CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Extracts of *Micromelum hirsutum*

The dried branches of *M. hirsutum* (4.5 kg) were grounded and sequentially extracted with hexane, dichloromethane and methanol to give the yields after solvent evaporation as in the followings, 14.50 g hexane extract (BH 0.32% of dried weight), 13.1 g dichloromethane extract (BD 0.29% of dried weight) and 171.0 g methanol extract (BM 3.80% of dried weight), respectively. The dried leaves of *M. hirsutum* (1.0 kg) were grounded and extracted with the same solvents as the dried and grounded branches to give the yields after solvent evaporation as follows, 36.0 g hexane extract (LH 3.60% of dried weight), 47.1 g dichloromethane extract (LD 4.7% of dried weight) and 170.0 g methanol extract (LM 17.0% of dried weight), respectively. These solvent extracts were prepared and characteristically identified by Associate Professor Dr. Nijsiri Ruangrungsi and Mr. Chaisak Chansriniyom, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. The identification characteristic or fingerprints of these extracts are in the Appendix A-1 to A-6. The abbreviations of the solvent extracts were as in the followings;

Solvent extracts	abbreviations
From branches:	
Methanol	BM
Hexane	BH
Dichloromethane	BD
From leaves:	
Methanol	LM
Hexane	LH
Dichloromethane	LD

1.2 Cells

- Human peripheral blood mononuclear cells (human PBMCs)

Human PBMCs were prepared from heparinized blood obtained from healthy male blood donors, age between 25 to 30 years old, at the National Blood Bank, Thai Red Cross Society, with informed consent. This study was approved by the Human Research Ethics Committee from the Faculty of Medicine, Chulalongkorn University. The cells were isolated from the whole blood by ficoll gradient centrifugation. They were maintained in complete RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS), 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2. The cells in the complete RPMI 1640 medium containing 5% FBS were used to assess activities of the solvent extracts.

- Human B-Lymphoma cells (Ramos cells)

The human B-Lymphoma cells, Ramos cells, were purchased from the American Type Cell Culture (ATCC) (Rockville, MD). The cells were maintained in the complete RPMI 1640 medium at 37°C in a humidified atmosphere of 5% CO_2.

