CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

All chemicals used were analytical grade or equivalent. The chemicals shown below are listed in groups according to the supplier.

Amersham (GE healthcare, UK)

IL-1β Human Biotrak Easy ELISA, TNF-α Human Biotrak Easy ELISA, IL-2 Human Biotrak Easy ELISA

BDH AnalaR® (Pooled, England)

Dimethyl sulfoxide, Sodium hydrogen carbonate

Fermentas (EU)

RevertAid™ First Strand cDNA synthesis kit

Gibco (New York, USA)

RPMI Medium 1640, Fetal bovine serum

Hyclone® (Waltham, MA, USA)

Penicillin/Streptomycin (10,000 U/10,000 μg/ml)

Invitrogen (USA)

SYBR GREENER qPCR UNIVERSAL

Macherey-Nagel (Germany)

Nucleospin® RNA II

Merck (Darmstadt, F.R. Germany)

Acetonitrile, Absolute ethanol, Silica gel

Promega (Wisconsin, USA)

GoTaq® Green Master Mix

Sigma-Aldrich® (USA)

Ficoll-hypaque, In vitro toxicology assay kit (Resazurin based)

Thermo Hypersil Co. (USA)

Hypersil® ODS-2 (5µm) column, 250×4.6 mm

T.P Drug Laboratories (1969) Co., LTD. (Thailand)

Gentamycin sulphate 80 mg/2 ml

Vivantis (Malaysia)

Agarose, DNA ladder

2.1.2 Xanthone

Extraction and isolation

The ethanol extract was first separated from mangosteens on a column (silica gel, Merck No. 7734, Mesh 70-230 ASTM). Gradient elution was conducted with n-hexane and then equalized with ethylacetate. Overall, the fractions generated from the column extraction were characterized using thin-layer chromatography (TLC) and then purified. Mangostin was eluted as a first moving yellow band. Mangostin readily crystallized out of elutes on keeping. Pure mangostin was obtained by recrystallization from acetone and gave a single spot on TLC.

HPLC analysis

Mangosteen extract was prepared at concentration of 100 µg/ml (dilution factor was 10) in methanol. The quantification was performed using four standard α-mangostin (Sigma-Aldrich®, USA) solutions in 25-200 µg/ml concentration range prepared by diluting the stock solution (1 mg/ml).

The compounds were separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system used was an isocratic comprising of 90% methanol. Flow rate was maintained at 1.0 ml/min for 30 min. Spectrophotometric detection was performed with a resolution at 319 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.3 Sesamin

Extraction and isolation

The seeds of *Sesamun indicum* Linn. were collected from Lampang province of Thailand and voucher specimen (BKF no. 138181) has been deposited at the Forest Herbarium, Royal Forestry Department, Ministry of Natural Resource and Environment, Bangkok, Thailand.

The air-dried and finely powdered seed of *Sesamum indicum* Linn. was sequentially percolated with hexane for 3 days at room temperature. This extraction with hexane was repeated 6 times, followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to obtain a crude hexane extract.

The hexane extract was separated by column chromatography over silica gel (Merck No. 7734, 500g). Elution started with hexane, gradually enriched with ethyl acetate in hexane up to 20% ethyl acetate in hexane. Fractions were collected, monitoring by TLC and combined. The solvents were evaporated to dryness to afford eight fractions (F1-F8). Fraction 7 (eluted with 15% ethyl acetate) was found to contain mainly colorless crystals.

Fraction F7 was rechromatographed on a silica gel column (Merck No. 7734, 200 g). Elution started with hexane, gradually enriched with various proportions of ethyl acetate in hexane up to 50% ethyl acetate. Fractions were collected and combined based on their TLC behavior. The solvents were evaporated to dryness to afford three subfractions f1-f3. Band f3 (eluted with 20% ethyl acetate) was further purified by crystallization with ethanol to yield colorless needle crystal which was identified by NMR as sesamin.

HPLC analysis

Sesamin was characterized using HPLC method compared with standard sesamin (Sigma-Aldrich®, USA). Sesamin was separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system used was a gradient mobile phase 70%: 50% acetonitrile. Flow rate was maintained at 1.0 ml/min for 20 min. Spectrophotometric detection was performed with a resolution at 280 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.4 *Andrographis paniculata*

Extraction and isolation

The air-dried and finely powdered from *Andrographis paniculata* (433.2 mg) was macerated in ethanol for 1 day at room temperature and their filtered. The ethanol extract was evaporated to dryness under reduced pressure to obtain a crude ethanol extract. Then re-constitution by deionized water and filtrated using Millipore filter 0.2 µm (Whatman®, Paradisc™ 25 mm).

