CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Scaphium scaphigerum* (G.Don) Schott & Endl

The water extract of *S.scaphigerum* fruits was prepared and identified by Thailand Institute of Scientific and Technological Research. The extract was lyophilized and stored in a desiccator at room temperature.

3.1.2 Cell culture

- Human peripheral blood mononuclear cells: PBMCs

Cells were prepared from healthy male blood donors and maintained in 96-well plate in complete RPMI 1640 medium at 37 °C, 97% humidity, 5%CO_2.

- Murine macrophage-like cell line: J774A.1 (ATCC Number TIB-67)

Cells were maintained in 25-cm^2 cultured flasks in complete DMEM at 37 °C, 97% humidity, 5%CO_2. The cells were subcultured three times weekly.

3.1.3 Instruments

The following instruments were used in this study.

aluminum foils (Diamond, USA), autoclave tape (3M, USA), autoclave (Hirayama, Japan), autopipette (Gilson, France), beakers: 50 ml, 1,000 ml (Pyrex, USA), biohazard lamina-flow hood (Science, Gelman), cell harvester (Nunc, Denmark), centrifuge, cylinders (Pyrex, USA), disposable gloves (Latex, USA), eppendorf (Corning, USA),

ELISA microplate reader (Multiskan EX, Germany), Flow cytometer (Becton Dickinson, USA), freezer – 80° C (Sanyo, Japan), glass pipettes: 1 ml, 5 ml, 10 ml (Witeg, Germany), hemocytometer (Boeco, Germany), incubator, light microscope (Olympus, Japan), microscope glass cover slips (Chance, England), 96 and 24 multi-well plates (Nunc, Denmark), parafilm (American National Can, USA), pH meter SA 520 (Orian, USA), pipette (Falcon, USA), pipette tip: 1000, 200, 20 µl (Molecular Bio- products, USA), plastic tube (Becton Dickinson), reagent bottles: 50,100,250,500 and 1000 ml (Duran, Germany), scintillation counter, spectrophotometer (V-530 UV/VIS Jasco, Japan), sterile membrane filters (Whatman, Japan), sterile polypropylene centrifuge tube: 15 ml, 50 ml. (Nunc, Denmark), sterile millipore 0.22 µM, 0.45 µM (Millex-GP, USA), refrigerator 4°C, -20°C (Sanyo, Japan), T- 25 and T-75 Tissue Culture flasks (Nunc, Denmark), vacuum pump, vortex mixer (Labnet, USA)

3.1.4 Reagents

The following reagents were purchased from any place using in this study.

- Reagents

absolute ethanol (Merck, Germany), calcium cholide (Merck, Germany), dimethyl sulfloxide (Sigma, USA), dulbecco's modified eagle's medium (DMEM) (Sigma, USA), fetal bovine serum (Hyclone, USA), glucose (Merck, Germany), [^{3}H] – thymidine, Hanks' balanced salts solution (HBSS) powder (Gibco, Germany), heparin (LEO, Denmark), HEPES (Hyclone,USA), Histopaque® -1077 (Sigma, Germany), hydrochloric acid: (Merck, Germany), L- glutamine (Gibco, Germany), lipopolysaccharide (Sigma, USA), methanol (Merck, Germany), MTT (Sigma, USA), nitro blue tetrazolium (NBT) (Sigma, USA), penicillin/streptomycin (Hyclone, USA), phytohemagglutinin (PHA) (Sigma, USA), potassium chloride (Merck, Germany), potassium hydrogen phosphate (Merck, Germany), potassium hydroxide (Merck, Germany), RPMI1640 medium (Sigma, USA), Scintillation fluid, sodium chloride (Sigma, USA), sodium hydroxide (Merck, Germany), di-sodium hydrogen phosphate monobasic (Merck, Germany), sodium

bicarbonate (Baker, USA), sodium hypochloride (Clorox, USA), 0.4 % Trypan blue dye (Sigma, USA), zymosan A (from *Saccharomyces cerevisiae*) (Sigma, USA)

- Reagent kit

FastImmune CD69 PE/CD3 PerCP kit (Becton Dickinson, USA), nitric oxide assay kit (Promega, USA)

3.2 Methods

3.2.1 Preparation of *Scaphium scaphigerum* fruits extract stock solution

Scaphium scaphigerum fruit extract was dissolved in double distilled water at the concentration of 10 mg/ml. and filtered through a 0.45 μm filter as the sterile stock solution. The solution was stored at -20°c until used.

