# CHAPTER IV

## MATERIALS AND METHODS

### Part I. Cell culture for propagation of virus

#### 1.1. Cell line and culture medium

A continuous cell line of African green monkey (*Cercopithecus aethiops)* kidney, called Vero cells, were obtained from Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. The cells were propagated in growth medium ( Modified Eagle Medium (MEM), Gibco, U.S.A. ) with 10% fetal calf serum (FCS, Gibco, U.S.A.) and 1% antibiotic (Gibco, U.S.A.) which contained penicillin, streptomycin and fungizone (see Appendix).

The cells were washed two times with phosphate buffered saline solution, pH 7.5 (PBS, see Appendix) and added 1-2 ml of trypsin - EDTA mixture. When the cells are detached, trypsin - EDTA was discarded, and 2-5 ml of growth medium was added. The cells were mixed thoroughly by a pipette and the viable cells were counted by trypan blue staining. The cell suspension was diluted in growth medium by experience, generally 1 : 3 to 1 : 5 and distributed in tissue culture flask. Then, the cells were incubated at 37°C , 5% $CO_2$ until the cell monolayers were confluent. The cells were possibly passaged in 1 : 3 to 1 : 10 split ratio (divisions) at each subculture. The subculture was done by trypsinization and dilution in growth medium.

The cells were frozen by the method as follow. The cell monolayers were trypsinized from one or more flasks of confluent monolayers and suspended in freezing medium (see Appendix) at a concentration of $1.5 \times 10^6$ cells/ml. The cell suspension was distributed in 2 ml cryovials, 1 ml per vial, then placed at 4°C at least for 3 hours. The vials were cooled slowly between 4°C and -25°C at the cooling rate of 1°C per minute.

When the temperature of vial reached -25°C, brought rapidly to -70°C or lower (liquid nitrogen). The cells were stored at -70°C or lower until used.

The cells were thawed by immersed the vial of frozen cells in a 37°C water bath, the cells were collected from vial by using sterile pipettes, dispersed in prewarmed medium. The cells were centrifuged by centrifugation (Sigma, Germany) at 2000 rpm for 20 minutes at 4°C. Then the old medium was discarded and the cells were placed in culture flasks with growth medium. The cells were incubated at 37°C , 5% $CO_2$ incubator. After 24 hours, the medium was replaced with fresh medium. The flasks were incubated at 37°C , 5% $CO_2$ for 3-6 days and subcultured regularly.

## Part II. Herpes simplex viruses

### 2.1. Herpes Simplex Viruses Standard Strain

HSV-1, strain KOS and HSV-2, strain 186 was obtained from Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. HSV-2, strain 186 was originally isolated, from a penile lesion, by Dr. Priscilla A. Schaffer of the Department of Virology and Epidemiology, Baylor College of Medicine, Texas Medical Center, Houston, Texas, U.S.A.

Virus stock was prepared from Vero cell monolayers infected with the virus at Multiplication of Infection (MOI) 0.1 - 1.0 plaque forming unit per cell (PFU/cell). After one hour of viral adsorption, the unadsorbed virus was removed; the culture was washed once with PBS, pH 7.5 (see Appendix) and replaced with MEM. The infected Vero cells were incubated at 37°C , 5% $CO_2$ for 24 to 48 hours or until the cell population showed cytopathic effect (CPE) more than 80%. Then, they were disrupted by being repeatedly frozen at -70°C and thawed at 37°C in water bath for three times. The disrupted cell suspension was pelleted by centrifugation (Sigma, Germany) at 2000 rpm for 20 minutes at 4°C. The supernatant was distributed in small aliquots into cryovials and stored at -70°C.

2.2. HSV-2 isolates

Between June 1997 through February 1998, all the patients with clinically suspected genital herpes at Bangrak Hospital, Bangkok and Venereal Diseases and AIDS Center 3 Chonburi, Thailand were interviewed for epidemic data, such as history of HSV infections and antiviral drugs uptake during they were sampled. Upon enrollment, a genital examination was performed and stage of genital lesion recorded. If vesicular lesions were present, aspirated fluid from a vesicle with a 26 or 27 gauge needle with tuberculin syringe and injected into a tube containing 2.0 ml of viral transport medium (MEM with 10% FCS and 2% antibiotic ). While ulcers presented, firmly rubbed sterile cotton swab against the base of the ulcers and took the swab into a tube of viral transport medium. The specimens were placed at 4°C container while rapid transportation to cell culture laboratory or collected at -70°C until they were isolated in cell culture. All specimens were cultured in Vero cell monolayer flask cultures (25 $cm^2$, NUNC, Denmark) at 37°C , humidified 5% $CO_2$ incubator and observed daily for development of CPE for 2-5 days.

