CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Experimental animals

Thirty male Wistar rats weighing between 250-300 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornprathom, Thailand. Animals were housed two per cage at the Faculty of Medicine, Srinakharinwirot University, Bangkok and acclimatized for at least 1 week before the experiment. All animals were allowed free access to food (C.P. company, Thailand) and drinking water. Light / dark period and temperature were controlled at 12/12 hour cycle and 25^{o} C, respectively. During the time of experimentation, body weight of each rat, food consumption, volume of drinking water were recorded every 7 days.

2. Instruments

1. Autopipettes (Gibson, France)
2. Centrifuge (Hettich Roto Magna, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Lyophilizer (Dura-Dry II MP, USA)
5. Metabolic shaker bath (Heto, Denmark)
6. pH meter (Beckman, USA)
7. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
8. Refrigerated superspeed centrifuge (Beckman, USA)
9. Refrigerated ultracentrifuge (Beckman, USA)
10. Sonicator (Elma, Germany)
11. Spectrophotometer (Jasco, Japan)
12. Surgical equipments
13. Timer
14. Ultra-low temperature freezer (Forma Scientific Inc., USA)
15. Vortex mixer (Clay Adams, USA)

3. Chemicals

Acetylacetone, 4-aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), cupric sulfate, dimethyl sulfoxide (DMSO), ethoxyresorufin (ER), Folin & Ciocalteu's phenol reagent, formaldehyde (37% solution formalin), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), methoxyresorufin (MR), nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), potassium phosphate monobasic anhydrous (KH_2PO_4), resorufin, sodium carbonate (Na_2CO_3), sodium citrate, sodium phosphate dibasic anhydrous (Na_2HPO_4) and Trisma® base were purchased from Sigma Chemical Co., USA.

Ammonium acetate was purchased from APS Finechem, Australia.

Acetic acid was purchased from J.T. Baker Inc., USA.

Carbon monoxide gas was purchased form T.I.G., Thailand.

Ethanol absolute and glycerol were purchased from Carlo Erba, USA.

Hydrochloric acid (HCl), diethyl ether, magnesium chloride ($MgCl_2$), methanol (Gradient grade), phenol, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4) and trichloroacetic acid (TCA) were purchased from Merck, Germany.

Sodium dithionite was purchased from Fluka Chemic, Japan.

Erythromycin stearate was obtained from Siam Pharmaceutical Co. Ltd., Thailand.

METHODS

Preparation of *M. loriformis* ethanolic extract

1.Four kilograms of dry powder of *M. loriformis* stem and leaves were purchased from Jaophaya Arpaipubate hospital, Prajinburi province, Thailand.

2. One hundred gram of the powder was extracted with 1L of 80% ethanol by percolator.

3. After 48 hours of percolation, the extracting fractions were collected, evaporated in a rotatory evaporator under reduced pressure at 52^{o} C and dried by freeze dryer.

4. The *M. loriformis* ethanolic extract was kept in tightly closed and light protected container at 2-8^{o} C.

Preliminary identification of *M. loriformis* ethanolic extract

1. Color reaction test (Jiratchariyakul W. and Soonthornchareonnon N.,1995)

1.1 Libermann-Burchard's test : identification for steroidal moiety

Half gram of the extract was shaked with 2 mL of chloroform. The chloroform fraction was evaporated in evaporating dish on a water bath. After cooling, 3 drops of acetic anhydride were added followed by 1 drop of concentrated sulfuric acid. Change of color was observed from pink, red, violet, navy blue and green respectively.

1.2 Test with ferric chloride TS : identification for phenolic compounds

One gram of the extract was added with 10 mL of hot distilled water followed by 2-3 drops of 10% sodium chloride. After the solution was filtered, 3-4 drops of 1% ferric chloride were added. A dark green solution was formed.

