CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and materials

All chemicals and materials used in this study are shown in Appendix A.

2.2 Screening for immunostimulating activity from Thai medicinal plants

2.2.1 Plant materials

Eleven Thai medicinal plants including *C. asiatica* and *R. nasutus* were used for screening of immunostimulating activity. Figure 2.1 shows pictures of nine medicinal plants besides *C. asiatica* and *R. nasutus* that were used for screening test. Whole fresh plants of *C. asiatica* and *Murdannia loriformis*, stems and leaves of *R. nasutus, Momordica charantia* fruits, bulbs of *Allium sativum*, stem parts of *Cymbopogon citratus,* and flowers of *Carthamus tinctorius* were obtained from local markets in Chiang Mai, Thailand. Four types of commercial dried plants, including *Eclipta alba* (stems and leaves), *Cyperus rotundus* (rhizomes), *Nymphaea lotus* (pollen) and *Nymphaea lotus* (plant embryos in lotus seeds) were obtained from Lampang province, Thailand. The pollen of *N. lotus* and plant embryos in *N. lotus* seeds was named as Dee-Buo and Ke-Sorn-Buo, respectively. *C. asiatica* and *R. nasutus* were authenticated by Dr. Chusie Trisonthi, Department of Biology, Chiang Mai University, Thailand.

2.2.2 Plant extraction

Whole fresh plants were washed with tap water and sliced into small pieces, dried and ground to a fine powder. One hundred grams of powder were extracted with 500 ml of either distilled water or 80% ethanol by stirring for 4 hours. The supernatants from the ethanol extraction were directly filtered through Whatman filter paper number 2, while supernatants from the water extraction were centrifuged at 5,000 rpm for 10 minutes before filtering. The filtrates were evaporated using a rotating evaporator (50-60°C) followed by lyophilization to dryness. The residue was dissolved in distilled water, adjusted to 50 mg/ml final concentration and sterilized by passage through a Millipore filter membrane (0.22 μm). A schematic diagram of the plant extraction protocol is shown in Figure 2.2. The yield of plant extracts is shown in Table 2.1.

Figure 2.1 Thai medicinal plants used for screening of immunostimulating activity (Farnsworth and Bunyapraphatsara, 1992).

