CHAPTER III

MATERIALS AND METHODS

Materials

Plant extract

The stems of *D. reticulata* collected from Erawan waterfall area, Kanchanaburi, Thailand. This plant was identified by Associate Professor Dr. Nijsiri Raungrungsi, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Air-dried and grounded stems of *D. reticulata* were extracted with dichloromethane and then with absolute ethanol. Ethanol was evaporated and the extract was stored in the solid form at 4°C. The stock solution of ethanol extract was prepared by dissolving in dimethylsulfoxide (DMSO) and stored at -20°C until used. It was diluted to optimal concentrations at the 0.2% DMSO constant final concentration.

Cell culture

J774A.1 cells were murine macrophages obtained from the American Type Culture Collection (ATCC). The cells were maintained in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 97 % humidity, 5% CO_2. They were subcultured three timed weekly during use.

Equipments and Instruments

The followings equipments and instruments were used in this study; CO_2 incubator (Thermo, USA), sterile laminar flow hood (ESSCO, USA), microplate reader (Labsystem, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), analytical balances (Satorius, Germany and Mettler Toledo, Switzerland), autopipettes (Gilson, USA), 24-well tissue culture plates

(Corning, USA), 96-well tissue culture plates (Corning, USA), cell scrappers (Greiner, UK), eppeddorf (corning, USA), 15 and 30 ml sterile polypropylene centrifuge tubes, T25 tissue culture flasks (Corning, USA)

Chemicals and reagents

The following reagents were used in this study: nitroblue tetrazolium (NBT) (Sigma, USA), lipopolysaccharide (LPS) (Sigma, USA), rezasurin (Sigma, USA), zymosan A from *Saccharomyces cervisiae* (Sigma, USA), DEPC (Molekula, UK), dimethyl sulfloxide (DMSO) (Sigma, USA), ImProm-IITM Reverse Transcription system (Promega, USA), nitric oxide assay kit (Promega, USA), Taq polymerase (Invitrogen, UK) , trypan blue dye (Sigma, USA), TRiZol reagent (Invitrogen, UK), dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), RPMI 1640 medium (sigma, USA), sodium bicarbonate (Baker, USA)

Methods

1. Determination of nitric oxide production

1. Incubate 135 µl/well of $1x10^5$ cells/ml J774A.1 cells in a 96-well plate for 24 h at 37°C, 97% humidity, 5%CO_2.
2. Add 15 µl/well of 6.25-100 µg/ml the ethanol extract, and then incubate for 24 h at 37°C.
3. Stimulate with 17 µl of 100 ng /ml LPS and further incubate for 24 h. 0.2% DMSO and 10 µM dexamethasone were used as the negative and the positive control, respectively.
4. Collect the supernatant for determining NO production in nitrite form by Griess reaction assay, while the treated cells were determined their viability by resazurin reduction assay.

   4.1 Griess reaction assay [70].

- Aliquot 100 µl of the supernatant from each well into 96-well plate.
- Add 20 µl sulfanilamide solution into each well and incubate for 10 min at room temperature in the dark.

- Add 20 µl NED solution to each well and further incubate for 10 min at room temperature in the dark.
- Determine the absorbance at 540 nm of the reaction mixture in each well by microplate reader
- Calculate the concentration of NO production from sodium nitrite standard curve using 1.56-100 µM standard nitrite solution in two-fold dilution.
- Calculate the percentage of NO inhibition by comparing LPS-stimulated condition using the following equation.

$$\% \text{ NO inhibition} = \left[\frac{\text{NO conc. of negative control} - \text{NO conc. of treatment}}{\text{NO conc. of negative control}} \right] X\ 100$$

4.2 Determination of cell viability

The viability of the cell treated with the ethanol extract were determined by resazurin reduction assay by the following procedure,

- Remove the supernatant of the treated cells.
- Add 100 µl fresh complete DMEM medium containing resazurin 50 µg/ml.
- Incubate for 2 h at 37°C, 97% humidity, 5% CO_2.

-Determine the reduction of resazurin to resorufin production in viable cells by measuring the absorbance of the reaction at 570 and 600 nm using a microplate reader.

- Calculate the percentage of viable cells by comparing with LPS-activated condition using the following formula;

$$\% \text{ viability} = \left[\frac{(OD_{570} - D_{600})_{sample}}{(OD_{570} - D_{600})_{LPS}} \right] X\ 100$$

2. Determination of phagocytic activity

The effect of the ethanol extract on phagocytosis of LPS-activated J774A.1 cells was determined by zymosan phagocytosis assay as in the following procedure [71];