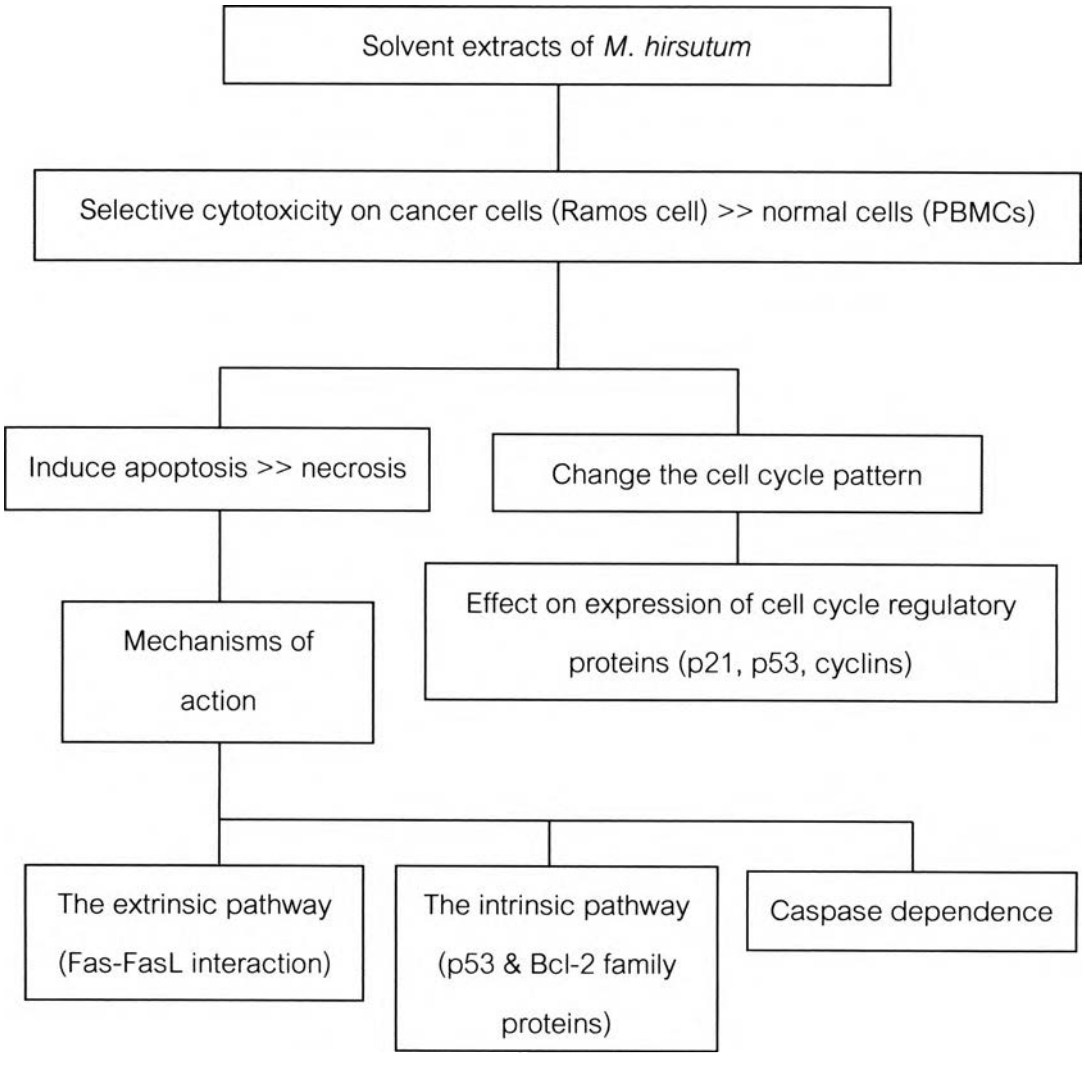
1.3 Equipments and Instruments

The following instruments were used in this study; analytical balance (GMPH, Sartorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hirayama, Japan), autopipette (Gilson, USA), biohazard lamina-flow hood (Science, Germany), centrifuge (Hettich, USA and Eppendorf, Germany), ELISA microplate reader (Labsystems multiskan, USA), fluorescence flow cytometer (Coulter, USA), gel electrophoresis system (Bio-Rad, USA), gel documentation (Bio-Rad, USA), hemocytometer (Brand, Germany), incubator (Thermo, USA), light microscope (Nikon, Japan), 24 and 96 multi-well plates (Corning, USA), PCR thermal cycler (Eppendorf, Germany), pH meter (Mettler tuledo, Switzerland), refrigerator 4°C and -20°C (Sanyo, Japan), T25 tissue culture flasks (Corning, USA) and vortex mixer (Scientific industries, USA).

1.4 Reagents

The following reagents and reagent kits were used in this study; annexin V apoptosis detection kit (Santa Cruz Biotechnology, USA), caspase inhibitor Z-VAD-FMK (Promega, USA), anti-Fas ligand antibodies (Biolegend, USA), fetal bovine serum (Gibco, USA), RPMI 1640 medium (Gibco, USA), L-glutamine (Gibco, USA), penicillin/streptomycin (Gibco, USA), etoposide (Ebewe Pharma, Austria), heparin (Leo, Denmark), Histopaque®-1077 (Sigma, USA), 0.4% trypan blue dye (Sigma, USA), Platinum® Taq DNA polymerase (Invitrogen, USA), Improme-II™ reverse transcription system (Promega, USA), agarose (Bio-Rad, USA), dNTP mix (Vivantis, Malaysia), absolute ethanol (Merck, Germany), TRIzol® Reagent (Invitrogen, USA), and diethyl pyrocarbonate (DEPC) (Molekula, UK)

Conceptual framework



2. Methods

2.1 Preparation of the stock solutions of *M. hirsutum* extracts

All solvent extracts were dissolved in DMSO to 50 mg/ml as the stock solutions. These solutions were stored at –20°C until use. Before used, the extracts were diluted in sterile double-distilled water to 5% DMSO solutions before treating cells at 1:10 ratio. This made the final solution of the extracts, at required concentrations, to be in constant 0.5% DMSO.