HPLC analysis

The methanol extract of *Andrographis paniculata* was separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system was deionized water: acetonitrile: ethanol in ratio 60:20:20 as mobile phase. Flow rate was maintained at 1.0 ml/min for 10 min. Spectrophotometric detection was performed with a resolution at 260 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.5 *Moringa oleifera*

Extraction and isolation

The fresh leaves of *Moringa oleifera*, obtained from Chiang Rai province, Thailand, were extracted by deionized water and ethanol.

The air-dried and finely powdered from *Moringa oleifera* was extracted by deionized water and ethanol.

This deionized water extraction was lyophilized to dryness under reduced pressure to obtain a crude water extract and the ethanol extraction was evaporated to dryness under reduced pressure to obtain a crude ethanol extract. Then re-constitution by deionized water or DMSO and filtrated using Millipore filter 0.2 µm (Whatman®, Paradisc™ 25 mm).

HPLC analysis

The water and ethanol extract of *Moringa oleifera* leaves and the water and ethanol extract of *Moringa oleifera* powder were separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system was deionized water: acetonitrile: ethanol in a ratio of 60:20:20 as mobile phase. Flow rate was maintained at 1.0 ml/min for 10 min. Spectrophotometric detection was performed with a resolution at 260 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.6 *Houttuynia cordata*

Extraction and isolation

The air-dried of *Houttuynia cordata*, obtained from Chiang Rai province, Thailand, was extracted by deionized water and ethanol.

The air-dried yeast fermented of *Houttuynia cordata,* obtained from Chiang Rai province, Thailand, was extracted by deionized water and ethanol.

The deionized water extraction was lyophilized to dryness under reduced pressure to obtain a crude water extract. The ethanol extract was evaporated to dryness under reduced pressure as well to obtain a crude ethanol extract. Then re-constitution by deionized water or DMSO and filtrated using Millipore filter 0.2 µm (Whatman®, Paradisc™ 25 mm).

HPLC analysis

The water and ethanol extracts of *Houttuynia cordata* and The water and ethanol extract of fermented *Houttuynia cordata* were separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system was deionized water: acetonitrile: ethanol in ratio 60:20:20 as mobile phase. Flow rate was maintained at 1.0 ml/min for 10 min. Spectrophotometric detection was performed with a resolution at 260 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.7 *Herricium erinaceus*

Extraction and isolation

The air-dried of *Herricium erinaceus* (28 g), obtained from Chiang Rai province, Thailand, was extracted by deionized water and ethanol.

The deionized water extract was lyophilized to dryness under reduced pressure to obtain a crude water extract, and the ethanol extract was also evaporated to dryness under reduced pressure to obtain a crude ethanol extract. Then re-constitution by deionized water and filtrated using Millipore filter 0.2 µm (Whatman®, Paradisc™ 25 mm).

HPLC analysis

The water and ethanol extracts of *Herricium erinaceus* were separated on a Hypersil® ODS-2 (5µm) column, 250×4.6 mm (Thermo Hypersil Co., USA). The solvent system was deionized water: acetonitrile: ethanol in ratio 60:20:20 as mobile phase. Flow rate was maintained at 1.0 ml/min for 10 min. Spectrophotometric detection was performed with a resolution at 260 nm. All runs were acquired and processed using ChromQuest 4.2.34 version 3.1.6 systems software.

2.1.8 *p*-hydroxycinnamaldehyde from acetone extract of *Alpinia galanga*

Extraction and isolation

Galanga powder (from fresh rhizomes of *A. galanga* Linn.) was obtained from The Common Life of Love and Unity of the Mountain People Foundation (CLUMP), specimen BKF no. 102287, from the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resource and Environment, Bangkok, Thailand.

Dried powder of *A. ganlanga* rhizomes was extracted with acetone with constant stirring for 7 days. To ensure complete extraction, 5 kg of powder was extracted with 17 L of acetone and the process was repeated 4 times. The filtrates were pooled and evaporated in a rotary evaporator under reduced pressure at 40 ^{o}C. The yield of the extract was determined. Dried residue was weighed and stored at -20 ^{o}C.

The acetone extract was further separated by column chromatography over silica gel (Merck No. 7734, 1.8 kg). Gradient elution was conducted initially with n-hexane, gradually enriched with ethyl acetate, followed by increasing amount of hexane: ethyl acetate in a ratio of 50:50. Fractions were collected, monitored by TLC and combined. The solvents were evaporated to dryness to yield seven fractions (F1-F7). Fraction F5 29.48 g (eluted with 10% ethyl acetate in hexane) was obtained as a semisolid.

Fraction F5 was rechromatographed on a silica gel column (Merck No. 7734, 150 g). Elution started with n-hexane, then with various proportions of ethyl acetate: n-hexane, followed by increasing amount of hexane: ethyl acetate in ratio 10:90. Fractions were collected and combined based on their TLC behavior. The solvents were evaporated to dryness to afford three subfractions f1-f3.

