CHAPTER III

MATERIALS AND METHODS

Materials

1. *Stephania venosa* (Bl.) Spreng

The water and the ethanol extracts of *S.venosa* tuber were kindly provided by Assistant Professor Dr. Pathama Leewanich, Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Thailand. Infrared spectra (IR) and ^{1}H Nuclear Magnetic Resonance (NMR) spectra of both extracts of *S.venosa* were kindly identified by Associate Professor Dr. Supaluk Prachayasittikul, Department of Chemistry, Faculty of Science, Srinakharinwirot University, Thailand (see appendix A). The water extract was kept at room temperature in a dessicator, while the ethanol extract was stored at 4 $^{\circ}$C. The water extract was dissolved in double distilled water, while the ethanol extract was dissolved in DMSO. The stock solutions of both extracts were prepared at the concentration of 200 mg/ml for cytotoxic assay and 100 mg/ml for proliferation and apoptotic assay. The stock solutions were filtered through a 0.45 μ membrane and stored at -20°C.

2. **Samples**

2.1 Whole blood

Ten milliliters of heparinized whole blood sample were taken from each healthy female blood donor aged between 30 - 50 years who attended the National Blood Bank, Thai Red Cross Society for blood donation. These volunteers should neither smoke nor drink alcohol and should not take any medications during 14 days prior to their blood donation. The blood samples of these subjects were used for preparing PBMCs in this study.

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2.2 Cell line

Jurkat cells clone E6-1 (Catalog No. TIB-152) was purchased from ATCC. Cells were grown in RPMI 1640 medium containing 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin in a humidified, 5 % CO_2 incubator at 37^0C. The confluent cells in a 25 cm^2 flask were subcultured every 2-3 days.

3. Equipments and Laboratory supplies

Pipette tip: 1000, 200, 20 μl (Molecular Bio- products, USA)

Aluminum foils (Diamond, USA)

Autoclave tape (3M, USA)

Beakers: 50 ml, 1,000 ml (Pyrex, USA)

Cylinders (Pyrex, USA)

Disposable gloves (Latex, USA)

Glass pipettes: 1 ml, 5 ml, 1 ml (Witeg, Germany)

Microscope glass cover slips (Chance, England)

96 and 24 multiwell plates (Nunc, Denmark)

Parafilm (American National Can, USA)

Pipette (Falcon, USA)

Reagent bottles: 50,100,250,500 and 1000 ml (Duran, Germany)

Sterile membrane filters (Whatman, Japan)

Sterile polypropylene centrifuge tube: 15 ml, 50 ml. (Nunc, Denmark)

T- 25 and T-75 Tissue Culture flasks (Nunc, Denmark)

Cryotube (Corning, USA)

Sterile Millipore 0.22 μM, 0.45 μM (Millex-GP, USA)

Eppendorf tube (Corning,USA)

Autoclave (Hirayama,Japan)

Autopipette (Gilson, France)

Biohazard Lamina- flow hood (Science, Germany)

ELISA Microplate Reader (Multiskan EX,Germany)

Freezer – 80° C (Sanyo,Japan)

Hemocytometer (Boeco, Germany)

Light microscope (Olympus, Japan)

pH meter SA 520 (Orian, USA)

Refrigerator 4°C, - 20°C (Sanyo,Japan)

Timer

Vacuum pump

Vortex (Labnet,USA)

Flow cytometer (Beckman coulter Epics XL, USA)

Spectrophotometer (V-530 UV/VIS Jasco, Japan)

4. Reagents

4.1 Reagents

Absolute ethanol (Merck, Germany)

Acetic acid (Merck, Germany)

Alamar blue (Biosource,USA)

Doxorubicin (Hearlern, Netherlands)

Etoposide (Pharmacia, Australia)

Sodium hypochloride (Clorox, USA)

Fetal bovine serum (Hyclone, USA)

Dimethyl sulfloxide (Sigma, USA)

Hanks' balanced salts solution (HBSS) Powder (Gibco, Germany)

Heparin (LEO, Denmark)

Histopaque® -1077 (Sigma, Germany)

Hydrochloric acid: (Merck, Germany)

L- Glutamine (Gibco, Germany)

Phytohemagglutinin (Sigma, USA)

Pokeweed mitogen (Sigma, USA)

Staphylococcal protein A (Fluka, Switzerland)

Potassium chloride (Merck, Germany)

Potassium hydrogen phosphate (Merck, Germany)

Sodium chloride (Sigma, USA)

Sodium hydroxide (Merck, Germany)

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Di- Sodium hydrogen phosphate monobasic (Merck, Germany)

Sodium bicarbonate (Baker, USA)

Glucose (Merck, Germany)

HEPES (Hyclone, USA)

Penicillin/Streptomycin (Hyclone, USA)

RPMI1640 (Gibco, Germany) with phenol red

RPMI1640 (Sigma, USA) with out phenol red

Calcium cholide (Merck, Germany)

0.4 % Trypan blue dye (Sigma, USA)

MTT (Sigma, USA)