2.2 Preparation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from heparinized blood by ficoll (Histopaque-1077) density gradient centrifugation as the followings:

1. Equally mix each blood sample with RPMI 1640 medium containing 2 µl/ml heparin.
2. Slowly layer 9 ml of the diluted blood sample on 5 ml ficoll solution in a 15 ml sterile polypropylene tube.
3. Centrifuge the tube at 3,200 rpm for 30 min at room temperature.
4. Carefully remove the top layer solution without disturbing the interface.
5. Collect cells at the interface into a new 15 ml sterile polypropylene tube.
6. Wash the cells twice with 12.5 ml RPMI 1640 medium (+ 2 µl/ml heparin) by centrifugation at 1,200 rpm for 10 min at room temperature.
7. Discard the supernatant and resuspend the pellet with 5 ml completed RPMI medium.
8. Determine viable cells by staining with 0.4% trypan blue dye solution at the ratio 1:1 and count the number of the cells on a hemocytometer. The cells were adjusted to required density with the completed RPMI 1640 medium.

In this study, PBMCs were used at the density of $1x10^6$ cells/ml, with more than 95%, viability

2.3 Determination of cytotoxicity activity of the *M. hirsutum* extracts

2.3.1 Preliminary determination of cytotoxicity activities of the extracts

All the solvent extracts from branches and leaves of *M. hirsutum* were preliminary screened for their cytotoxicity against Ramos cells using resazurin assay **[109]**. The assay was performed in triplicate with 2 independent experiments (n=2)

1. Treat $1x10^6$ cells/ml Ramos cells (10% FBS medium) with solvent extracts at the concentration 10 and 50 µg/ml for 42 h at 37°C. 0.2% DMSO and 1 µg/ml doxorubicin were used as the negative and positive controls, respectively.
2. Add resazurin to final concentration 5 µg/ml, further incubate for 6 h at 37°C.
3. Determine the production of resorufin product in viable cells by measuring OD at 570 and 600 nm with a microplate reader.
4. Calculate the percentage of toxicity of the extracts by the following equation;

$$\% \text{ Cytotoxicity} = \frac{[\Delta OD_{0.5\%DMSO} - \Delta OD_{sample}] \times 100}{OD_{0.5\%DMSO}}$$

Where $\Delta OD = absorbace_{570\ nm} - absorbance_{600\ nm}$

The extracts which had cytotoxicity against Ramos cells higher than 50% at 50 µg/ml were further assessed.

2.3.2 Determination of cytotoxic activities at IC50's of the extracts

The extracts which had cytotoxicity against Ramos cells higher than 50% at 50 µg/ml were assessed for their cytotoxic activities at IC50's. The assay was performed in triplicatie with 4 independent experiments (n=4) in the following procedure.

1. Treat $1x10^6$ cells/ml Ramos cells or PBMCs with 5 concentrations of the extract for 18 and 42 h at 37°C.
2. Add resazurin to final concentration 5 µg/ml, further incubate for 6 h at 37°C.
3. Determine the production of resorufin product in viable cells by measuring OD at 570 and 600 nm with a microplate reader.

4. Calculate the percentage of toxicity of the extracts.
5. Calculate the concentration of each extract which caused 50% cytotoxicity (IC50) from the percentage of toxicity after 24 h exposure.
6. Compare the cytotoxicity of the solvent extracts between on Ramos cells and on PBMCs
7. Select the solvent extracts which had cytotoxicity on tumor cells higher than PBMCs for the next studies.