When HSV CPE appeared, the culture was frozen and thawed three times, and the suspension is centrifuged at 2000 rpm, 4°C for 20 minutes. The supernatant was distributed in small aliquots into vials, the code of virus and the collected date were labeled. Then, viruses were stored at -70°C until they were used in this research.

When the primary culture of isolates developed CPE less than 80% area of the monolayers. Viruses were propagated again by inoculation on Vero cell monolayers at MOI 0.1 - 1.0 PFU/cell. The cultures were incubated at 37°C, humidified 5% $CO_2$ incubator for one hour by gentle mixing every 15 minutes. After one hour of virus adsorption, the non adsorbed virus was discarded. The infected cells were incubated at 37°C, humidified 5% $CO_2$ incubator for 1-5 days. The cultures were examined for HSV CPE everyday. When CPE appeared, the viruses were kept at -70°C or lower, so,

primary or secondary isolated cultures were used in this study and the primary culture was the first culture to choose. Before using, all virus isolates were identified and titrated.

2.3. HSV-2 Identification

All virus isolates were identified by using polyclonal HSV-2 antibody directed identified specific antigens in enzyme immunoassay (EIA) which followed and applied from the technique of Punnarugsa, V., et al. (89) and Gerard, P.R., et al. (90). Before this process was done, the optimal concentration and volume of antibody coating, virus and conjugate were titrated. The microtiter plate (96 wells NUNC, Denmark) was coated with 100 µl of 2 fold dilution, beginning 1 : 1000 to 1 : 8000 rabbit anti-herpes simplex virus type 2, MS ( DAKO, Glostrup, Denmark) and incubated at 4°C, overnight. The plate was washed with PBS-T (see Appendix) for 5 minutes, 3 times. And 150 µl of blocking solution (see Appendix) was added in every wells, the plate was incubated at 37°C for one hour. Then, the plate was washed with PBS-T, 3 times, 100 µl of the appropriate virus in diluent (see Appendix) was added, the plate was incubated at 37°C for one hour. Then, the plate was washed 3 times with PBS-T and 100 µl of peroxidase-conjugate rabbit anti-herpes simplex virus type 2 (DAKO, Glostrup, Denmark) was added. The plate was incubated at 37°C for one hour, washed 3 times with PBS-T and substrate OPD (see Appendix) was added, the plate was incubated at room temperature in the dark for 20 minutes. After incubation the reaction was stopped by adding 50 µl of 4N $H_2SO_4$ and the OD of each well at 492 nm was read and recorded by using Microplate Reader (BIO-RAD Model 3550, U.S.A.). The results were interpreted by using the cut off value obtained from negative, positive and reagent control (the diluent was used instead of virus). The negative result was the well with OD less than cut off value and the positive result was the well with OD equal to or more than cut off value. For the validity, the OD of reagent control have to be less than the cut off value and the OD of positive control have to be more than 2 times of reagent control.

In addition, the size of HSV isolated plaque was identified and compared with the size of the plaques from HSV-1 strain KOS and HSV-2 strain186, the size of plaque from HSV-2 are bigger than HSV-1.

2.4. Titration of virus

All viruses were titrated in 96-well tissue culture plate (96-W-C, NUNC, Denmark). Virus was prepared in serial 5-fold dilution in maintenance medium, 50 microliter (μl) of each dilutions of virus were added in the well (triplicated ) and followed by addition of Vero cell suspension 50 μl ($6 \times 10^5$ cells/ml). The plate was incubated at 37°C , humidified 5% $CO_2$ incubator for 3 hours , in order to complete cell attachment upon the surface of well and virus adsorbed into cell. Then 100 μl of overlay medium (see Appendix) was applied. The medium was discarded 3 days later and the infected cells were stained with 1% crystal violet in 10% formalin for 30 minutes. The plates were washed by tap water, air - dried and the number of plaques was counted and the virus titer as plaque forming unit per milliliter (PFU/ml) was calculated .

Before this process was done, the appropriate concentration and volume of antibody coating, virus and conjugate were titrated in ELISA technique.

Part III Study of antiviral activity of acyclovir against HSV-2 strain 186

ACV (Wellcome, Bangkok, Thailand) is white and pure lyophilized powder, was diluted in the two fold dilution with MEM at the concentration 0.0625 - 1.5 μl/ml.

