CHAPTER III

MATERIALS AND METHODS

1. Crude Extraction

Each plant was chopped into small pieces and extracted twice with 95% ethanol for 3-4 days. The extract was filtered through filter paper (Whatman No.1) and the filtrate was evaporated on a water bath at 70°C. In order to increase the solubility, each ethanol extracts were subjected for complex form by polyvinylpyrrolidone (PVP K30, Fluka). These complex were called ethanol fraction.

Table 4. Plant materials and ratio between ethanol extract : PVP used in this study.

No.	Plants	Part of use	Ethanol extract : PVP (w/w)
1.	*Caesalpinia sappan* (ฝาง)	Leaves and stems	1 : 4
2.	*Derris scandens* (เถาวัลย์เปรียง)	Leave and stems	1 : 4
3.	*Duranta repens* (เทียนหยด)	Leaves	1 : 4
4.	*Gossypium herbaceum* (ฝ้าย)	Leaves and stems	1 : 4
5.	*Homalomena aromatica* (เต่าเกียด)	Dry bulb	1 : 4
6.	*Houttuynia cordata* (พลูคาว)	Whole plant	1 : 4
7.	*Litchi chinensis* (ลิ้นจี่)	Leaves	1 : 4
8.	*Loranthus pentandrus* (กาฝากมะม่วง)	Leaves	1 : 6
9.	*Santalum album* (แก่นจันทน์)	Heart wood	1 : 4
10.	*Phyllanthus amarus* (ลูกใต้ใบ)	Whole plant	1 : 5
11.	*Phyllanthus emblica* (มะขามป้อม)	Fruits	1 : 4
12.	*Rhinacanthus nasutus* (ทองพันชั่ง)	Leaves and stems	1 : 4
13.	*Saussurea lappa* (โกฐกระดูก)	Rhizome	1 : 6

The ethanol extract of *Derris scandens*, *Santalum album*, and *Rhinacanthus nasutus* were partitioned with hexane until they were completely separated into two layers. The hexane extract of these medicinal plants were evaporated on a water bath at 50°C. By using the same method, the chloroform extract, ethyl acetate extract and butanol extract were obtained but the butanol extract were evaporated on the water bath at 70 °C instead of 50°C. Each extract was subjected for complex forming with polyvinylpyrrolidone to give hexane, chloroform, ethyl acetate, and butanol fraction, respectively.

Table 5. Ratio between solvent extract : PVP used in this study.

No.	Plants	Fraction	Ethanol extract : PVP (w/w)
1.	*Derris scandens* เถาวัลย์เปรียง	Hexane	1 : 4
		Chloroform	1 : 4
		Ethyl acetate	1 : 4
		Butanol	1 : 4
2.	*Rhinacanthus nasutus* ทองพันชั่ง	Hexane	1 : 12
		Chloroform	1 : 10
		Ethyl acetate	1 : 6
		Butanol	1 : 6
3.	*Santalum album* แก่นจันทน์	Hexane	1 : 10
		Chloroform	1 : 4
		Ethyl acetate	1 : 6
		Butanol	1 : 4

2. Cell culture

A human hepatocellular carcinoma cell line, PLC/PRF/5, was kindly provided from Department of Biotechnology, National Institute of Health, Ministry of Public Health, Nontaburi, Thailand.

One milliliter of PLC/PRF/5 seed cultures were thawed at 37°C in water bath and mixed with 10 ml of 2% fetal bovine serum (FBS, HyClone®, USA) in Minimal essential medium (Eagle's MEM, Nissui, Japan) (maintenance medium) (see appendix). The cell suspension was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and added 15 ml of 10% FBS in MEM (growth medium) to dispersed the accumulated cell. The cell suspension was transfered into a new 75 cm^2 tissue culture flask (Corning®, USA). Then the culture flask was incubated in a 5% CO_2 incubator at 37°C until the cell monolayers were confluent.