3.2.2 Preparation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were prepared from healthy male blood donors from the National Blood Bank, Thai Red Cross Society for blood donation. The donors were aged between 20 - 35 years. Ten milliliter of their blood was withdrawn and transferred into a tube containing 1% heparin and mixed. Using Histopaque – 1077, human PBMCs were isolated from whole blood sample according to the following procedures:

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

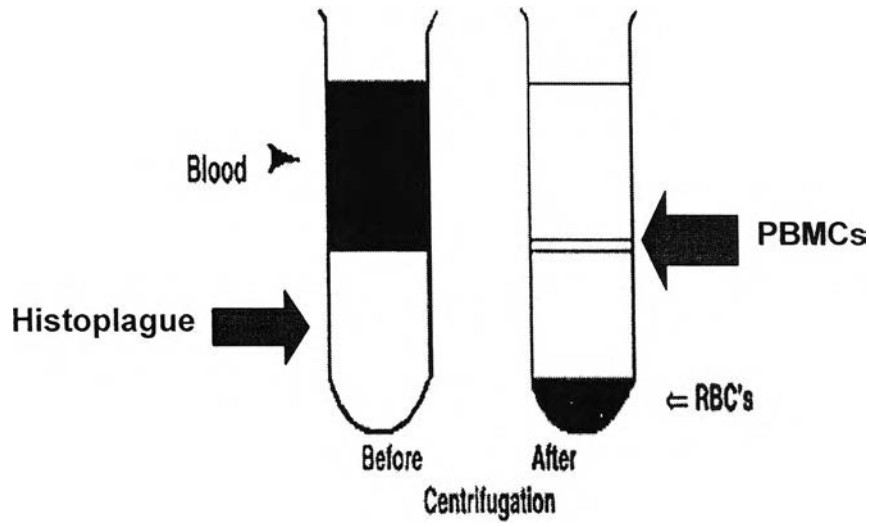
2. Mix the blood 1:1 with Hanks' balanced salts solution (HBSS) containing 2 μl/ml heparin at room temperature.

3. Layer 9 ml of the blood/ HBSS mixture onto the top of Histopaque 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 layer from each tube without disturbing the interface.

6. Collect cells at the interface (buffy layer containing 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. Remove the supernatant and resuspend the pellet in 5 ml complete RPMI medium (RPMI 1640 media + 10 % fetal bovine serum + L- gutamine 0.5 %).

9. Determine viable cells by using trypan blue exclusion and count the human PBMCs on hemocytometer and adjust to the required density with complete RPMI 1640 medium.

The isolated PBMCs with cell viability more than 90 % at density of $1x10^6$ cells per ml in complete RPMI 1640 medium were used in this study.

3.2.3 Mitogenic activity assay

The mitogenic effect of the *S. scaphigerum* fruit extract on human PBMCs was determined by the MTT and tritiated thymidine incorporation assay.

- MTT assay

The *S. scaphigerum* fruit extract at the final concentrations of 10, 30, 100, 300 and 500 μg/ml were used in this assay according to the following procedures:

1. Pipette 90 μl of PBMCs at the density of $1x10^6$cells/ml in completed RPMI 1640 medium into each well of a 96-well plate.

2. Incubate the cells overnight at 37 °C, 97% humidity, 5%CO_2.

3. Add 10 μl of each concentration of *S. scaphigerum* fruit extract into each well. Completed RPMI 1640 medium and 10 μg/ml PHA were used as the negative and positive controls, respectively.

4. Incubate cells for 48 hour at 37°C, 97% humidity, 5%CO_2.

5. Add 10 μl of MTT solution (5 mg/ml in PBS) in each well.

6. Incubate the cells for 3 hr at 37 °C, 97% humidity, 5%CO_2.

7. Centrifuge the plate at 2,000 rpm 25 °C for 5 minutes.

8. Remove the supernatant from each well and add 100 μl of DMSO to solubilize the formazan crystal.

9. Incubate the plate in the dark room at temperature for 3 hr.

10. Measure the optical density (OD) of the plate at 570 nm and 650 nm by 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 %stimulation.

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

- Tritiated thymidine incorporation assay

From MTT assay, the three concentrations of the *S.scaphigerum* fruit extract were used in this assay. The procedures of the assay were as follow:

1. Aliquot 180 μl of PBMCs at the density of 1x 10^6cells/ml in completed RPMI 1640 medium into each well of a 96-well plate.

2. Incubate cells overnight at 37 °C, 97% humidity, 5%CO_2.

3. Add 20 μl of each concentration of the extract into each well. Completed RPMI 1640 medium and 10 μg/ml PHA were used as the negative and positive controls, respectively.

4. Incubate the cells for 42 hour at 37°C, 97% humidity, 5%CO_2.

5. Add 25 μl of [^{3}H]-thymidine (20 μCi / well in RPMI 1640 medium) into each well and incubate the plate for 6 hour.

7. Harvest the cells on glass fiber filters

8. Transfer each filters into each vial containing 2 ml scintillation fluid.

9. Count the radioactivity in each vial as counts per minute (CPM) with β-scintillation counter and calculate the %stimulation by the following equation:

$$\% \text{ stimulation} = \left(\frac{\text{CPM (sample)} - \text{CPM (control)}}{\text{CPM (control)}} \right) \times 100$$

3.2.4 T cell activation assay

The effect of the *S. scaphigerum* fruit extract on T cell activation was determined by detecting CD69 expression detection on $CD3^+$ T cells.

