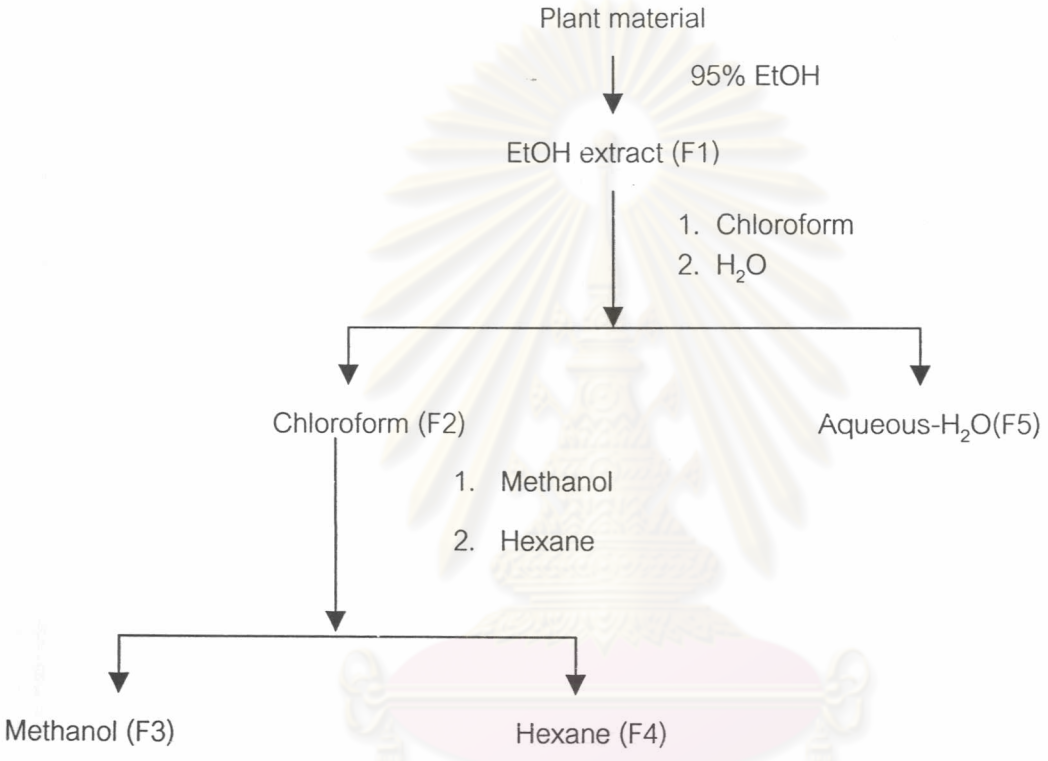

Figure 4 Extraction scheme of medicinal plants extraction

6. Virus assay

Titration of virus was performed by the following plaque assay. Serial ten-fold dilutions of virus in MEM were added onto Vero cell monolayers in a 96-well tissue culture plate (Nunc, Denmark) (25 μl/well; 3 wells/dilution). The virus was allowed to adsorb for 1 hour in a 5% CO_2 incubator at 37°C before adding 100 μl of overlay medium (MEM + 2%tragacanth) (see appendix) in each well. The plate was incubated for 3-4 days in a 5% CO_2 incubator at 37°C. The number of plaques was counted under an inverted microscope and the virus titer was calculated as plaque forming unit per milliliter (PFU/ml). To confirm the plaque numbers, the medium was discarded and the infected cells were fixed with10% formalin and stained with 1% methylene blue for 30 minutes.

7. Cytotoxicity Test

Cytotoxicity was determined by adding serial two-fold dilutions of the test sample in the growth medium to Vero cell monolayers quadruplicately (100 μl/well). The cells were incubated in a 5% CO_2 incubator at 37°C for 3 days and then were examined under the microscope for observing cell cytotoxicity. the cell were fixed with formalin and stained with methyiene blue for 30 min and the stain was solubilized with 3% hydrochloric acid. The OD was measured at 550 nm using microtiter plate reader. the cytotoxic data were ploted as dose-response curves, from which the 50% cytotoxic concentration(CC_{50}) was obtained.

8. Anti-HSV-1 and HSV-2 Activities of plant extracts

The antiherpes simplex virus activities were investigated by the methods modified from Hayashi et al.,1996 using inactivation, prophylactic activity, and plaque reduction assay.

8.1 Inactivation assay

To determine a neutralizing activity of plant extract or ACV(as control) to virus, inactivation was performed by the following method. Twenty-five microliters of virus was incubated with 25 µl of sample dilution in a 5% CO_2 incubator at 37°C for 1 hour. Mixture were then added onto monolayer cells in triplicated wells and incubated in a 5% CO_2 incubator at 37°C for 1 hour. The overlay medium containing plant extract or ACV in appropriate concentration was added to the cultures after the mixture was discarded. Cultures were incubated in a 5% CO_2 incubator at 37°C for 3-4 days. The number of plaques was counted and confirmed by staining with methylene blue as previously mentioned.