5.2 Reagent kit

Annexin V Apoptosis Detection Kit (Santa Cruz Biotecnology, USA)

Methods

1. The PBMC preparation

Using Histopaque-1077, PBMCS were isolated from 10 ml whole blood specimen according to the following protocol:

1. Pipette 5 ml of Histopaque-1077 at room temperature into each 15 ml polypropylenes centrifuge tubes.

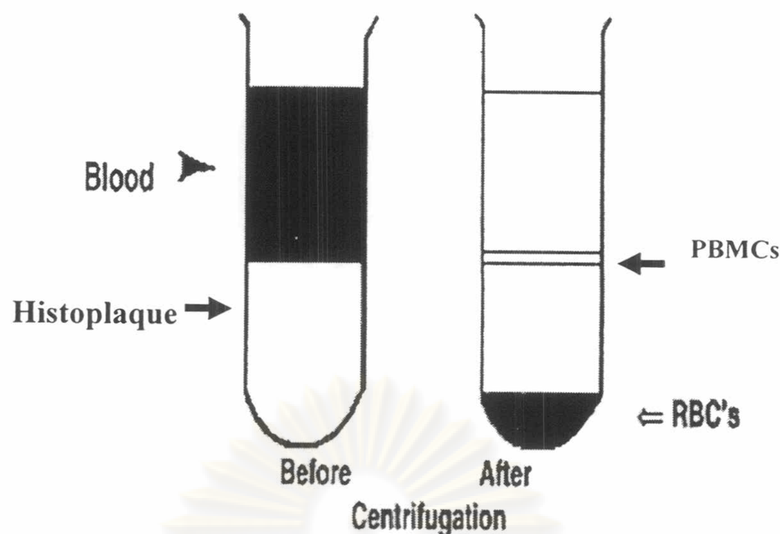
2. Mix the blood 1:1 with Hanks' Balanced Salts Solution (HBSS) containing 2 μl/ml heparin at room temperature.

3. Layer 9 ml of the blood/ HBSS mixture onto the top of histoplaque in each tube. Be careful not to mix the two parts together, and cap the tubes tightly.

4. Centrifuge the tubes at 400 g for 30 minutes at room temperature.

5. Carefully remove the top plasma layer from each tube without disturbing the interface. Discard this layer into a container of sodium hypochloride.

6. Collect the interface (white ring of PBMCs) from each tube. (See the following picture)



7. Immediately transfer the PBMCs to a new sterile polypropylene 15 ml centrifuge tube. Wash the cells twice with 12.5 ml HBSS (+2 μl/ml heparin + 1% fetal bovine serum). Collect the pellet by centrifugation at 250 g for 10 min at room temperature.

8. Resuspend the pellet in 5 ml complete RPMI 1640 medium (RPMI 1640 media + 10 % fetal bovine serum + L- glutamine 0.5 %).

9. Count the PBMCs on hemocytometer and adjust to the required density with complete RPMI medium.

2. Cytotoxicity assay

The cytotoxic effect of the water and the ethanol extracts of *S.venosa* tuber on normal human PBMCs was determined by the study of cell viability using the trypan blue dye exclusion and alamarBlue reduction assay.

2.1 Trypan blue dye exclusion assay

The assay was performed in triplication as follows:

2.1.1 An aliquot of 190 μl PBMCs and Jurkat cells suspended in completed RPMI1640 medium at the density of 5 x 10^5 cells/ml was pipetted into each well of a 96-well plate.

2.1.2 Subsequently, 10 μl of the water and the ethanol extracts of *S.venosa* tuber were added to give the final concentration of 1, 3, 10, 30, 100, 300 and 1000

μg/ml. Medium and 0.1 % DMSO were used as the negative controls while 1.5 μg/ml doxorubicin was used as positive control, respectively.

2.1.3 The plate was incubated at 37 °C, 97% humidity, 5%CO_2 for 48 hrs.

2.1.4 The cell viability was evaluated by trypan blue dye exclusion assay as follows.

- Take a clean hemocytometer slide and fix the coverslip in place.
- Aliquot 50 μl of the suspended cells in each well into an eppendorf tube.
- Add 50 μl of 0.4 % trypan blue solution
- Leave the mixture for 4 minute. (Do not leave them for a longer period of time, or else viable cells will deteriorate and take up the dye).
- Pipette an aliquot of the stained cells on the hemocytometer, place the slide on the microscope. The number of unstained (viable) and stained (dead) cells were counted separately.
- Calculate the percentage of cell viability and the concentration of the extracts, which gave 50% inhibition of cell growth (IC_{50}).

2.1.5 The IC_{50} of *S.venosa* cytotoxicity was calculated.

$$\%\text{ cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

2.2 AlamarBlue reduction assay

2.2.1 An aliquot of 190 μl suspended PBMCs and Jurkat celsl in completed RPMI1640 medium at the density of 5×10^5 cells/ml was pipetted into each well of a 96-well plate.

2.2.2 Subsequently, 10 μl of the water and the ethanol extracts of *S.venosa* tuber were added to give the final concentrations of 1, 3, 10, 30, 100, 300 and 1000 μg/ml. Medium and 0.1 % DMSO were used as negative controls while 1.5 μg/ml doxorubicin was used as positive control, respectively.