- CD69 detection

Three concentrations of the extract were used in this assay. The procedures of the assay were as follow:

1. Aliquot 500 µl of PBMCs at the density of 1x 10^6cells/ml in completed RPMI 1640 medium into each tube.

2. Incubated the cells overnight at 37 °C, 97% humidity, 5%CO_2.

3. Add 50 µl of *S. scaphigerum* fruit extract at each concentration of the extract into each tube. Completed RPMI 1640 medium and 10 µg/ml PHA are used as the negative and positive controls, respectively.

4. Incubated cells for 24 hour at 37°C, 97% humidity, 5%CO_2

5. Centrifuge the tubes at 1500 rpm for 5 minutes at 4 °C.

6. Carefully remove supernatant and add 5 µl of anti CD3 perCP/CD69 PE into each tube.

7. Mix and incubate for 20 minutes at 4 °C.

8. Add 2 ml of washing buffer (PBS containing bovine serum and sodium azide) into each tube and centrifuge at 1500 rpm for 5 minutes at 4 °C.

9. Add 500 µl of 1% formaldehyde.

10. Detect CD69 and CD3 on the PBMCs by using Flow cytometer.

3.2.5 Macrophage function assay

Murine macrophages, J774A.1, were used for determining the effect of *S. scaphigerum* fruit extract on the nonspecific immune response. Effects of the extract on phagocytosis and nitric oxide production of these cells were evaluated by phagocytosis zymosan and Griess reaction assay, respectively.

- Zymosan phagocytosis assay

Three concentrations of the extract were used in this assay. The procedures of the assay were as follow:

1. Aliquot 180 µl of J774A.1 cells at the density of $5x10^5$cells/ml in completed DMEM into each well of a 96-well plate.

2. Incubate cells overnight at 37 °C, 97% humidity, 5%CO_2.

3. Add 20 µl of *S. scaphigerum* fruit extract at each concentration into each well. Completed RPMI 1640 medium and 5 µg/ml LPS were use as the negative and positive controls, respectively.

4. Incubate the plate for 24 hour at 37°C, 97% humidity, 5%CO_2.

5. Carefully remove supernatant and wash twice with DMEM.

6. Add 800 µg/ml of formazan and 600 µg/ml of NBT in each well and incubate further for 60 minutes at 37°C, 97% humidity, 5%CO_2.

7. Wash the cells with 200 µl methanol three times.

8. Add 120 µl of 2M KOH and fix the cells with 140 µl of DMSO.

9. Measure the optical density of the plate at 570 nm by microplate reader. The percentage of NBT reduction or % phagocytosis was calculated by the following equation:

$$\% \text{ phagocytosis} = \left(\frac{OD_{570nm}\,(\text{sample}) - OD_{570nm}\,(\text{control})}{OD_{570nm}\,(\text{control})} \right) \times 100$$

- Griess reaction assay

Three concentrations of the extract were used in this assay. The procedures of the assay were as follow:

1. Aliquot 500 µl of J774A.1 cells at the density of $5x10^5$cells/ml in completed DMEM into each well of a 24-well plate.

2. Incubated the cells overnight at 37 °C, 97% humidity, 5%CO_2

3. Add 50 µl of *S. scaphigerum* fruit extract at each concentration into each well. Completed DMEM and 1 µg/ml LPS were used as the negative control and positive control, respectively.

4. Incubated the plate for 24 hour at 37°C, 97% humidity, 5%CO_2

5. Carefully remove 150 µl of supernatant into each well of 96- well plate.

6. Add 20µl of the sulfanilamide solution into each well of sample and wells containing the dilution series for the nitrite standard reference curve.

7. Incubate the plate in the dark for 10 minutes at room temperature.

8. Add 20µl of the NED solution to each well.

9. Incubate the plate in the dark for 10 minutes room temperature.

10. Measure the optical density of the plate at 550 nm by microplate reader.

11. Determine nitrite concentration from nitrite standard reference curve.

Preparation of a nitrite standard reference curve

1. Prepare 100 ml of a 100 µM nitrite solution in ultrapured water as stock solution.

2. Add 150 µl of the nitrite solution in serial dilutions (0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µM.) into each well.

3. Follow the procedures (6) to (10) above.

5. Plot the standard curve of the nitrite reference solutions.

3.2.6 Data analysis

All data were presented as mean values ± S.E.M. Difference among means were analyzed by using one – way analysis of variance (ANOVA). All statistical analysis was performed according to the statistic program, SPSS. A value of $p < 0.05$ was considered to be significant.