CHAPTER III

MATERIALS AND METHODS

1.Materials

1.1 Extracts of *Glycosmis parva*

The hexane, ethyl acetate, butanol and water extracts of branches and leafs from *Glycosmis parva* were prepared and identified by Associate Professor Dr. Nijsiri Ruangrungsi and Mr. Chaisak Chansriniyom Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The TLC fingerprints of the extracts used in study are in Appendix A-1 which represent G1, G2, G5 and G6 respectively.

All extracts, except the water extracts, were dissolved in DMSO at 50 mg/ml as the stock solutions. These solutions were stored at –20°C until use. When they were used, they were diluted in a sterile double-distilled water to 2% DMSO solutions before treating cells at 1:10 ratio. These made the final solution of the extracts, at required concentrations, to be in 0.2% DMSO.

The stock solutions of the water extracts were prepared in double distilled water, sterilized through 0.22 um filters, and stored at –20°C until use.

1.2 Macrophages

The murine macrophage cells J774A.1, were purchased from the American Type Culture Collection (ATCC). The cells were grown in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a CO_2 incubator at 37°C. They were subcultured by scraping when the cells were 80% confluence. They were used in this study with their viability more than 85%.

1.3 Chemicals and reagents

The following reagents were used in this study:Chloroform (Sigma, USA), DEPC (Molekula, UK), disposable cell scraper (Greiner bio-one, USA), dimethyl sulfloxide (DMSO) (Sigma, USA), Dulbecco's modified eagle's medium (DMEM) (Gibco, USA), fetal bovine serum (Gibco, USA), hydrochloric acid (Merck,Germany), ImProm-IITM

Reverse Transcription system (Promega, USA), lipopolysaccharide (Sigma, USA), nitric oxide assay kit (Promega, USA), penicillin/streptomycin (Gibco, USA), primer (Bio Basic, Canada), Taq polymerase (Invitrogen, UK) , trypan blue dye (Sigma, USA), TRiZol reagent (Invitrogen, UK)

1.4 Equipment and Instruments

The followings equipments and instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hiclave ^{TM} , HVE-50, Hirayama, Japan), autopipette (Gilson, USA), biohazard laminar flow hood (ESSCO, USA), centrifuge machine (Hettich, USA), ELISA microplate reader (Labsystems multiskan, USA), gel electrophoresis (Bio-Rad, USA), hemacytometer (Brand, Germany), light microscope (Nikon, USA), 96 and 24 muti-well plate (Corning, USA), spectrophotometer (Shimadzu, Japan), thermocycle machine (Eppendorf, USA) , vortex mixer (Scientific industries, USA)

2.Methods

2.1 Effect of *Glycosmis prava* solvent extracts on NO production in LPS-stimulated J774A.1 cells

J774A.1 cells, at a density of 2 x 10^5 cells/ml were grown in a 96 well plate at 37 °C for 24 h. The cells were pre-treated with the hexane, ethyl acetate, butanol and water extracts of branches and leaves from *G. prava* at concentration 3.125-100 µg/mL, for 24 h before being stimulated with 100 ng /ml LPS for the next 24 h. The non pre-treated LPS –stimulated cells and the 0.2% DMSO-treated cells were used as the control and the untreated control, respectively. The supernatants of the treated cells was collected for nitric oxide content determination and the cells were assessed for cytotoxic of the extracts.

The assay for nitric oxide content was perform in the dark at room temperature by using Griess reagents as in the following procedures. In 96-well plate, 100 µl of supernatants were reacted with 20 µl of sulfanilamide reagent for 10 min, then 20 µl of N-1-napthylenediamine dihydrochloride (NED) reagent was added. The plate was incubate further for 10 min and measured by microplate reader at 540 nm. The nitric

oxide content in each well was determined as nitrite content (μM) by using nitrite standard curve. The percentages of nitric oxide inhibition of the extracts were calculated by comparing with the non pre-treated LPS-stimulated condition.

$$\% \text{ NO inhibition} = \left(\frac{\text{NO conc. of Negative Control - NO conc. of Treatment}}{\text{NO conc. of Negative Control}} \right) \text{ X } 100$$

The 50% inhibitory concentration (IC_{50}) on NO production of the extracts was also calculated. These concentrations of the extracts were used in the next experiments.