2.4 Determination of apoptotic induction

Apoptotic induction activities of the solvent extracts which had cytotoxic activity against Ramos cells higher than PBMCs were evaluated at 2-3 optimal concentrations based on their IC50 values. Apoptotic cells were detected by using annexin V-FITC which specifically binds to exposed phosphatidylserine (PS) on the outer cell membrane of early apoptotic cells. The assay was performed in duplicate with 3 independent experiments (n=3).

1. In 24 well plate, treat $1x10^6$ cells/ml/well Ramos cells with 2-3 concentrations of the solvent extracts at 37°C for 12 and 24 h. 0.5% DMSO and etoposide were used as the positive and negative control, respectively.
2. Collect the treated cells in each well to a micro-centrifuge tube and centrifuge at 12,000 rpm 25°C for 1 min.
3. Removed the supernatant, wash the cells twice with 500 µl cold PBS, and separate the cells by centrifugation at 12,000 rpm, 25°C for 1 min.
4. Discard the supernatant, resuspend the cell pellet in 100 µl of the assay buffer and transferred into a flow cytometer tube.
5. Add 1 µl of 0.05 µg/ml PI and 0.5 µl of annexin V-FITC in each tube.
6. Incubate the tubes in the dark condition at room temperature for 15 min.
7. Subsequently add 400 µl of the assay buffer into each tube and immediately analyze $1x10^4$ cells/sample by fluorescence flow cytometer.
8. Assess types of the cells as in the followings; the annexin V-FITC$^-$/PI$^-$ cells as viable cells, the annexin V-FITC$^+$ cells as apoptotic cells, the PI$^+$ cells as

nonapoptotic cells, and the annexin V-FITC^{+}/PI^{+} cells as late apoptotic or secondary necrotic cells.

In this study, the solvent extracts which induced cells death mainly by apoptosis were further assessed in the next study.

2.5 Determination of apoptotic induction mechanism

The solvent extracts which induced cell death mainly by apoptosis were further investigated for their mechanisms of apoptotic induction. The dependency on caspase activation was evaluated by using a pan-caspase inhibitor, Z-VAD-FMK. Their effect through extrinsic pathway of apoptosis was determined by using anti-Fas ligand antibody to inhibit the interaction between PAS and FAS ligand. The effect of the extract through the intrinsic pathway of apoptosis was evaluated by determining the mRNA expression of pro-apoptotic and anti apoptotic proteins in the BCL-2 family.

2.5.1 Determination of the effect of the solvent extracts through caspase activation

1. In a 24-well plate, pre-treat 1x10^{6} cells/ml/well Ramos cells with 50 µM Z-VAD-FMK for 1 h at 37^{o}C.
2. Treat the cells with 2-3 concentrations of the solvent extracts for 12 h at 37^{o}C.
3. Detect types of cell death by performing as the step 2nd - 8th of the topic 2.4.

2.5.2 Determination of effect of the solvent extracts through Fas-Fas ligand interaction

4. In a 24-well plate, pre-treat 1x10^{6} cells/ml/well Ramos cells with 200 ng/ml FasL antibody for 1 h at 37^{o}C.
5. Treat the cells with 2-3 concentrations of the solvent extracts for 12 h at 37^{o}C.
6. Detect types of cell death by performing as the step 2nd - 8th of the topic 2.4.

2.5.3 Determination of the expression of proteins involving in mitochondrial dependent pathway

The mRNA expressions of proteins involve in apoptosis including *p53* gene, pro-apoptotic Bcl-2 (*BAX and BAK*) and anti-apoptotic Bcl-2 (*BCL-XL and BCL-2*) genes were determined as in the following procedures;

2.5.3.1 Treatment Ramos cells with the extract

Treat $1x10^6$ cells/ml Ramos cells with 2-3 concentrations of the solvent extracts for 12 h at 37°C.