8.2 Prophylactic activity assay

To determine an activity of plant extract or ACV(as control) to viral adsorption or penetration, prophylactic activity assay or pretreatment assay was performed by the following method. Twenty-five microliters of sample was added onto monolayer cells in triplicated wells and incubated in a 5% CO_2 incubator at 37°C for 1 hour. After each sample dilution was discarded, the cells were infected with 25 µl of virus and incubated in a 5% CO_2 incubator at 37°C for 1 hour. The overlay medium containing crude extract or ACV in appropriate concentration was added to the cultures after the unadsorbed virus was discarded. The cultures were incubated in a 5% CO_2 incubator at 37°C for 3-4 days. The number of plaques was counted and confirmed by staining with methylene blue as previously mentioned.

8.3 Plaque reduction assay

To determine an activity of crude extract or ACV(as control) to intracellular viral replication, plaque reduction assay or post-treatment was performed by the following method. Twenty-five microliters of virus was added onto monolayer cells in triplicated wells and incubated in a 5% CO_2 incubator at 37°C for 1 hour. After the unadsorbed virus was discarded, 25 µl of each sample dilution was added and incubated in a 5% CO_2 incubator at 37°C for 1 hour. Each sample dilution was removed from the plate and then, the overlay medium containing crude extract or ACV in appropriate concentration

was added to the cultures. The cultures were incubated in a 5% CO_2 incubator at 37°C for 3-4 days. The number of plaques was counted and confirmed by staining with methylene blue as previously mentioned.

The antiviral activity of 3 medicinal plants against HSV-1 and 2 were screened and compared with that of ACV in term of 50% effective concentration (EC_{50}). The EC_{50} were determined by an equation, $Y = a + b \log X$ or $Y = a + b X$, whereas Y is the amount of plaque (% of control); X is the concentration of extract; and a, b are constant values.

9. Preliminary test for anti-HSV-1 and 2 activities of plant extracts

Preliminary test for the mechanism study of antiviral activities of the plant extracts were performed by the methods modified from Piret et al., 2002.

9.1 Post-binding assay

The effect of plant extracts or ACV(as control) on HSV attached to Vero cells. Were examined confluent Vero cells seeded in 24-well tissue culture plates were maintained at 4°C for few minutes. Cells were first incubated with viruses 50-100 PFU for 2 hours at 4°C to allow a stable attachment of the virus without fusion with cell membrane. Unbound viruses were removed, Cells were then washed with PBS or with low pH citrate buffer or with PBS containing increasing concentrations of plant extracts for 1 minute at 4°C. Cell were washed once with PBS and overlaid with overlayer medium. Cells were incubated for 2 days at 37°C in a 5% CO_2 atmosphere. Cell were then fixed, washed, and stained. The amount of virus which had penetrated into cells after the shift at 37°C was evaluated following the determination of the numbers of PFU.

9.2 Penetration assay

The effect of plant extracts or ACV(as control) on the rate of penetration of HSV into Vero cells were investigated cofluent Vero cells seeded in 24-well tissue culture

plates were incubated with viruses 50-100 PFU for 2 hours at 4°C. After removal of unbound viruses. The plates were shifted to 37°C to allow penetration of virus into cells. At selected times after the temperature shift (0,15,30, and 60 min), cells were treated for 1minute with PBS or with PBS containing plant extracts. Cells were overlaid with overlayer medium and incubated for 2 days at 37°C in a 5% CO_2 atmosphere. Cells were then fixed, washed, and stained. The amount of virus which had penetrated into cells was evaluated following the determination of the numbers of PFU.

9.3 Virus yield inhibition assay

In a preliminary experiment, activities of plant extracts or ACV (as control) by virus yield inhibition assay were determined. The amounts of 50-100 PFU of viruses were added into 24-wells tissue culture plate of confluent Vero cell monolayer and allowed to adsorb for 1 hour at 37 °C. Unadsorbed virus was discarded and the cells were washed twice with PBS. It wased observed that this procedure reduced the amount of unadsorbed virus to a negligible amount. The infected cells were then maintained in serial 2-fold concentration of plant extracts or ACV(as control), each in triplicate at 37°C for 1, 8, 24 48 and 72 hours. After 1, 8, 24, 48 and 72 hours of infection, the cells were observed for CPE, frozen and thawed twice, then pooled and centrifuged at 1000 rpm for 10 minutes. The supernatants were stored at –70 °C for plaque assay. Antiviral activity was expressed as 50% inhibitory concentration (IC_{50}).