The cytotoxicity of the extractes was performed by incubating the treated cells in 96 wells plate with 50 μg/ml resazurin at 37°C for 2 h. The amount of resorufin, the product from resazurin reduction in viable cells, was determined using microplate reader by subtracting the OD at 570 from the OD at 600 nm. The percentage of cytotoxicity of the extracts was calculated by using the following formular ;

$$\% \text{ cytotoxic} = \left(\frac{\text{delta OD (negative control) - delta OD(sample)}}{\text{delta OD (negative control)}} \right) \text{ X } 100$$

The extracts that inhibit NO production at the non - cytotoxic concentrations (2 or 3 concentrations/extract) were assessed for their effects on mRNA expression of interested genes involved in macrophage stimulation.

2.2 Effects of the extracts on mRNA expression of cytokines, iNOS and COX-2 in LPS-stimulated J774A.1 cells

J774A.1 cells, at the density of 2×10^5 cells/ml, were grown in a 24 well plate at 37°C for 24 h. The cells were pretreated with the extracts (2 or 3 concentrations) at 37°C for 24 h, then treated with LPS 100 ng/ml at 37°C for 4 h for assessing cytokine expression. The same procedure was performed as above but the cells were stimulated with LPS for 24 h for iNOS and COX-2 expression. The non pre-treated LPS-stimulated cells and the 0.2% DMSO-treated cells were used as the control and the untreated control, respectively.

The treated cells were collected for total RNA preparation, cDNA production, and TNF-α, Il-1β, IL-6, iNOS and COX-2 expression determination.

Total RNA preparation

The cells were lysed and homogenized in 1 ml of TRIzol® Reagent at room temperature sample for 5 min. The homogenized samples were transfer to eppendorf tube. Two hundred µl chloroform was added into each tube. The tubes were vigorously shaked by hand for 15 seconds , incubated further at room temperature for 2-3 min, and separated for supernatant by centrifugation 12,000g for 15 min. at 4 °C. The supernatants were carefully collected into fresh eppendorf tubes. 0.5 ml of isopropyl alcohol was added into each tube. The tubes were incubated at room temperature for 10 min. The RNA pellets were separated by centrifugation 12,000g for 10 min. at 4 °C. The supernatant were discarded. The pellets were washed with 75% ethanol. Each wash the pellets were separated by centrifugation at 7,500g for 5 min. at 4°C. After washing, the pellets were air-dried and dissolved in RNase free-water. The RNA content was determined by spectrophotometer at 260 nm and calculated by the following formular

RNA(µg) = Absorbance at 260 nm X 40 X dilution factor

The RNA sample were store at -70 °C until use.

cDNA synthesis by reverse transcription

For each sample tube, 1.5 µg total RNA was pre-heat with Oligo dT_{15} primer in Nuclerse – Free Water at 5 µl final volume at 70 °C for 5 min. The tube were immediately chilled on ice for 5 min. Fifteen µl reverse transcription mixture containing; 25 mM $MgCl_2$, mixed dNTP, ribonuclease inhibitor and reverse transcriptase were added into each tubes. The tubes were incubated at 25 °C for 5 min, then 42 °C for 1 hour 30 min. and finally at 70 °C for 15 min. The samples were stored at -20 °C until use.

Amplification of interested cytokines, iNOS and COX-2 cDNA by polymerase Chain Reaction (PCR)

For each PCR tube, 1 µl of cDNA sample was mixed with PCR reaction mixture containing primer, mixed dNTP, Taq polymerase in PCR buffer. The PCR was performed by the following conditions; denaturation for 30 sec at 94 ˚C, annealing for 45 sec at 55˚C, extension for 1 min at 72 ˚C and final extension for 7 min at 72 ˚C at the end of 25th cycles. The PCR products were run by 1.5 % agarose gel electrophoresis at 100 volt for 45 min in TBE buffer , using 6 µl plus 2 µl loading dye for each sample. The gel was stained with ethidium bromide for 4 min and destained with TBE buffer for 30 min. The PCR products were analyzed and semicontitated by a gel documentation.

7. Statistical analysis

All data were presented as mean and standard deviation or standard error of means. Data analysis was performed on SPSS 17.0. If the test of homogeneity of variances showed that there was no significant deviation of variances in the data, the analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) post hoc test was used. The p-value of less than 0.05 was considered statistically significant.