Fraction f2 (0.41 g) was re-chromatographed on a silica gel column (Merck No. 7734, 30 g) by gradient eluting with pure n-hexane then with various proportions of ethyl acetate: n-hexane, followed by increasing amount of hexane: ethyl acetate in a ratio of 90:10. Fractions were collected and combined based on their TLC behavior. The solvents were evaporated to dryness to afford three subfractions fr1-fr3.

Fraction fr2 (0.34 g), eluted with 10% ethyl acetate in hexane, was further purified by crystallization with methanol to yield white solid powder (0.34 g) which was identified by MS/NMR as *p*-hydroxycinnamaldehyde.

HPLC analysis

The characterization of *A. galanga* acetone extract was carried out using a Shimadzu CLASS-VP V5.02 with the column ODS C18 (4.6 x 250 mm) and UV-Vis Diode array as a detector, controlled by computer using the software provided by the manufacturer. 20 µl of each fraction were injected into the column using CH_3CN: H_2O (20:80) as a mobile phase. The flow rate and retention times were 1 ml/min and 30 min.

2.2 Methods

2.2.1 Peripheral blood mononuclear cells (PBMC) isolation and culture

Peripheral blood mononuclear cells (PBMC) are isolated from whole blood sample using different density gradient centrifugation procedures by Ficoll-hypaque (Sigma-Aldrich®, USA) (69). At the end of the centrifugation step, the following layers are visually observed from the top to bottom: plasma/platelets, PBMC, Ficoll-hypaque and erythrocytes/granulocytes. Briefly, the whole blood sample 10 ml in heparinized vaccutainer tube was diluted with sterile PBS ratio 1:1. Then it was underlayed by Ficoll-hypaque (Sigma-Aldrich®, USA) 10 ml and spin at 4,000 rpm for 30 minutes. The PBMC layer was collected and washed 2 times to get rid of some contaminants by sterile PBS.

PBMC (8×10^5 cells/ml in 24-wells plates) were cultured in RPMI medium 1640 (Gibco, New York, USA) supplemented with Penicillin/Streptomycin (100 U /100 μg/ml) and gentamicin 40 mg/ml at 37°C in a humidified incubator containing 95% air 5% CO_2 (8).

2.2.2 Phytochemicals preparation

In this study, phytochemical extracts were divided into two groups. The first group; xanthone, sesamin, *Moringa oleifera* powder extract, *Moringa oleifera* ethanol extract, *Houttuynia cordata* ethanol extract, fermented *Houttuynia cordata* ethanol extract, and *p*-hydroxycinnamaldehyde were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO added to cells never exceeded 0.1% (44). The second group; *Andrographis paniculata* extract, *Moringa oleifera* water extract, *Herricium erinaceus* extract, *Houttuynia cordata* water extract, and fermented

Houttuynia cordata water extract were dissolved in deionized water. To evaluate the effects of phytochemicals, their various concentrations (0.5, 1.0, 5.0, and 10.0 µg/ml) were added to the cultures, whereas the untreated sample was used as a control. Treatments were performed in triplicate experiments.

2.2.3 Influenza type A H1N1 induced PBMC treatment

To determine the effects of phytochemicals on cytokine release from influenza type A H1N1 induced PBMC. Influenza type A H1N1 (Hemagglutinin = 128), obtained from Prof. Yong Poovarawan, Faculty of Medicine, Chulalongkorn University, was optimized condition by dilute in embryonated egg, which is shown in the appendix in Figure 31. Then, added to the PBMC culture (8×10^5 cells/ml in 24-wells plates), whereas the untreated sample was used as a control. Cells were co-treated with phytochemicals in a concentration of 0.5, 1.0, 5.0, and 10.0 µg/ml then incubated at 37 ^{o}C with 95% air 5% CO_2 for 24 h, which modified method from reference (69). After 24 h of treatment, human IL-1β, TNF-α, IL-2 in culture medium and gene expression were analysed using ELISA and real-time qPCR, respectively.

2.3 Analytical methods

2.3.1 Cell viability by alamarBlue® assay

Cell viability was determined by the colorimetric detection on changes of the reducing environment through the mitochondrial conversion of resazurin (oxidize form, blue) to resorufin (reduced form, red). PBMC (2 x 10^5 cells/ml) were plated in 96-well plates and incubated at 37 ^oC with 95% air 5% CO_2 for 24 h. Cells were treated with phytochemicals in concentrations of 0.5, 1.0, 5.0, and 10 µg/ml. For background control, RPMI medium without cells was used. Then, 10% of alamarBlue® solution (Sigma-Aldrich®, USA) was added into culture medium and incubated at 37 ^oC with 95% air 5% CO_2 for 24 h. The absorbance was measured at 540 nm and 620 nm by a microplate reader.