2.2.3 After the incubation period of 48 hrs, 15 μl of alamarBlue was added to each well and the plate was re-incubated for 24 hrs. The plate was then measured

the absorbance at 570 nm (reduced form) and 600 nm (oxidized form) using microplate reader. Specific absorbance (specific OD), obtained by subtracting the absorbance at 600 nm from that of 570 nm, was used in the calculation for % cytotoxicity of the extracts.

$$\% \text{ cytotoxicity} = \left(\frac{\text{specific OD (control)} - \text{specific OD (sample)}}{\text{specific OD (control)}} \right) \times 100$$

3. **Proliferation assay determined by using MTT reduction colorimetric assay**

The water and the ethanol extract of *S.venosa* tuber at the 0.5 IC_{50}, IC_{50} and $2xIC_{50}$ from cytotoxic assay were used in the study.

1. An aliquot 90 µl of PBMCs in completed RPMI 1640 medium at the density 1×10^6 cells/ml was pipetted into each well of a 96-well plate.

2. The plate was incubated overnight at 37 °C, 97% humidity, 5%CO_2

3. Added 10 µl of the final concentration 10 µg/ml PHA, 50 µg/ml PWM or 25 µg/ml SPA into each well.

5. Subsequently, 10 µl of the water and the ethanol extracts of *S.venosa* tuber were added to give the final concentrations equivalent to the IC_{50}, two fold lower and two fold higher concentrations (100, 200 and 400 µg/ml of the water extract; 20,40 and 80 µg/ml of the ethanol extract). Medium and 0.1 % DMSO were used as the negative control well while PHA, SPA and PWM were used as positive controls, respectively.

6. The plate was incubated for 48 hrs at 37 °C, 97% humidity, 5%CO_2.

7. Ten µl of MTT solution (5 mg/ml in PBS) was added in each well.

8. The plate was incubated for 3 hrs at 37 °C, 97% humidity, 5%CO_2.

9. Then the plate was centrifuged at 2,000 rpm 25 °C for 5 minutes.

10. The supernatant was removed from each well and 100 µl of DMSO was added to solubilize the formazan crystal.

11. The plate was incubated in the dark at room temperature for 3 hr.

12. The plate was then measured the absorbance at 570 nm and 650 nm (using microplate reader). Specific absorbance (Specific OD), obtained by subtracting

the absorbance at 650 nm from that of 570 nm, was used in the calculation for % antiproliferative.

$$\% \text{ antiproliferative} = \left(\frac{\text{specific OD(control)} - \text{specific OD(sample)}}{\text{specific OD (control)}} \right) \times 100$$

5. Apoptotic activity by annexin V staining assay

1. An aliquot of 960 μl PBMCs suspended in complete RPMI1640 medium at the density 3×10^5 cells/ml was pipetted into each well of a 24-well plate.

2. Subsequently, 40 μl of the water and the ethanol extracts of *S.venosa* tuber were added to give the final concentrations equivalent to the IC_{50} , two fold lower and two fold higher concentrations (100,200 and 400 μg/ml of the water extract; 20,40, 80 and 160 μg/ml of the ethanol extract). Medium and 0.1 % DMSO were used as the negative control well while 10 μg/ml of etoposide was used as positive control, respectively.

3. The plate was incubated for 12 hr at 37 °C, 97% humidity, 5%CO_2.

4. The PBMCs in each well were collected to 1 ml microcentrifuge tube and centrifuged at 13,400 rpm 25 °C for 1 minute.

5. The supernatant was removed. The PBMCs were washed with cold PBS 1 ml and centrifuged at 13,400 rpm 25 °C for 1 minute twice.

6. The supernatant was removed and the cell pellet was resuspended in 100 μl of 1x assay buffer and transferred to flow cytometry tube.

7. One μl of PI (0.05 μg/ml) and 0.5 μl of Annexin V-FITC (0.2 μg/ml) were added in the tube.

8. The tube was incubated in the dark at room temperature for 15 minutes.

9. Subsequently, 400 μl of 1x assay buffer was added and cells were analyzed immediately by flow cytometry. For each sample 10,000 cells were measures compared the control group.

10. Assessment of apoptosis and necrosis

Phosphatidylserine externalization of apoptosis was determined by two-color flow cytometric analysis of Annexin V-FITC binding and PI uptake using FACS

analysis. The proportions of Annexin V-FITC$^-$/PI$^-$ cells corresponds to living cells, Annexin V-FITC$^+$/PI$^-$ cells corresponds to early apoptosis, Annexin V-FITC$^+$/PI$^+$ cells corresponds to late apoptosis or secondary necrosis and that of PI$^+$ cells corresponds to necrosis *(59-61)*.

7. Statistical analysis

All data were presented as means and standard error of means (mean $\pm$ SEM). One way analysis of variance (one-way ANOVA) was used to compare the significance between the control and the treatment groups. The p-value less than 0.05 was set for the significant difference.

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