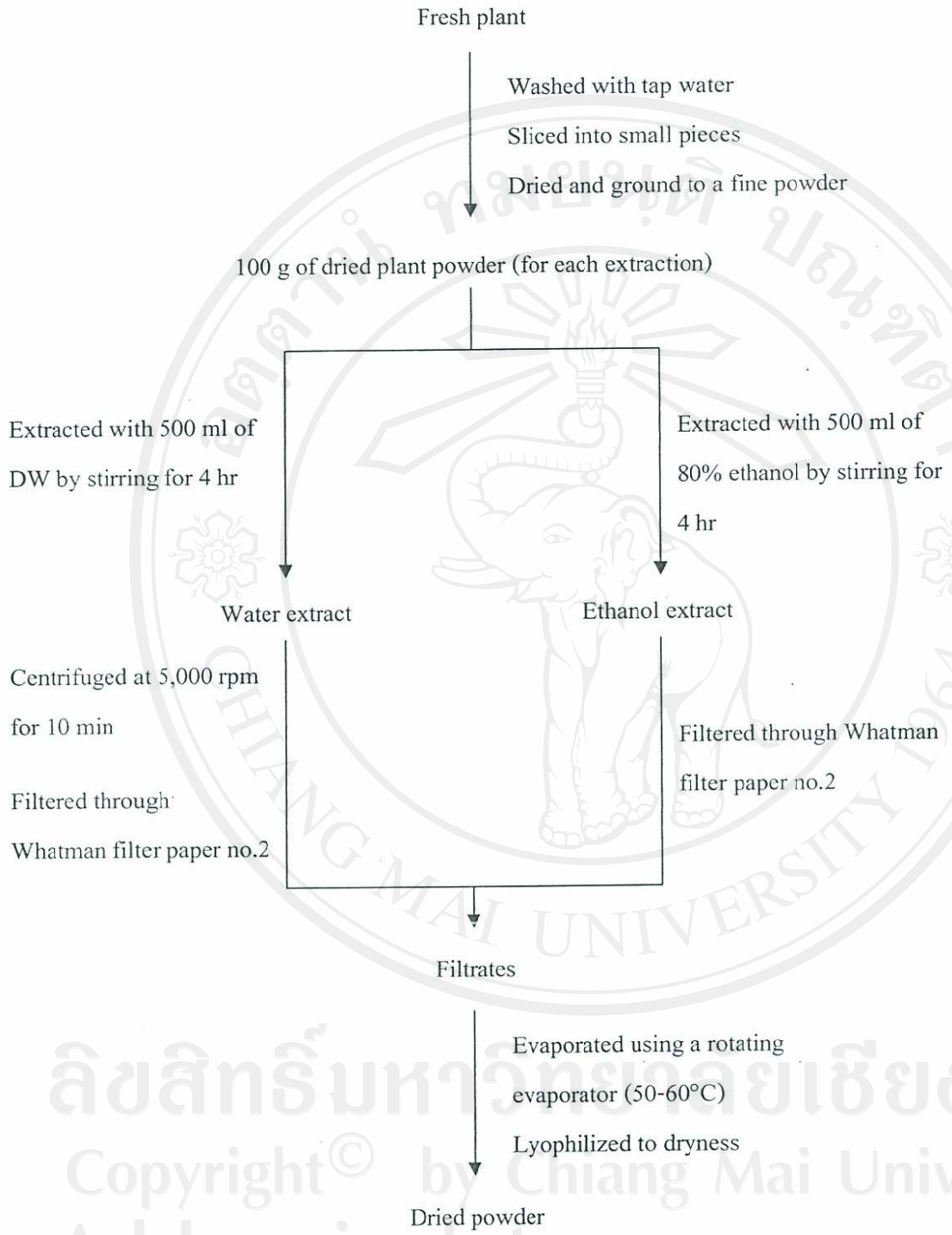

Figure 2.2 A schematic diagram of the plant extraction protocol

Table 2.1 The percentage of plant extract (% yield) after water and 80% ethanol extraction.

The % yield was calculated from gram of dried weight plant powder (100g).

Medicinal plant	% Yield	
	Water extraction	80% Ethanol extraction
C. asiatica	5.36	5.13
R. nasutus	8.18	7.54
M. loriformis	1.18	1.42
M. charantia	0.92	1.27
A. sativum	15.07	22.6
C. citratus	3.32	2.87
C. tinctorius	39.35	25.4
E. alba	13.6	5.2
C. rotundus	14	7.3
N. lotus (Dee-Buo)	29.1	21.4
N. lotus (Ke-Sorn-Buo)	15	7.5

2.2.3 Preparation of peripheral blood mononuclear cells (PBMCs)

The PBMCs were separated from whole blood of three healthy donors by Ficoll-Hypaque gradient centrifugation. Blood were collected from healthy donors and mixed with an equal volume of phosphate buffer saline (PBS). Diluted blood was layered over a Histopaque 1.077 density gradient and centrifuged at 300-400xg for 30 min at room temperature. The interface layer consisting of mononuclear cells was collected and washed three times with tissue culture medium (RPMI-1640). After washing, PBMCs were suspended in RPMI-1640 containing 10% FBS. The viability of PBMCs as determined by the trypan blue exclusion test was more than 98%. The concentration of PBMCs was finally adjusted to $1.0x10^6$ cells/ml.