The percentage of difference between the treated and control cells in cytotoxicity was calculated by an equation as follow:

$$\% \text{ difference between treated and control cells} = \frac{[(O2 \times A1)-(O1 \times A2)]}{[(R1 \times N2)-(R2 \times N1)]} \times 100$$

O1 = 80,586 molar extinction coeffient of oxidized alamarBlue® at 540 nm

O2 = 117,216 molar extinction coeffient of oxidized alamarBlue® at 620 nm

A1 = Absorbance of treated cell at 540 nm

A2 = Absorbance of treated cell at 540 nm

R1 = 155,677 molar extinction coeffient of reduced alamarBlue® at 540 nm

R2 = 14,562 molar extinction coeffient of reduced alamarBlue® at 620 nm

N1 = Absorbance of control cell (media plus alamarBlue® but no cells) at 540 nm

N2 = Absorbance of control cell (media plus alamarBlue® but no cells) at 620 nm

The percentage of viability of treated cell relative to control cell was calculated by an equation as follow:

$$\% \text{ of control} = \frac{\text{treated cell}}{\text{Untreated control cell}} \times 100$$

2.3.2 A double sandwich based Enzyme-linked immunosorbent assay (ELISA) kit for cytokine markers

After 24 h of treatment, culture medium was determined using human IL-1β, TNF-α, and IL-2 Biotrak Easy ELISA kit according to a manufacturer's instruction (Amersham, GE healthcare, UK). The assay system is based on a solid phase ELISA, which utilizes an antibody for (h)IL-1β or TNF-α or IL-2 bound to the wells together with a biotinylated antibody to (h)IL-1β or TNF-α or IL-2 and streptavidin conjugated to horseradish peroxidase. Briefly, 150 µl of standard diluent buffer and 100 µl of deionized water were added to each well of an eight-well strip plate coated with polyclonal antibody to (h)IL-1β or TNF-α or IL-2. Then, 50 µl of samples 0.5, 1.0, 5.0, and 10.0 µg/ml was added and incubated for 3 h at room temperature, then washed with washing buffer 400 µl/well for 4 times. After incubation, 100 µl of premixed TMB substrate was added to each well and incubated at room temperature for 15 minutes in the dark then added 100 µl of the stop solution. The optical density was measured at 450 nm using the Titertek Multiskan M340 multiplate reader, and the values were converted to concentration by using a standard curve.

The cytokines released from culture medium of influenza type A H1N1 induced PBMC were estimated using the calculation:

$$\% \text{ cytokine relative to embryonated egg control} = \frac{\text{Sample medium}}{\text{Embryonated egg control}} \times 100$$

2.3.3 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by using Nucleospin® RNA II (Machere-Nagel, Germany). Reverse transcription reaction was performed using 0.5 μg of total RNA and reverse-transcribed into cDNA using RevertAid™ First Strand cDNA synthesis kit (MBI Fermentas, Germany), then amplified 35 cycles using two oligonucleotide primers derived from published GAPDH sequence (NM_002046); a forward primer of GAPDH sequence 5'-TGGTATCGTGGAAGGACTCAT-3' and a reverse primer of GAPDH sequence 5'- GTGGGTGTCGCTGTTGAAGTC-3'. Genimic cDNA was amplified for 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 53 °C for 1.15 min. and elongation at 72 °C for 1.15 min. in GoTaq® Green Master Mix (Promega,Wisconsin, USA). The PCR products were subjected to 1.5% agarose gel electrophoresis.

2.3.4 Real-time quantitative polymerase chain reaction (Real-time qPCR)

Real time qPCR was used to examine expression of cytokine markers in the influenza type A H1N1 induced PBMC culture. For determination of cytokine maker genes expression, SYBR Green detection was used and the values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time quantitative polymerase chain reaction was performed in a DNA Engine (ABi 7500) using SYBR

GREENER qPCR UNIVERSAL (Invitrogen, USA). The sequence primers of cytokine markers IL-1β (70), TNF-α (70), and IL-2 (71)) were shown in the appendix in Table 1.

Relative expression levels for each primer set were normalized to the expression of GAPDH by the $2^{-\triangle CT}$ method (72).

$$\text{Fold of relative to GAPDH} = \frac{\text{Expression coefficient of sample}}{\text{Expression coefficient of untreated control}}$$

2.3.5 Statistical method

Statistical analysis was performed using one-way ANOVA. Data are expression as the mean ± S.D. of triplicate samples. The *p* values of < 0.05 were considered significant.