CHAPTER III

MATERIALS AND METHODS

3. 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 (see appendix).

The cells were washed once with phosphate buffered saline solution (PBS) and added 1 ml of trypsin-EDTA mixture (see appendix). 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-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 (see appendix). 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. Crude extracts of medicinal plants

Table 3 Plant materials used in this study

No.	Plants [Thai name]	Family	Part of use	Collection date
1	*Andrographis paniculata* [ฟ้าทะลายโจร] [1]	Acanthaceae	Leaves and stems	2/08/01
2	*Bridelia ovata* [มะกา] [1]	Euphorbiaceae	Leaves	2/08/01
3	*Cissus quadrangularis* [เพชรสังฆาต] [1]	Vitidaceae	Whole plant	2/08/01
4	*Citrus reticulata* [ส้มเขียวหวาน] [2]	Rutaceae	Pericarp	3/08/02
5	*Clinacanthus siamensis* [ลิ้นงูเห่า] [1]	Acanthaceae	Leaves	2/08/01
6	*Cocos nucifera* [มะพร้าว] [2]	Palmae	Endosperm	26/03/02
7	*Costus speciosus* [เอื้องดิน] [1]	Costaceae	Leaves and stems	2/08/01
8	*Momordica charantia* var. *maxima* [มะระ] [2]	Cucurbitaceae	Fruits	02/07/01
9	*Momordica charantia* var. *minima* [มะระขี้นก] [2]	Cucurbitaceae	Fruits	02/07/01
			Seeds	02/07/01
10	*Nephelium lappaceum* [เงาะ] [2]	Sapindaceae	Pericarp	23/10/01
11	*Orthosiphon aristatus* [หญ้าหนวดแมว] [1]	Labiatae	Leaves	02/08/01
12	*Phyllanthus amarus* [ลูกใต้ใบ] [1]	Euphorbiaceae	Whole plant	02/08/01
13	*Schefflera leucantha* [หนุมานประสานกาย] [1]	Araliaceae	Leaves	02/08/01
14	*Thunbergia laurifolia* [รางจืด] [1]	Acanthaceae	Leaves and stems	02/08/01
15	*Vitis vinifera* [องุ่น] [2]	Vitaceae	Seeds	17/12/01
16	*Barleria lupulina* [เสลดพังพอน] [1]	Acanthaceae	Leaves	14/08/01
17	*Clinacanthus nutans* [พญายอ] [1]	Acanthaceae	Leaves	24/07/01

[1] From Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok.

[2] From market.

F1: Ethanol extract; F2: Hexane extract; F3: Aqueous-ethanol extract; F4: Ethyl acetate extract; F5: Aqueous-ethyl acetate extract

4. Acyclovir

Acyclovir powder (Sigma, Lot. No. 117F0756, USA) was prepared into a stock solution in concentration of 400 µg/ml in PBS. The ACV stock solution was distributed into small aliquots and stored at –20°C until used.

5. Crude Extraction

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

A volume of F1 was partitioned with an equal volume of hexane. If the mixture was not completely separated into two layers, the water was added until they were completely separated. The hexane extract was evaporated under reduced pressure at 40°C to give fraction 2 (F2). The ethanol-water extract was evaporated on the water bath until dried, to give an aqueous-ethanol extract (fraction 3, F3).

A volume of F3 was partitioned with an equal volume of ethyl acetate. A quantity of water was added until the 2 layers were completely separated. The ethyl acetate layer was evaporated under reduced pressure at 40°C to give the ethyl acetate extract called fraction 4 (F4). The aqueous fraction was also evaporated to give fraction 5 (F5). 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 used. The extraction scheme was shown in Figure 4.

Some fractions were formed complex with polyvinylpyrolidone (PVP:extract = 7:1 w/w) in order to increase the solubility. Maximum PVP concentration used in study was 7,000 µg/ml and had not effected to Vero cell (Chutinan Kantasuk, 1992).