1. Incubate 135 µl/well of $1x10^5$ cell/ml J774A.1 cells in a 96-well plate for 24 h at 37C°, 97% humidity, 5%CO_2.
2. Add 15 µl/well the ethanol extract to the final concentrations 25, 50 and 100 µg/ml and incubate for 24 h at 37°C, 97% humidity, 5%CO_2.
3. Stimulate the cells with 100 ng /ml LPS and incubate for 24 h at 37°C.
4. Carefully remove supernatant and wash the cells twice with DMEM.
5. Add 800 µg/ml of zymosan and 600 µg/ml of NBT in each well and further incubate for 1 h at 37°C, 97% humidity, 5%CO_2.
6. Wash the cells with 200 µl methanol three times.
7. Add 120 µl 2M KOH and 140 µl DMSO.
8. Determine the oxidize NBT production by measuring the absorbance at 570 nm using microplate reader
9. Calculate the percentage of phagocytosis inhibition of the ethanol extract on LPS-activated J774A.1 cells by the following equation

$$\% \text{ Phagocytosis inhibition} = \left(\frac{OD_{control} - OD_{sample}}{OD_{control}} \right) X\ 100$$

Where $OD_{control}$ = absorbance of LPS-activated condition without the extract
OD_{sample} = absorbance of LPS-activated condition with the extract

3. Determination of mRNA expression of cytokines, iNOS and COX-2

The effect of the ethanol extract on the mRNA expression of proinflammatory cytokines (TNF-α, IL-1, and IL-6), iNOS and COX-2 in LPS-activated J774A.1 cells was determined as in the following procedure;

3.1 Treat J774A.1 cells with the ethanol extract

- Incubate 500 µl/well of $1x10^5$ cells/ml J774A.1 cells in a 24-well plate for 24 h at 37°C, 97% humidity, 5%CO_2.

- add 45 µl/well of 25-100 µg/ml the ethanol extract and further incubate for 24 h. 0.2% DMSO and 10 µM dexamethasone were used as the negative and the positive control, respectively.
- Stimulate the cells with 100 ng /ml LPS for 4 h at 37°C for determination of pro-inflammatory cytokine expression and for 24 h at 37°C for determination of iNOS and COX-2 expression.
- Remove the supernatant from the treated cells

3.2 Isolate total RNA from the treated cells

- Lyses and homogenize the treated cells in each well with 1 ml Trizol® reagent.
- Incubate for 5 min at room temperature.
- Transfer the homogenized samples to eppendorf tubes.
- Add 200 µl chloroform into each tube, vigorously shake by hand for 15 seconds, incubated for 3 min at room temperature, and centrifuge at 12,000 g for 15 min at 4°C.
- Carefully pipette the aqueous phase from each tube into a new eppendorf tube.
- Add 0.5 ml isopropanol into each tube, mix, and incubate for 30 min at 4^0C.
- Separate the RNA pellets by centrifugation 12,000 g for 10 min at 4 °C.
- Remove the supernatant and wash the pellets twice with 1 ml of 75% ethanol by centrifugation at 7,500g for 5 min. at 4°C.
- Air dry the pellet and then dissolve it in RNase free-water.
- Determine the total RNA concentration and contamination of each tube by measuring its absorbance at 260 nm and 280 nm by Nanodrop.
- Store the total RNA samples at -20°C until used.

3.3 Synthesis cDNA by reverse transcription

- Mix 1.5 µg total RNA of each sample with 1 µl oligo dT_{15} primer in nuclease-free water in a 0.2 ml eppendorf tube.
- Heat the tubes at 70°C for 5 min and then immediately keep the tubes on ice for 5 min.

- Prepare reverse transcription mixture solution containing 25 mM $MgCl_2$, mixed dNTP, Ribonuclease Inhibitor, and reverse transcriptase.
- Add 15 μl of the mixture solution into each tube.
- Generate cDNA by putting the tubes in a thermocycler machine using the following conditions; 25 °C for 5 min, then 42°C for 1 h and 30 min, and finally 70°C for 15 min.
- Store the cDNA samples at -20 °C until used.

3.4 Amplify cDNA's of pro-inflammatory cytokines, iNOS and COX-2 by PCR

- Mix 1 μl of cDNA of each sample with 24 μl PCR reaction mixture containing primer, mixed dNTP, Taq polymerase in PCR buffer in a 0.2 ml PCR tube.
- Perform the PCR in the thermocycler machine using the following conditions; 94°C for 2 min., followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72 °C for 60 sec, and final extension at 72 °C for 7 min.
- Mix 6 μl PCR product with 2 μl loading dye and run on 1.5% agarose gel electrophoresis at 100 volt for 60 min in TBE buffer.
- Stain the agarose gel with 0.5 μg/ml ethidium bromide in TBE buffer for 5 min and de-stained with the TBE buffer for 20 min.
- Identify and analyze the density of PCR products using a gel-documentation.

Statistical analysis

Data were expressed as mean ± standard error (mean ± S.E.). One-way ANOVA followed by Turkey's post hoc test was used for comparison between control and treatment groups. All statistical analysis was performed according to the statistic program, SPSS version 17. P-value < 0.05 was considered statistically significant difference.