2.2.4 Lymphocyte activation assay

One hundred micro liters of human PBMCs suspension were pipetted into each well of 96-well tissue culture plates; to these were added 100 µl of media containing different concentrations of 80% ethanol or water extracts and 10 µl containing sub-optimal concentration of PHA or PWM. One triplicate series of wells was used as a negative control (without extracts and mitogen) and as a positive control (with mitogen). The plates were incubated for 3 days at 37°C in a 5% CO_2 incubator. Cell proliferation was estimated by adding 0.2 µCi of [^{3}H]-thymidine per well during the final 18 hours of culture. After 18 hours, cells were harvested on glass filter fibers. Filters were then allowed to dry at room temperature on the bench top. [^{3}H]-thymidine incorporation into cells was measured as count per minute (CPM) using a liquid scintillation counter (β-counter). The cytotoxicity of extracts to human PBMCs was tested by trypan blue exclusion test. Stimulation index (SI) was calculated as following formula.

$$SI = \frac{\text{(Mean CPM of treated cultures - Mean CPM of negative control)}}{\text{(Mean CPM of positive controls - Mean CPM of negative controls)}}$$

2.3 Effects of *C. asiatica* and *R. nasutus* extracts on lymphocytes

2.3.1 Effects of *C. asiatica* and *R. nasutus* extracts on lymphocyte mitogenesis

This part of the study was to confirm the immunomodulating activity of *C. asiatica* and *R. nasutus* extracts. Plants were collected in three independent batches and extracted as described in section 2.2.2. Batch 1 and Batch 3 of plants were collected during the winter season (November to January), whilst batch 2 was collected on May. Human PBMCs were cultured with three different batches of extracts (200 μg/ml) with or without PHA or PWM 3 days at 37°C in a 5% CO_2 incubator. Cell proliferation was estimated by adding 0.2 μCi of [^{3}H]-thymidine per well during the final 18 hours of culture. [^{3}H]-thymidine incorporation into cells was measured as count per minute (CPM) using a liquid scintillation counter (β-counter) as described in section 2.2.4.

Batch 3 of *C. asiatica* and *R. nasutus* extracts (100-400 μg/ml) were confirmed their effects on lymphocyte proliferation by testing in three independent donors. Moreover, the purified compounds from *C. asiatica*, asiaticoside and asiatic acid (0.1-10 μg/ml), were also tested. The human lymphocyte activation assay was conducted as described above.

2.3.2 Effects of *C. asiatica* and *R. nasutus* extracts on cytokine production

2.3.2.1 Culture condition

Human PBMCs were prepared as described in section 2.2.3. Human PBMCs ($5x10^5$ cells/ml) were cultured in RPMI-1640 for 18 hr in the presence or absence of 500 μg/ml plant extracts. For stimulation of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α), PHA (5 μg/ml) and LPS (10 ng/ml) were used, respectively. Culture supernatants were collected and stored at –80°C until tested, the procedures described in following section.

2.3.2.2 Determination of human IL-2 and TNF-α

ELISA kits were used to measure IL-2 and TNF-α secretion in culture supernatant following the manufacturer's instructions. The 96-well plate was coated with 100 μg/well of capture antibody (anti-human TNF-α or anti-human IL-2) and incubated overnight at 4°C. The plate was washed 3 times with washing buffer (0.05% PBS-Tween 20), inverted and blotted on absorbent paper to remove any residual washing buffer. The wells were then blocked with assay diluents providing with the kit and incubated for 1 hr at room temperature. The plate was washed 3 times with washing buffer and 100 μl/well of standard (recombinant TNF-α or IL-2) and samples (culture supernatant from section 2.3.2.1) were added into appropriate wells. The plate was incubated at room temperature for 2 hr. After 5 times washing, 100 μl/well of detection antibody (biotin-conjugated anti-human TNF-α or IL-2) was added to the wells and incubated for another 1 hr at room temperature. The plate was washed 7 times with washing buffer, then added 100 μl/well of avidin conjugated horseradish peroxidase (Avidin-HRP) were added and incubated at room temperature for 30 minutes. After 7 times of washing, 100 μl/well of TMB substrate solution was added and incubated for 15 minutes at room temperature. Fifty micro liters of stop solution (2 N H_2SO_4) was then added to each well. The absorbance was read at 450 nm by using the ELISA plate reader. The concentration of TNF-α or IL-2 in culture supernatant was calculated by using standard curves for each.