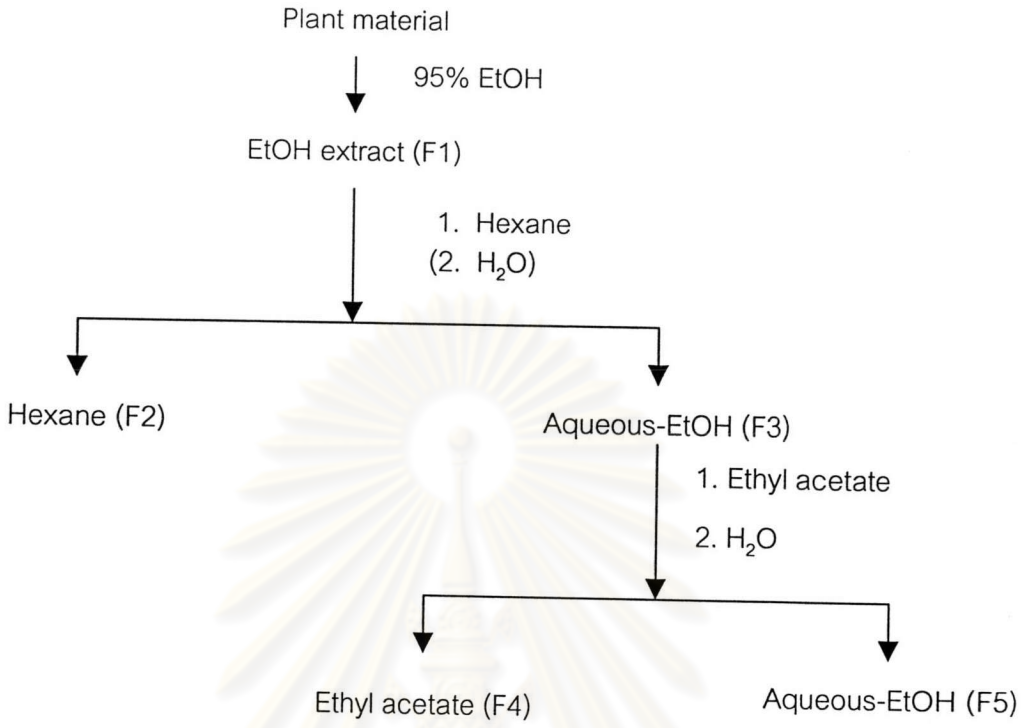

Figure 4 Extraction scheme of medicinal plants extraction

6. Determination of A Viral Titer

Titration of virus was performed by the following plaque assay. Serial five-fold dilutions of virus in MEM were added onto Vero cell monolayers in a 96-well tissue culture plate (Nunc, Denmark) (50 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 (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 stained with 1% crystal violet in 10% formalin for 30 minutes.

7. Cytotoxicity Test

Cytotoxicity test was performed according to the study by Chutinan Kuntasuk (1992). Serial two-fold dilutions of the test sample in the growth medium were added to Vero cell monolayers quadruplicately (100 µl/well). The cells were incubated in a 5% CO_2 incubator at 37°C for 7 days and then were examined under the microscope for observing CPE. The cytotoxicity of the sample was expressed as median cytotoxic dose (CD_{50}) which was calculated as described by Reed and Muench (1938) (see appendix)

8. Study of Anti-HSV-2 Activities

In this study, plaque reduction assay (PRA) was performed to determine anti-HSV-2 activity of all crude extracts and ACV.

8.1 Inactivation

To determine a neutralizing activity of crude extract or ACV to virus, inactivation was performed by the following method. Two hundred microliters of virus was incubated with 200 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 crude 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 crystal violet as previously mentioned.

8.2 Pre-treatment

To determine an activity of crude extract or ACV to viral adsorption or penetration, pre-treatment was performed by the following method. Fifty 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 50 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 crystal violet as previously mentioned.

8.3 Post-treatment

To determine an activity of crude extract or ACV to intracellular viral replication, post-treatment was performed by the following method. Fifty 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, 50 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 crystal violet as previously mentioned.

The antiviral activity of 17 medicinal plants against HSV-2 were screened and compared with that of acyclovir in term of median effective dose (ED_{50}). The ED_{50} concentrations 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.