2. Thin layer chromatography (TLC) (วีณา จิรัจฉริยากูล, 2536)

System of TLC

1. Adsorbent : silica gel GF254 (Merck)
2. Developing solvent system : chloroform : methanol : water (15:7:1)
3. Detection : spray with 10% aqueous sulphuric acid, oven at 110^{o} C for 2-3 min. Spot became violet-red color.

4. *M. loriformis* ethanolic extract : 0.1 g of the extract was dissolved with 1 mL of chloroform : methanol (1:1). Ten microlitre of the solution was spotted on TLC plate
5. Reference solutions included
    1. Five microlitre of the solution of 0.1% β-sitosterol in chloroform : methanol (1: 1)
    2. Five microlitre of the solution of 0.1% sitosteryl glucoside (3-β-D-glucopyranosyl-24ξ-ethyl-cholest-5-ene) in chloroform : methanol (1: 1)

Effects of *M. loriformis* ethanolic extract on hepatic CYP and clinical blood chemistry

1. Animal treatment

The protocol of animal treatment used in this study was approved by the Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Appendices, page 115).

Thirty rats were randomly divided into 3 treatment groups. Each group contained 10 rats as followings:

1. Control group: Rats were given orally with 1 ml/kg/day distilled water for 30 days.
2. *M. loriformis* treatment group I: Rats were given orally with 0.1 g/kg/day of *M. loriformis* ethanolic extract for 30 days.
3. *M. loriformis* treatment group II: Rats were given orally with 1 g/kg/day of *M. loriformis* ethanolic extract for 30 days.

Note - The oral dosages of *M. loriformis* ethanolic extract (0.1 and 1.0 g/kg/day) used in this study were the doses that significantly inhibited azoxymethane-induced aberrant crypt focus formation both in the initiation stage (21-51%) and post-initiation stage (12-27%) in rat colon (Intiyot *et al.*, 2002).

- *M. loriformis* ethanolic extract was freshly dissolved with distilled water to make a concentration of 0.1 and 1 g/mL before administration to rats.
- The obtained solutions were not solely clear and appeared to be brown in color. The solutions were thoroughly mixing at the time of drawing into gavage tube for oral administration to rats.

- During the treatment period, body weight, food consumption and volume of drinking water were recorded every week.

2. Blood sampling for determination of hematology and clinical blood chemistry

At the end of the treatment, animals were fasted for 10 hours before anesthesized with diethyl ether by inhalation. Blood was drawn from left ventricle. Whole blood samples were used for hematological assays. The remaining blood samples were centrifuged for collecting serum samples which were used for determining various clinical blood chemistry.

2.1 Hematology

Hemoglobin (Hb), hematocrit (Hct), platelet count, white blood cell (WBC) count, red blood cell (RBC) count, % differential WBCs, RBC indices (mean corpuscular volume, MCV; mean corpuscular hemoglobin, MCH; mean corpuscular hemoglobin concentration, MCHC), and RBC morphology were determined by Professional Laboratory Management Corp Co., Ltd.

2.2 Clinical blood chemistry

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (SCr), total cholesterol, triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), glucose and uric acid in serum samples were analyzed by the Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok. In addition, total bilirubin, direct bilirubin and electrolytes (sodium, potassium, chloride) were determined by Professional Laboratory Management Corp Co., Ltd.

3. Liver microsome preparation

Rat liver microsomes were prepared according to the method described by Lake (1987) with some modifications.

Reagents

1. 0.1 M Phosphate buffer, pH 7.4

One litre of 0.1 M phosphate buffer, pH 7.4 consisted of 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4, and 11.50 g of KCl. The solution was adjusted to pH 7.4 with NaOH or HCl.