The appropriate concentration and volume of Vero cells, virus and ACV used in this study, were followed and modified from the method of Pengsuparp, et al. (20).

Vero cells were suspended $6 \times 10^5$ cells/ml with MEM.

Virus (HSV-2 strain 186) was diluted to 1200 PFU/ml (30 PFU/25 μl)

The study of antiviral activity was done by plaque reduction assay which divided in 3 treatments (19).

3.1. Pre - treatment

Pre - treatment is the method to study antiviral activity by inhibition of virus invading into cells. The method was done by adding 25 µl each dilution of ACV in 96 well microtiter plate in triplicate and 50 µl Vero cells ($6 \times 10^5$ cells/ml) was added. The cell- mixture was incubated at 37°C , humidified 5% $CO_2$ incubator for 1 hour and 25 µl (30 PFU) of the virus was dispensed in the well. The plate was incubated at 37°C , humidified 5% $CO_2$ incubator for 3 hours. Then, 75 µl of the overlay medium was added in each well and 25 µl of the same dilution of ACV was dispensed in the well; the plate was incubated at 37°C , humidified 5% $CO_2$ incubator for 2-3 days or until the plaques were formed. The medium was removed from the plate and the cells were stained with 1% crystal violet in 10% formalin for at least 30 minutes and the plaques were counted and 50% effective dose ( $ED_{50}$ , the concentration which reduce 50% the number of viral plaques) of ACV was calculated against each virus.

3.2. Post - treatment

Post - treatment is the method to study antiviral activity by inhibition of viral replication in host cells (Vero cells). This method was similar to the pre - treatment, but there was the difference in mixing the virus and Vero cell at 37°C for one hour before adding ACV. Then, the procedures as mentioned in the pre - treatment were followed by incubation at 37°C for 2-3 days, staining, counting the number of plaques and calculation of $ED_{50}$ of ACV against the viruses.

3.3. Inactivation

Inactivation is the method to study antiviral activity of ACV against viruses by exposure of the virus directly to ACV. The procedure was similar to pre - treatment and post - treatment, but the viruses were mixed with each dilution of ACV for one hour before adding the cells. Then, the procedures as mentioned in the pre - treatment and post - treatment were followed and the $ED_{50}$ of ACV against the viruses was calculated.

## Part IV. Study of antiviral activity of acyclovir against HSV-2 isolates

The procedure of this study was similar to that mentioned in the study of antiviral activity of ACV against HSV-2 strain 186. The HSV-2 isolates were diluted to 30 PFU/25μl, were used instead of HSV-2 strain 186, but the treatment used in this study was chosen from the treatment which showed the lowest ACV concentration of $ED_{50}$ from part of Part III (Study of antiviral activity of ACV against HSV-2 strain 186). The $ED_{50}$ of ACV against all HSV-2 isolates were evaluated.

## Part V. Study of antiviral activity of medicinal plant extracts against HSV-2 strain 186 and HSV-2 isolates

5.1. Source of plant materials

The phasalad leaves ( the leaves which are not young and mature) of *Cerbera odollam* Gaerth., *Clausena excavata* Burm. F., *Coleus amboinicus* Lour. and *Thevetia peruviana* Schum. and the leaves and apex of *Phyla nodiflora* (L.) Greene. were collected from Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand in May 1997. The plants were identified by comparison with herbarium specimens in the Department of Pharmacognosy and Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand and comparison with the details in FLORA OF JAVA (81). The criteria for identification of phasalad leaves was compared by the color and position of leaves in each plant.

5.2. Extraction

One hundred grams of fresh leaves from *Cerbera odollam* Gaerth., *Clausena excavata* Burm. F., *Coleus amboinicus* Lour. and *Thevetia peruviana* Schum. and the leaves and apex of *Phyla nodiflora* (L.) Greene. were chopped into small pieces (4-5 mm). Each plant was extracted repeatedly in methanol 3 times (1L, 3 days, each). The filtrates were pooled and some quantities of filtrate volume were evaporated under reduced pressure at temperature not exceeding 40°C. In this study, the methanol extract is called fraction 1 (F1).

The methanol fraction was partitioned between chloroform and water by the ratio 2 : 1. The chloroform fraction (approximately 500 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give a chloroform extract (F2).

The aqueous fraction was obtained from methanol - water (approximately 300 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give a an aqueous - methanol extract (F3).