2.4 Effects of *C. asiatica* and *R. nasutus* extracts on macrophages

2.4.1 Cell culture

The mouse macrophage cell line J774.2 (ECACC reference number: 85011428) was grown in DMEM without phenol red supplement with 10% (v/v) FBS, 100 μg/ml streptomycin and 100 unit/ml penicillin and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown to confluence in sterile tissue culture flasks and gently detached by scraping. Cell number and viability using trypan blue exclusion, were assessed on a haemocytometer. Cells were cultured in triplicate at a density of $1x10^6$ cells/ml in 96-well flat-bottomed tissue culture plates for 2 hr at 37°C in 5% CO_2 incubator. Cells then were activated with various concentrations of plant extracts with or without LPS. Culture supernatants were removed for nitrite and TNF-α determination 24 hr after activation. Total RNA was isolated from the remaining cells to permit measurement of iNOS and TNF-α gene expression by reverse transcription-polymerase chain reaction (RT-PCR).

2.4.2 Nitrite assay

Nitric oxide (NO) synthesis was determined by assaying culture supernatants for nitrite, the stable reaction product of NO and molecular oxygen. Nitrite concentration was determined by the Griess reaction, which converts nitrite into a purple-colored azo compound. Accurate concentration of nitrite can be determined by photometric measurement of the colored azo compound. The reaction is shown in Figure 2.3. One hundred μl of culture supernatants were incubated with equal volumes of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochroride in 2.5% H_3PO_4) at room temperature for 10 min and absorbance (540 nm) was determined in a spectrophotometer. Nitrite concentration was determined by using sodium nitrite (0-125 μM) as a standard (Appendix E).

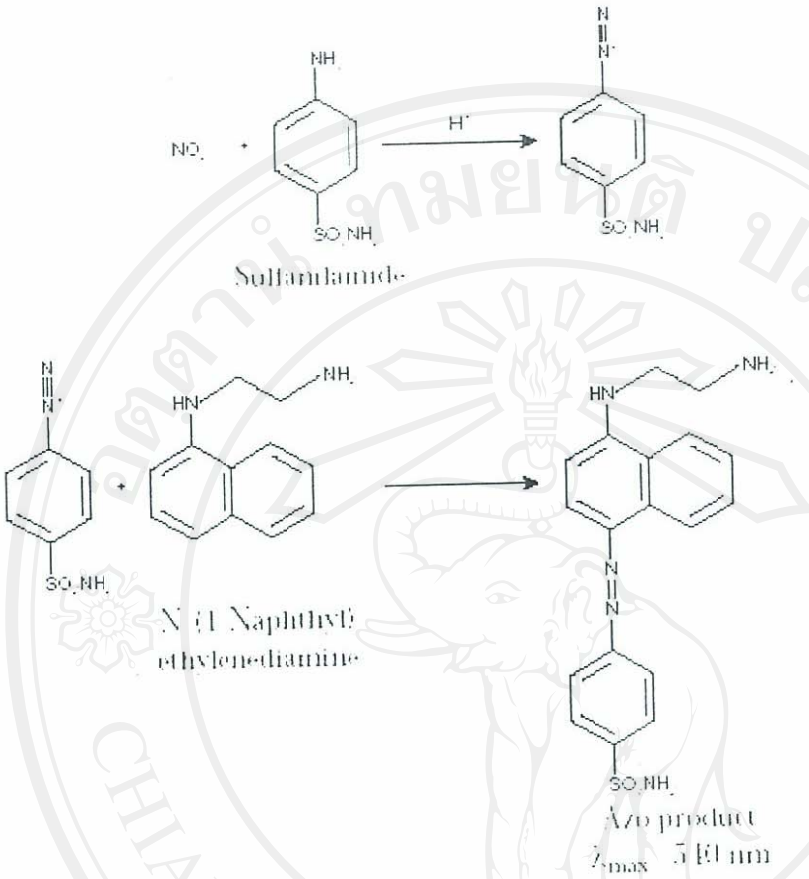

Figure 2.3 The chemistry of Griess reagent.