2.5.3.2 Isolation of total RNA

1. Collect the treated cells into 15 ml centrifuge tubes and separate the cells by centrifugation at 1,200 rpm 25°C for 10 min.
2. Removed the supernatant, lyse and homogenize the cells in 1 ml of TRIzol® reagent, transfer the lysate to 1.5 ml eppendorf tubes and incubate for 5 min at room temperature.
3. Add 0.2 ml chloroform, vigorously shake the tubes for 15 sec., and incubate at room temperature for 2-3 min.
4. Centrifuged at 12,000 rpm for 15 min at 4°C, and transfer the aqueous phase to fresh eppendorf tubes.
5. Add 0.5 ml isopropyl alcohol, incubate at room temperature for 10 min, and centrifuge 12,000 rpm for 10 min at 4°C.
6. Removed the supernatant, wash the RNA pellet with 1 ml 75% ethanol, mix by vortexing, separate the pellet by centrifugation 7,500 rpm at 4°C for 5 min.
7. Removed the supernatant and air-dry the RNA pellet for 5-10 min.
8. Dissolve the pellet in DEPC-treated water, incubate at 55-60°C for 10 min.
9. Determine the concentration and protein contamination of total RNA by spectrophotometer at 260 and 280 nm. The total RNA samples should have optical densitiy ratio; OD_{260}:OD_{280} > 1.8.
10. Stored the total RNA samples at -70°C until use.

2.5.3.3 Preparation of complementary DNA (cDNA) by reverse transcription-polymerase chain reaction

1. Mix 1 µl Total RNA of each sample and 1 µl oligo dT15 primer in 0.2 ml PCR tube.
2. Heat the tubes at 70°C for 5 min and immediately chill on ice for 5 min.

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

2.5.3.4 Determine mRNA expression of pro- and anti-apoptotic genes by PCR

PCR was performed using gene-specific primers (Table 1) for p53, BCL-2, BCL-XL, BAX and BAK. The GAPDH gene was used as the internal control. PCR was carried out in a 50 µl reaction mixture containing PCR buffer, 0.5 µl cDNA of p53, GAPDH, BAK and BCL-XL or 1 µl cDNA of BAX and BCL-2, 1.5 mM $MgCl_2$, 0.2 mM mixed dNTP, 0.4 µM of each primers and 1 unit of platinum® Taq DNA polymerase. The PCR conditions were: 94°C for 2 min., followed by 35 cycle of 30 sec. denaturation at 94°C, 30 sec. annealing at appropriate Tm, 1 min extension at 72°C, and finally 10 min extension at 72°C. PCR products were analyzed by electrophoresis in 1.5% agarose gel at 100 V. The gel was stained with ethidium bromide in 1xTBE buffer. PCR products were imaged and determined their densities by gel documentation. The densities of the PCR products were expressed as % of internal control (ratio of the band density divided by that of the housekeeping gene (GAPDH) x 100).

Table 1: Primers for RT–PCR and their annealing temperatures.

Gene	Primer sequences	Tm °C	PCR product (bp)
GAPDH	Forward: 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3' Reverse: 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'	60	530
p53	Forward: 5'-CAT GAG CGC TGC TCA GAT AG-3' Reverse: 5'-CTG AGT CAG GCC CTT CTG TC-3'	56	643
BAK	Forward: 5'-TGA AAA ATG GCT TCG GGG CAA GGC-3' Reverse: 5'-TCA TGA TTT GAA GAA TCT TCG TAC C-3'	55	642
BAX	Forward: 5'- TGG-AGC-TGC-AGA-GGA-TGA-TTG - 3' Reverse: 5' - GAA-GTT-GCC-GTC-AGA-AAA-CAT-G - 3'	60	96
BCL-XL	Forward: 5'- CAA TGG ACT GGT GAG CCC A -3' Reverse: 5'- AGT TCA AAC TCG TCG CCT G -3'	55	307
BCL-2	Forward: 5'-GGT GCC ACC TGT GGT CCA CCT-3' Reverse: 5'-CTT CAC TTG TGG CCC AGA TAG G-3'	58	458

2.6 Determination of the effect of the solvent extracts on Ramos cell cycle

1. Treat $1x10^6$ cells/ml Ramos cells with 2-3 concentrations of the solvent extracts in a 24-well plate at 37°C for 1.5 h.