2. 0.1 M Phosphate buffer, pH 7.4, containing 20% v/v glycerol
3. 0.9% w/v NaCl

Procedure

1. After removing from the body, rat livers were quickly perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale.
2. The livers were rinsed with ice-cold 0.9% w/v NaCl, and blotted dry with gauzes.
3. The whole livers were weighed, cut into pieces, and homogenized with 3 times of its weight by phosphate buffer, pH 7.4.
4. The liver homogenates were centrifuged at 10,000 *g* for 30 minutes at 4 ^{o}C, using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei, and mitochondria.
5. The supernatants were transferred to ultracentrifuge tubes and further centrifuged at 100,000 *g* for 60 minutes at 4 ^{o}C, using refrigerated ultracentrifuge.
6. The pellets (microsomal subfractions) were resuspended with 5 mL of 0.1 M phosphate buffer, pH 7.4 containing 20% v/v glycerol. The microsomal suspensions were aliquoted, kept in microtubes, and stored at –80 ^{o} C until the time of enzyme activity assays.

4. Determination of protein concentrations

Liver microsomal protein concentrations were determined according to the method modified from the method of Lowry *et al.*, (1951).

Reagents

1. 2% w/v Na_2CO_3
2. 0.5 M NaOH
3. 2% w/v Sodium citrate
4. 1% w/v Cupric sulfate
5. 1 mg/mL BSA in 0.5 M NaOH
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent. The solution was prepared freshly in a sufficient amount for all tubes in the assay (6.5 mL of reagent was required for each tube). This reagent

comprised 2% w/v Na_2CO_3, 0.5 M NaOH, 2% w/v sodium citrate, and 1% w/v cupric sulfate solutions in a 100: 10: 1: 1 ratio, respectively.

Procedures

- All standard and unknown samples were prepared in duplicate.

1. The following reagents were added into each standard tube:

Standard tube	0	50	100	150	200	250	300	(μg)
1 mg/ml BSA	0	50	100	150	200	250	300	(μl)
0.5 M NaOH	500	450	400	350	300	250	200	(μl)

After addition of these reagents, each tube was mixed thoroughly.

2. To each unknown sample tube, 490 μl of 0.5 M NaOH and 10 μl of microsome were added and then mixed thoroughly.
3. Six and a half milliliter of freshly prepared working protein reagent was added to each tube.
4. The tubes were allowed to stand at room temperature for 10 minutes. Then, 200 μl of Folin & Ciocalteu's phenol reagent was added to each tube and immediately vortexed for a minimum of 30 seconds.
5. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbance of the solution was measured by spectrophotometer at 500 nm using the 0 μg standard tube as a blank.

Calculations

The average absorbance of each standard was plotted against its amount of protein. The best-fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve. The protein concentration was expressed in a unit of mg/ml or μg/μl by dividing its amount of protein with the volume of microsome used in the reaction.

5. Spectral determination of total CYP contents

Total CYP contents in microsomes were determined spectrophotometrically according to the method of Omura and Sato (1964).

Reagents

1. 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol
2. Solid sodium dithionite
3. Carbon monoxide

Procedures

1. Microsomes were diluted to 2 mg/ml with 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol.
2. Of the total volume of 5 ml diluted samples, a few grains of sodium dithionite were added with gentle mixing, then 2.5 ml each was transferred to the sample and reference cuvettes.
3. Both cuvettes were placed in a spectrophotometer, adjusted to zero and corrected to a baseline between 400 nm to 500 nm.
4. The sample cuvette was bubbled with carbon monoxide (approximately 1 bubble/second) for about 1 minute, immediately placed in the spectrophotometer again and scanned from 400 nm to 500 nm. The absorbance difference between 450 nm and 490 nm was recorded.

Calculations

Total CYP contents were calculated based on the absorbance difference between 450 nm and 490 nm as well as an extinction coefficient of 91 $mM^{-1}cm^{-1}$. Using Beer's law and an assuming a cuvette path length of 1 cm, total CYP contents were given by:

$$\text{Total CYP contents (nmol/mg protein)} = \frac{\text{Absorbance difference (450-490 nm) x 1000}}{\text{91 x concentration (mg/ml) of diluted sample}}$$

6. Determination of CYP activities

6.1 Alkoxyresorufin O-dealkylation assays

The catalytic activities of CYP1A1, CYP1A2, CYP2B1/2 were determined by measuring the rate of O-dealkylation of ER, MR, and BR & PR, respectively, using the method of Burke and Mayer (1974) and Lubet *et al.* (1985) with some modifications.