2.4.3 TNF-α quantification

The ELISA kit, using recombinant mouse TNF-α as standard, was used to measure TNF-α secretion in culture supernatants following the manufacturer's instructions with a limit of detection of >8 pg/ml. The 96-well plates were coated with 100 μg/well of anti-mouse TNF-α and incubated overnight at 4°C. The plates were washed 3 times with 0.05% PBS-Tween 20, blocked with assay diluents, and incubated for 1 hour at room temperature. One hundred μl/well of standard (recombinant mouse TNF-α) and samples (culture supernatant from section 2.4.1) were added into appropriate wells. The plates were incubated at room temperature for 2 hours. After washing, 100 μl/well of biotin-conjugated anti-mouse TNF-α was added to the wells and incubated for another 1 hour at room temperature. The plates were washed, 100 μl/well of avidin conjugated horseradish peroxidase (Avidin-HRP) were added, and plates were incubated at room temperature for 30 minutes. After washing, 100 μl/well of TMB substrate solution was added and plates were incubated for 15 minutes at room temperature. Fifty μl of stop solution (2N H_2SO_4) was then added to each well. The absorbance was read at 450 nm by using the ELISA plate reader. The concentration of TNF-α in culture supernatant was calculated by using a standard curve.

2.4.4 Total RNA isolation

J774.2 mouse macrophages were collected from section 2.4.1. Total RNA was isolated from stimulated cells with RNAgents® Total RNA Isolation System (Promega, USA) following the manufacturer's instructions. Cells were homogenized in chilled denaturing solution for 5 minutes following by adding 2M sodium acetate, pH 4.0 and mixed thoroughly. The tubes were then added phenol: chloroform: isoamyl alcohol, mixed vigorously and chilled on ice for 15 minutes. The top aqueous phase, which contained the RNA, was carefully removed and transferred to a fresh tube. DNA and proteins will remain in the organic phase and at the interface. An equal volume of isopropanol was added to the aqueous phase and the samples were incubated at -20°C for 30 minutes to precipitate the RNA. The RNA pellet was obtained by centrifugation at 10,000xg for 10 minutes at 4°C. The RNA pellet was washed by adding 1 ml of ice-cold 75% ethanol, breaking the pellet with RNase-free pipette tip, and centrifuged at 10,000xg for 10 minutes at 4°C. The pellet was then air-dried by leaving in the RNase-free environment for 5-20 minutes. The RNA was resuspended in Nuclease-free water, and the amount of RNA was quantified by UV spectroscopy and stored at -20°C. Before use, RNA samples were treated with deoxyribonuclease I (Invitrogen, USA) to eliminate DNA contamination.

2.4.5 Reverse transcription-polymerase chain reaction (RT-PCR)

The reverse transcription reaction was carried out using ThermoScript™ RT system (Invitrogen, USA). The reaction was performed in 5x cDNA synthesis buffer (250 mM Tris acetate (pH 8.4), 375 mM potassium acetate and 40 mM magnesium acetate), using 10 mM dNTPs, 0.1M DTT, 40 units/μl RNaseOUT™, 15 units/μl ThermoScript™ RT, 50 ng/μl random hexamers and 1 μg total RNA. All tubes are incubated in a thermal cycler: 25°C for 10 min; 50°C for 30 min; 85°C for 5 min. After cDNA generation, the tubes were plated on ice for 5 min prior to PCR.