3. Cytotoxic assay

The cells were washed twice with phosphate buffered saline solution (PBS) and added 2.5 ml of 0.04% trypsin (GIBCO™) (see appendix). When the cells were detached, trypsin was discarded, and the growth medium was added into the detached cells. The cells were tapped slightly and mixed thoroughly by pipetting up and down. The viable cells were counted by staining with 0.2 % trypan blue (Fluka). The cell suspension was diluted in the growth medium to a concentration of $2x10^5$ cell/ml and distributed 100 µl to each well of a 96-well flat-bottomed tissue culture plate (Costar®, Corning, USA). Then the cells were incubated in the CO_2 incubator at 37°C for 24 hrs. Prepared the serial two-fold dilutions of the test sample by diluted with the maintenance medium. This solution was sterilized by filtration (0.20 µm filter membrane, Minisart®, Sartorius, Germany).

The culture medium in the 96-well plate was discarded after incubation for 24 hours. Then, 100 µl of test samples was added into each well (triplicate). For control well, 100 µl of maintenance medium without test sample was added (triplicate). The cells were incubated in the CO_2 incubator at 37°C for 24 hrs.

To perform 3-(4,5-dimethyl-thiazoyl-2-yl)2,5-diphenyltetrazolium bromide (MTT, USB™, USA) assay, the culture medium was discarded from the well. Fifty microliter of MTT in PBS (2 mg/ml) were added to each well and incubated for 3 hours. After that, 100 µl of dimethyl sulfoxide (DMSO, Merck, Germany) were added to each well and mixed using multichannel micropipette to dissolve the formazan crystal. Absorbance

(optical density; OD) reading on each well was performed at 570 nm (single wavelength) by spectrophotometer (BIO-RAD Model 550 Microplate reader) (Won Ho Kim et al.,1993). Percent viability (% viability) and 50% cytotoxic concentration (CC_{50}) were calculated (see appendix).

4. Anti-HBsAg assay

The cells were washed twice with PBS and added 2.5 ml of 0.04% trypsin. Then the cells were detached. Trypsin was discarded and the growth medium was added. The cells were tapped lightly and mixed thoroughly by a pipette. The viable cells were counted by staining with 0.2% trypan blue. The cell suspension was diluted in the growth medium to a concentration of 10^5 cell/ml and distributed 1 ml to each well of a 24-well plastic plate (Costar®, USA). The cells were then incubated in the CO_2 incubator at 37°C for 24 hrs. The test samples were prepared to serial two-fold dilutions by diluting with the maintenance medium. This solution was sterilized by filtration (0.20 μm filter membrane).

After 24 hours, the culture medium in a 24-well tissue culture plate was discarded. One milliliter of a test sample was added into each well. Each sample was done duplicate. For control well, one milliliter of maintenance medium without test sample was added and was also done duplicate. The cells were incubated in the CO_2 incubator at 37°C for 24 hrs.

To determine an anti-HBsAg activity, the culture medium was harvested from the well. The amounts of HBsAg were assessed by the enzyme linked immunosorbent assay (ELISA)(Murex HBsAg Version 3, Abbott, Thailand). Absorbance reading on each well was performed at 450/620 nm by spectrophotometer (Lucy 2 ELISA reader, Lucysoft). Percent inhibition (% Inhibition) and 50% inhibition concentration (IC_{50}) were calculated (see appendix).

4.1 ELISA (Murex HBsAg Version 3)

4.1.1 Reagent

4.1.1.1 Antibody Coated Wells One plate (GE34/9F80-01) of 96 wells coated with mouse monoclonal antibody to HBsAg.

4.1.1.2 Sample Diluent One bottle containing 16 ml of green/brown buffer containing cetergents and proteins of goat and bovine origin. Mix by inversion before use. Contains 0.05% ProClin 300® preservative.

4.1.1.3 Negative Control One bottle containing 2.5 ml of normal human serum non-reactive for HBsAg and antibodies to HIV-1 and HIV-2, HCV and HTLV-I/II. The serum is diluted in a buffer containing protein of bovine origin. Contains 0.05% Bronidox® preservative.