Reagents

1. 0.1 M Tris buffer, pH 7.4

2. 20 mM K_3PO_4

3. Resorufin and Alkoxyresorufins

   a) 0.5 mM MR (MW = 227)

   MR 1.135 mg was dissolved and made up to 10 ml with DMSO.

   b) 0.5 mM BR (MW = 303)

   BR 1.515 mg was dissolved and made up to 10 ml with DMSO.

   c) 0.5 mM ER (MW = 241)

   ER 1.205 mg was dissolved and made up to 10 ml with DMSO.

   d) 0.5 mM PR (MW = 283)

   PR 1.415 mg was dissolved and made up to 10 ml with DMSO.

   e) 0.5 mM Resorufin (MW = 235)

   Resorufin 1.175 mg was dissolved and made up to 10 ml with DMSO.

4. NADPH regenerating system

   NADPH regenerating system comprised the solutions as following:

   a) 0.1 M NADP, pH 7.4

   NADP 0.765 g was dissolved and made up to 10 ml with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contained 1 mmol of NADP)

   b) 0.5 M G6P, pH 7.4

   G6P 1.41 g was dissolved and made up to 10 ml with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contained 5 mmol of G6P)

   c) 0.3 M $MgCl_2$, pH 7.4

   $MgCl_2$ 609.93 mg was dissolved and made up to 10 ml with 20 mM K_3PO_4. The solution pH was adjusted to 7.4 with HCl or NaOH. (10 µl contained 3 mmol of $MgCl_2$)

   d) G6PD

   G6PD was diluted to 100 units per ml with 20 mM K_3PO_4, pH 7.4 (10 µl contained 1 unit of G6PD)

On the experiment, the mixture of 0.1 M NADP, 0.5 M G6P and 0.3 M $MgCl_2$ solutions was freshly prepared in the ratio of 1: 1: 1, respectively. For the reaction volume of 1 ml, 30 µl of this mixture was used for microsomal preincubation and 10 µl of G6PD was added to initiate the reaction.

Procedures

1. For the final reaction volume of 1.5 ml, microsomal sample was diluted with 0.1 M Tris buffer, pH 7.4 to measure out 300 μg of protein.
2. The following solutions were added for the reaction preincubation
    a) 0.1 M Tris buffer, pH 7.4
    b) 0.5 mM Alkoxyresorufin 15 μl
    c) The mixture of NADPH regenerating system 45 μl containing
        - 0.1 M NADP 15 μl
        - 0.5 M G6P 15 μl
        - 0.3 M $MgCl_2$ 15 μl
    d) Varied volume of diluted microsomal suspension containing 300 μg of microsomal protein
3. Three tubes were used for each microsomal sample. One was a sample blank and the other two were samples.
4. Each tube was preincubated in a 37°C shaking water bath for 2 minutes.
5. The reaction was started by adding 15 μl of G6PD. Sample blank was added with 15 μl of 0.1 M Tris buffer instead of G6PD.
6. After 5 minute incubation, the reaction was stopped with methanol 1.5 ml (gradient grade).
7. The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.
8. A resorufin standard curve was constructed using the duplicated resorufin concentrations of 0.003, 0.006, 0.013, 0.025, 0.050 and 0.075 nmol/ml.
9. The procedure was verified by varying amount of microsomal protein used in the reaction (100, 200, 300 μg of microsomal protein/ml of the reaction mixture). The liver microsome was prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Soucek and Gut, 1992). The reaction was performed as mentioned above using BR as a substrate. Correlation coefficient (r^2) between amount of microsomal protein and fluorometric absorbance was 0.9992 (Appendices, page 112).