PCR reactions were carried out in 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), using 2.5 mM dNTPs, 2.5 units HotStarTaq DNA polymerase, 0.4 μM gene specific primers and titrated 18S primer: competimer pair (Ambion, USA). The determination of the optimal ratio of 18S primer: competimer is shown in appendix F. PCR was performed using the MasterCycler Gradient (Eppendorf). The PCR reaction was carried out using the following temperature profile: initial activation, 95°C for 15min following by 29 cycles of denaturation, 94°C 30s; primer annealing, 59°C (for iNOS) or 57°C (for TNF-α) for 30s; and primer extension, 72°C for 30s. The final extension step was performed at 72°C for 10 min. After the PCR reactions were completed each PCR product was electrophorosed through a 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

2.4.6 Determination of macrophage-mediated cytolytic activity

J774.2 mouse macrophages were first incubated in DMEM medium alone or in medium supplemented with extracts for 24 hours in 96-well tissue culture plates. After that the incubation medium was removed and macrophages were washed with complete DMEM, containing 10% FBS. For the measurement of cytolytic activity, macrophages were co-incubated with B16F10 target cells in the present of 1 μg/ml LPS at 37°C, in a 5% CO_2 incubator. The effector (macrophages) to target cell (B16F10) ratio was 10:1. After 18 hours incubation, the supernatant was discarded, and remaining viable adherent cells were stained with 0.2% crystal violet in 2% ethanol for 10 minutes. Microtiter plates were rinsed with tap water and 0.1% sodium lauryl sulfate was added to each well to solubilize the stained cells. An absorbance is measured at 540 nm with an ELISA plate reader. The cytolytic activities of the macrophages are expressed as the percentage of target cell cytolysis as below:

$$\% \text{ Cytolysis} = \left\{1 - \frac{\text{O.D. of [(target + macrophages) - macrophages]}}{\text{O.D. of target (non-treat)}}\right\} \times 100$$

2.4.7 MTT assay

J774.2 mouse macrophages were cultured in 96-well tissue culture plate and treated with various concentrations of *C. asiatica* and *R. nasutus* extracts for 48 hr. Cell viability was determined by the colorimetric MTT cleavage assay. The reaction of MTT assay is shown in Figure 2.4.

MTT (3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide) was dissolved in phosphate buffer saline (PBS) at 5 mg/ml and filter sterilized to remove any insoluble residue. Twenty micro liters of filter sterilized stock MTT solution was added to each well and cultures were incubated for 4 hr at 37°C. Plates were centrifuged at 450xg and supernatant was removed and 100 µl of acid (0.04 N HCl)/isopropanol added to the wells and mixed thoroughly to completely dissolve crystalline material. An absorbance was measured at 540 nm by ELISA plate reader.

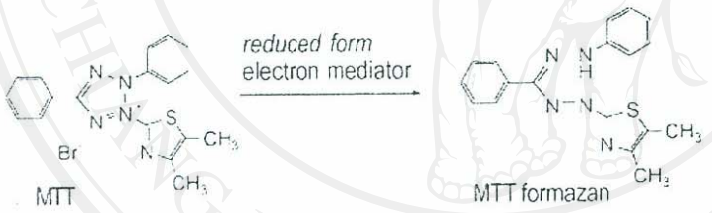

Figure 2.4 The reaction of MTT assay.

2.5 Effects of *C. asiatica* and *R. nasutus* extract on specific antibody production *in vivo*

2.5.1 Animals

Male Balb/c mice (8-12 weeks of age, weighing 20-25g) were obtained from the animal resource facilities of the faculty of medicine, Chiang Mai University, Thailand and the animals were kept in an air controlled room with a 12-h light/dark cycle and fed with normal mouse chow. Mice were divided into treated and control groups, 6 mice each. In treated groups, mice were fed with different concentrations of *C. asiatica* or *R. nasutus* extract/kg bw and the control mice received distilled water throughout the whole experiment.