2. Removed the supernatant, wash the cells twice with 2 ml of RPMI 1640 medium and separate the cells by centrifugation at 1,200 rpm for 10 min at 25°C.

3. Discard the supernatant and resuspend the cells pellet in 1 ml of the completed RPMI 1640 medium containing 10% FBS.

4. Incubate the treated cells for 48 h at 37°C.

5. Collect the cells in each well to a flow cytometer tube and centrifuged at 1,500 rpm 4°C for 5 min.

6. Removed the supernatant, wash the cells twice with 500 µl cold PBS, and separate the cells by centrifugation at 1,500 rpm at 4°C for 5 min.

7. Discard the supernatant and resuspend the cell pellet in 150 µl cold PBS.

8. Fix the cells by slowly adding 350 µl absolute ethanol and continuously shaking.

9. Incubate for 15 min at -20°C, and then collect the cells by centrifugation at 1,500 rpm for 5 min at 4°C.

10. Wash the cells twice with 500 µl cold PBS and collect the cells by centrifugation at 1,500 rpm for 5 min at 4°C.

11. Resuspend the cells in 500 µl assay buffer,

12. Add 5 µl of 4 mg/ml RNase and incubate for 30 min at room temperature.

13. Add 5 µl of 0.05 µg/ml PI and incubate the tube in dark condition for 15 min at room temperature.

14. Analyze the cells (100,000 cells/sample) by fluorescence flow cytometer.

2.7. Determination of the effect of solvent extracts on the mRNA expression of regulatory proteins in the cell cycle

The extracts which changed Ramos cell cycle were further investigated for their effect on the mRNA expression of regulatory proteins in the cell cycle, including p21, p53 and various cyclins (*CYCLIN A, CYCLIN B1, CYCLIN D1 and CYCLIN E*), as in the following procedures;

1. Treat Ramos cells as in the 1st steps - 4th step of the topic 2.6.
2. Collect the treated cells for total RNA isolation and cDNA generation as in the topic 2.5.3.2 and 2.5.3.3
3. Generate PCR products of p21, p53, CYCLIN A, CYCLIN B1, CYCLIN D1 and CYCLIN E genes by using gene-specific primers, performing PCR reactions, and detecting the PCR products in the similar steps as in the topic 2.5.3.4.

Table 2: Primers for RT–PCR and their annealing temperatures.

Gene	Primer sequences	Tm °C	PCR production (bp)
GAPDH	Forward: 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3' Reverse: 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'	60	530
P21	Forward: 5' - GCG ATG GAA CTT CGA CTT TGT - 3' Reverse: 5' - GGG CTT CCT CTT GGA GAA GAT-3'	54	352
P53	Forward: 5'-CAT GAG CGC TGC TCA GAT AG-3' Reverse: 5'-CTG AGT CAG GCC CTT CTG TC-3'	56	643
CYCLIN A	Forward: 5'-GTC ACC ACA TAC TAT GGA CAT G-3' Reverse: 5'-AAG TTT TCC TCT CAG CAC TGA C-3'	53	300
CYCLIN B1	Forward: 5'- CGG-GAA-GTC-ACT-GGA-AAC-AT - 3' Reverse: 5'- AAA-CAT-GGC-AGT-GAC-ACC-AA - 3'	55	177
CYCLIN D1	Forward: 5' - CTG-GCC-ATG-AAC-TAC-CTG-GA - 3' Reverse: 5' - GTC-ACA-CTT-GAT-CAC-TCT-GG - 3'	54	482
CYCLIN E	Forward: 5'-AAT AGA GAG GAA GTC TGG-3' Reverse: 5'- AGA TAT GCA ACC TGC ATG-3'	55	440

2.8 Statistical analysis

Data were presented as mean plus or minus standard error (mean±S.E.). Statistical comparisons were made by one-way ANOVA followed by Turkey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 17. Any p-value < 0.05 was considered statistically significant.