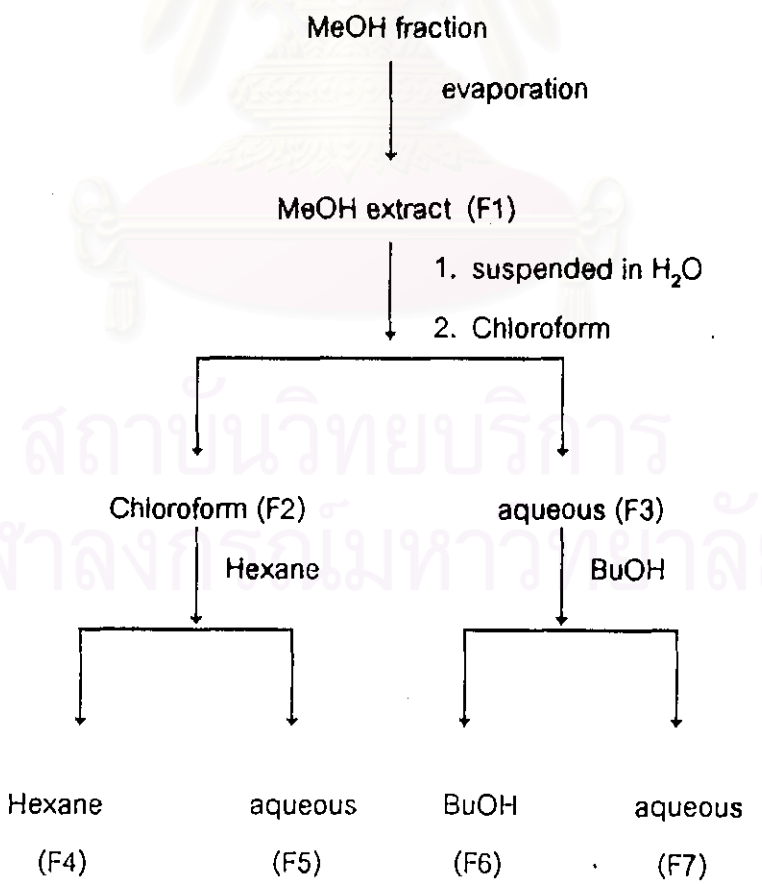
Some volume of the chloroform fraction (F2, approximately 300 ml) was partitioned between hexane and water by the ratio 2 : 1. The hexane fraction (approximately 250 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give a hexane extract (F4).

The aqueous fraction was obtained from hexane - water (approximately 300 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give an aqueous - hexane extract (F5).

Some of the aqueous fraction was obtained from methanol - water (F3, approximately 300 ml ) was partitioned between butanol (BuOH) and water by the ratio 2 : 1. The butanol fraction (approximately 250 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give a butanol extract (F6).

The aqueous fraction was obtained from butanol - water (approximately 300 ml) was evaporated under reduced pressure at temperature not exceeding 40°C until dried, to give an aqueous - butanol extract (F7).

Scheme 1. Separation of medicinal plants extraction



5.3. The cytotoxicity assay

All fractions of medicinal plant extracts were tested for cytotoxicity of Vero cells. The fractions were prepared to be the stock in concentration 10 mg/ml in DMSO and the stock was diluted to 1 mg/ml (1000 µg/ml) in 10% DMSO by sterilized distilled water and was diluted to 200, 80, 40, 20 and 8 µg/ml in 2.5% DMSO in MEM.

Vero cell monolayers, $5 \times 10^6$ cells/ml, 50 µl were dispensed in 96 well microtiter plate (NUNC, Denmark) and exposed to 50 µl of each dilution of extraction, starting at 200 µg/ml. Then, 100 µl MEM with 10% FCS and 1% antibiotic was added. DMSO, 2.5% was used as a reagent control and the wells containing the cells and medium (MEM) (triplicated) were used as a cell control.

The cells were examined microscopically for the presence of cytotoxic effects everyday for 1-5 days.

The results of cytotoxicity were confirmed by soluble - formazan method (20). The principle of the test is the viable cells not damaged can change tetrazolium reagent to colored formazan (91) and the OD at 450 nm was read by microplate reader (BIO-RAD model 5220, U.S.A.)

5.4. Study of antiviral activity of medicinal plant extracts against HSV-2 strain 186

The procedure in the study of antiviral activity of ACV against HSV-2 strain 186 was followed in this study. The HSV-2 strain 186 diluted to 30 PFU/25 µl was used. The medicinal plant extracts were diluted in various concentrations, beginning at the concentration of less than cytotoxicity. Only the treatment from the study of antiviral activity of ACV against HSV-2 strain 186 which elicited the lowest $ED_{50}$ of ACV was used in this study and $ED_{50}$ of all medicinal plant extracts against HSV-2 strain 186 were evaluated.