2.5.2 Humoral antibody response to BSA

One week after the administration of the crude water extract, pre-immunized blood were collected from the mice, allowed to clot for 30 minutes and centrifuged at 1000xg for 15 minutes at 4°C. Serum was stored at –20°C for use as a non-immunized control. On the next day, mice were immunized with 1 mg of bovine serum albumin (BSA) in PBS by intra-peritoneal (i.p.) injection. Two weeks after the first immunization, blood was collected, and serum was stored at –20°C for assay of the primary response. On the next day of bleeding, mice were boosted with BSA, the same as the first immunization. Two weeks after the booster, blood was collected and serum was stored at –20°C for assay of the secondary response. A schematic diagram of animal treatment protocol is shown in Figure 2.5.

2.5.3 Measurement of antibody production in serum

Anti-BSA IgG and IgM antibodies in serum were measured by the ELISA method. The 96- well plates were coated with 2 % BSA in PBS pH 7.4 and incubated overnight at 4°C. Plates were blocked by 5 % skimmed milk for 2 hr at 37°C. Diluted serum samples or PBS control were added directly into the wells and incubated for 1 hr at 37°C. After plates were washed six times with 0.05 % Tween 20 in PBS, horseradish peroxidase (HRP)-conjugated anti-mouse IgG or IgM were added and incubated for 1 hr at 37°C. This reaction were developed by adding TMB substrate for 15 min at room temperature in dark and optical density was measured at 450 nm using an ELISA plate reader.