4.1.1.4 Positive Control One bottle containing 2 ml of inactivated human serum positive for HBsAg but negative for antibodies to HIV-1 and HIV-2 and HCV. The serum is diluted in a buffer containing protein of bovine origin. Contains 0.05% Bronidox® preservative.

4.1.1.5 Conjugate One bottle containing 6 ml (GE34/9F80-01) or two bottles each containing 16 ml (GE36/9F80-05) of horseradish-peroxidase labelled goat antibody to HBsAg in a red buffer containing proteins of bovine and goat origin. Mix by inversion before use. Contains 0.05% ProClin 300® preservative.

4.1.1.6 Substrate Diluent One bottle containing 35 ml of a colorless solution of tri-sodium citrate and hydrogen peroxide.

4.1.1.7 Substrate Concentrate One bottle containing 35 ml of 3,3,5,5-tetramethylbenzidine (TMB) and stabilizers in a pink solution. To prepare the substrate solution add a volume of colorless substrate diluent to an equal volume of pink substrate concentrate in either a clean glass container o a new polystyrene vessel.

4.1.1.8 Wash Fluid One bottle containing 125 ml of 20 times working strength glycine/borate wash fluid. Contains 0.2% Bronidox® preservative.

4.1.2 Procedure

4.1.2.1 Prepare substrate solution and wash fluid.

4.1.2.2 Use only the number of wells required for the test.

4.1.2.3 Add 25 µl of sample diluent to each well.

4.1.2.4 Add 75 µl of sample or controls to the wells.

4.1.2.5 Cover the plate with a lid and incubate for 60 minutes at 37 °C.

4.1.2.6 Add 50 µl of conjugate to each well.

4.1.2.7 Shake the plate using a plate shaker for 10 seconds or manually agitate by gently tapping the sides for 10 seconds.

4.1.2.8 Cover the plate with the lid and incubate for 30 minutes at 37 °C

4.1.2.9 At the end of the incubation time wash the plate 5 times. After washing is completed invert the plate and tap out any residual wash fluid onto absorbent paper.

4.1.2.10 Immediately after washing the plate, add 100 µl substrate solution to each well.

4.1.2.11 Cover the plate with the lid and incubate for 30 minutes at 37 °C while color develops. A purple color should develop in wells containing reactive samples.

4.1.2.12 Add 50 µl stop solution (1 M H_2SO_4 in distilled water) to each well.

4.1.2.13 Within 15 minutes read the absorbance of each well at 450 nm using 620 nm as the reference wavelength.

5. Chromatographic techniques

Butanol extract of *Derris scandens* and hexane extract of *Rhinacanthus nasutus* were partially purified with column chromatographic techniques as describe below.

5.1 Thin Layer Chromatography (TLC)

Technique	:	one way ascending
Stationary phase	:	TLC aluminium sheets Silica gel 60 F_{254} , layer thickness 0.2 mm (Merck)
Solvent systems	:	Various solvent systems depending on material.
Distance	:	6.5 cm
Temperature	:	28 – 35 °C (room temperature)
Detection	:	1) UV light (254 and 365 nm)
		2) 10% sulfuric acid in ethanol and heating at 110 °C

5.2 Column Chromatography (CC)

Column	:	Flat bottom glass column (various diameter)
Stationary phase	:	Silica gel 60 (No.9385, Merck,Germany) particle size 0.040-0.060 mm (230-400 mesh ASTM)
Packing method	:	Wet packing
Sample loading	:	The sample was dissolved in a small amount of the eluent, then loaded on the top of the column.
Technique	:	Short column chromatography
Solvent system	:	Various solvent systems depending on materials.
Detection	:	Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm. The TLC plate was then sprayed with 10% sulfuric acid in ethanol and heated at 110 °C

Hexane extract of *Rhinacanthus nasutus* was partially purified with column chromatographic techniques as describe below.

5.3 Gel Filtration Chromatography

Gel filter	:	Sephadex LH-20 (Sigma)
Packing method	:	Gel filter was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.
Solvent system	:	Methanol
Sample loading	:	The sample was dissolved in a small volume of the eluent and applied on top of the column.