5.5. Study of antiviral activity of medicinal plant extracts against HSV-2 isolates

The procedure from the study of antiviral activity of medicinal plant extracts against HSV-2 strain 186 in 5.4. was followed in this part, but, the only medicinal plant extracts which showed active antiviral activity against HSV-2 strain 186 in 5.4. were performed. The HSV-2 isolates diluted to 30 PFU/25μl, were used. Only the treatment from the study of antiviral activity of ACV against HSV-2 strain 186 which elicited the lowest $ED_{50}$ of ACV was performed in this part and the $ED_{50}$ of active medicinal plant extracts against HSV-2 isolates were evaluated.

## Part VI. Study of antiviral activity for inhibiting viral entry into cell, growth inhibition of viral replication in the cell and direct viral demolishment of medicinal plant extracts against HSV- 2 strain 186 and HSV-2 isolates

HSV-2 strain 186 and 6 HSV-2 isolates chose by simple random sampling (chose from every count six) were performed. The appropriate quantity of viruses in this study, were piloted and diluted to 50 PFU/25 μl.

The methanol extract (F1) of 5 medicinal plant extracts were chosen to be extract representatives and ACV was tested as the control. The concentration of the medicinal plant extracts showing the highest concentration of no cytotoxicity to Vero cell was indicated in the results from 5.3. The antiviral activity was examined by plaque reduction assay which divided into 3 treatments, similar to the study in Part III, but the incubation time was varied as follow:

6.1. Pre - treatment

Pre - treatment is the method to study antiviral activity by inhibition of virus invading into cells. The method was done by adding 50 μl of active extracts to Vero cell monolayers in 96 well microtiter plate in triplicated. The cell - mixture was incubated at 37°C, humidified 5% $CO_2$ incubator for 1, 2, and 3 hour and the extracts were removed.

Then, the cells were washed one time with PBS and 25 µl (50 PFU) of the viruses were dispensed in the well. The plate was incubated at 37°C , humidified 5% $CO_2$ incubator for one hour. Then, the viruses were removed and the cells were washed one time with PBS and 100 µl MEM and 100 µl of the overlay medium were added in each wells, the plate was incubated at 37°C, humidified 5% $CO_2$ incubator for 2-3 days or until the plaques were formed. The medium was removed from the plate, the cells were stained with 1% crystal violet in 10% formalin for at least 30 minutes and the plaques were counted comparing to virus control (no active extracts) and cell control ( no virus). ACV was also used as standard positive control.

6.2. Post - treatment

Post - treatment is the method to study antiviral activity by inhibition of viral replication in host cells (Vero cells). The method was done by mixing the viruses and Vero cell monolayers at 37°C for one hour and the viruses were removed. Then, the F1 extracts were added into the cells. After incubation at 37°C, in humidified 5% $CO_2$ incubator for 1, 2 and, 3 hours, the extracts were removed, cells were washed one time with PBS and 100 µl MEM and 100 µl of the overlay medium were added in each wells. The plate was incubated at 37°C, humidified 5% $CO_2$ incubator for 2-3 days or until the plaques were formed. The medium was removed from the plate, the cells were stained with 1% crystal violet in 10% formalin for at least 30 minutes and the plaques were counted comparing to the virus control (no active extracts) and cell control ( no virus). ACV was also used as standard positive control.

6.3. Inactivation

Inactivation is the method to study antiviral activity of F1 extracts against viruses by direct viral demolishment. The procedure are similar to pre - treatment and post - treatment, but the viruses were mixed with active extracts at 37°C for 1, 2, and 3 hours before adding the monolayers. After incubation at 37°C in humidified 5% $CO_2$ incubator for one hour, the viruses were removed and the cells were washed one time with PBS

and 100 µl MEM and 100 µl of the overlay medium were added in each well, the plate was incubated at 37°C in humidified 5% $CO_2$ incubator for 2-3 days or until the plaques were formed. The medium was removed from the plate, the cells were stained with 1% crystal violet in 10% formalin for at least 30 minutes and the plaques were counted comparing to virus control (no active extracts) and cell control ( no virus). ACV was also used as standard positive control.

## Part VII. Statistical analysis

The chi-square test ($\chi^2$), t - test and ANOVA were used to compare the differences among groups. A p value $\leq .05$ was considered significant.