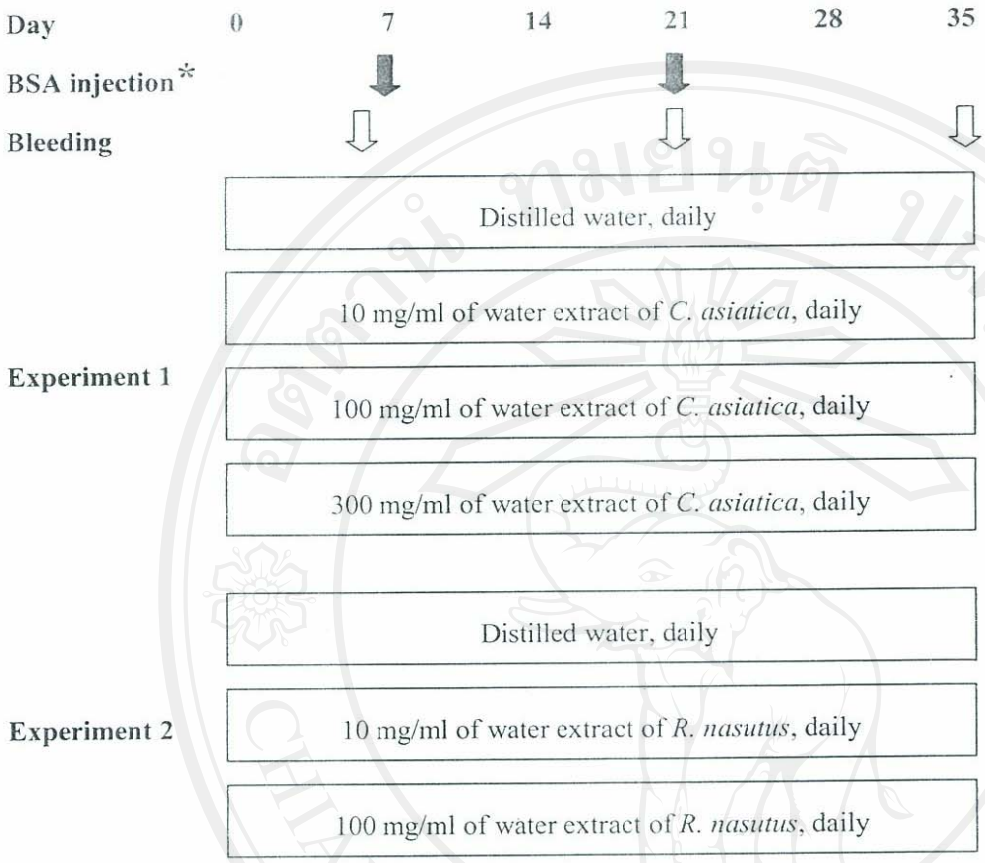

Figure 2.5 A schematic diagram of animal treatment protocol. Experiment 1; effect of water extract of *C. asiatica* on specific antibody production, Experiment 2; effect of water extract of *R. nasutus* on antibody production. * Mice were injected with 1 mg of BSA solution in 0.1 ml of PBS.

2.6 Effect of mycotoxin mixtures on the immune cells

2.6.1 Effect of mycotoxin mixtures on lymphocytes

The mitogen-stimulated human lymphocyte proliferation assay was used to study the effect of mycotoxin mixtures on lymphocytes. Human PBMCs from three healthy donors were isolated as described in section 2.2.3. Human PBMCs ($1x10^5$ cells/well) were cultured in the presence of various concentrations of mycotoxins with either PHA or PWM. The concentrations of mycotoxins used in this study were based on the result from each mycotoxin on lymphocyte activation assay. For the mixture of DON with either AFB1 or FB1, the 50% inhibition concentration of lymphocyte proliferation (IC50) of DON was cultured with various concentrations of either AFB1 or FB1. For the mixture of AFB1 and FB1, the IC50 of AFB1 was cultured with various concentrations of FB1. The lymphocyte activation assay was performed as described in section 2.2.4. The percentage of inhibition of lymphocyte proliferation was calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Mean CPM of mitogen controls} - \text{Mean CPM of treated cultures}) \times 100}{\text{Mean CPM of mitogen controls}}$$

2.6.2 Effect of mycotoxin mixtures on macrophages

Effects of individual mycotoxins and mycotoxin mixtures on macrophage function, including the production of NO and TNF-α and the expression of iNOS and TNF-α gene were evaluated. Using J774.2 mouse macrophages as a model, cells were cultured with mycotoxins or mycotoxin mixtures in the presence of LPS (50ng/ml) in 6-well tissue culture plates. The concentrations of mycotoxin used in this experiment were based on a preliminary study of the effect of individual mycotoxins. Culture supernatants were removed for nitrite and TNF-α determination 24 hours after activation. Determination of NO and TNF-α level were described in section 2.4.2 and section 2.4.3, respectively.

2.7 Modulation effect of *C. asiatica* and *R. nasutus* extraxts on mycotoxin induced-immunomodulation

These experiments were performed in order to test whether the plants that showed immunostimulating activity could alter the immunomodulation caused by mycotoxins. DON and AFB_1 were shown to be toxic to the immune cells including lymphocytes and macrophages. Thus they were used in the following experiments.

2.7.1 Effects on lymphocytes

Human PBMCs, isolated as described in section 2.2.3, were pretreatmented with various concentrations of water extract of *C. asiatica* and *R. nasutus* for 4 hours. DON (100ng/ml) or AFB_1 (5μg/ml) and mitogen (PHA or PWM) were added to the plates. The plates were further incubated for 3 days. The proliferation of human PBMCs was determined by [^{3}H]-thymidine incorporation assay as described in section 2.2.4.

2.7.2 Effects on macrophages

J774.2 mouse macrophages were cultured with various concentrations of water extract of *C. asiatica* and *R. nasutus*. Cells were washed with DMEM medium and cultured with new medium containing either DON (200 ng/ml) or AFB_1 (5 μg/ml) in the presence of LPS (50ng/ml). Culture supernatants were removed for nitrite and TNF-α determination 24 hours after activation. Determination of NO and TNF-α level were described in section 2.4.2 and section 2.4.3, respectively.

2.8 Statistical analysis

All data were expressed as mean ± SD. The Mann-Whitney U test was used to assess the statistical significance of differences. A p value of <0.05 was considered significant.