Calculations

Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of incubation (5 minutes) and an amount of microsomal protein (300 μg) used in the reaction. The units were expressed as pmol/ mg protein/ min.

6.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP2E1 was determined based on the rate of aniline 4-hydroxylation, using the method of Schenkman *et al.* (1967). Aniline hydrochloride was used as a specific substrate of CYP2E1.

Reagents

1. 10 mM Aniline hydrochloride

   Aniline HCl 129.6 mg was dissolved and made up to 100 ml with double distilled water. The solution was stored in a dark bottle.

2. 6% w/v TCA

   TCA 60 g was made up to 1 L with double distilled water.

3. 20% w/v TCA

   TCA 200 g was made up to 1 L with double distilled water.

4. 1% w/v Phenol

   Phenol 20 g and NaOH 40 g were made up to 2 L with double distilled water.

5. 1 M Na_2CO_3

   Anhydrous Na_2CO_3 106 g was made up to 1 L with double distilled water.

6. 10 μM 4-aminophenol

   4-Aminophenol 0.0365 g were dissolved and made up to 10 ml with double distilled water. This aminophenol solution 0.1 ml was added into TCA 15 g and made up to 250 ml with double distilled water.

7. NADPH regenerating system (The preparation was described in 6.1)

Procedures

1. To make a final volume of 2 ml reaction incubation, each preincubation mixture composed of microsome containing 5 mg protein, 500 μl of aniline HCl, 30 μl of NADPH regenerating system and Tris buffer, pH 7.4 qs to 1980 μl.

2. All tubes were preincubated in a shaker bath at 37°C for 2 minutes.
3. The catalytic reaction was started by adding 20 μl of G6PD. For sample blank, 20 μl of Tris buffer, pH 7.4 was used instead of G6PD.
4. After the microsomal samples were incubated for 30 minutes, the reaction was terminated by adding of 1 ml of 20% TCA. The tubes were placed on ice for 5 minutes.
5. The solution was centrifuged at 3,000 r.p.m. for 10 minutes. One milliliter of supernatant was transferred to another tube, then 1 ml of phenol and 1 ml of Na_2CO_3 were added to each tube and mixed homogeneously.
6. All tubes were allowed to stand at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 630 nm.
7. For standard curve, 1 ml of each standard 4-aminophenol solution (0, 2, 4, 6, 8 and 10 μM) was carried out instead of the supernatant in step 5.
8. The procedure was verified by varying amount of rat microsomal protein used in the reaction (2.5, 5.0, 7.5 mg of microsomal protein/ 2 ml of the reaction mixture). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 0.9985 (Appendices, page 113).

Calculations

Rate of aniline 4-hydroxylation was calculated by dividing the amount of 4-aminophenol formed (nmol), with the mg of microsomal protein used and 30 minutes of the incubation period. The units were expressed as nmol/ mg protein/ min.

6.3 Erythromycin N-demethylation assay

The catalytic activity of CYP3A was determined based on the rate of erythromycin N-demethylation, using the method of Nash *et al.*, (1953) and Friedli G. (1992). Erythromycin stearate was used as specific substrate of CYP3A.

Reagents

1. Formaldehyde standard (M.W. 30, 37% solution formalin)
2. 20 mM K_3PO_4, pH 7.4
3. 10 mM Erythromycin stearate (M.W = 1,018.4)

Erythromycin stearate 0.1018 g was dissolved and made up to 10 ml with double distilled water

4. NADPH regenerating system (The preparation was described in 6.1)
5. .12.5% w/v TCA

   TCA 12.5 g was made up to 100 ml with double distilled water.
6. Nash reagent

   Nash reagent comprised 30 g of ammonium acetate, 0.4 ml of acetylacetone, 0.6 ml of glacial acetic acid and double distilled water qs. to 100 ml.

Procedures

All standard and unknown samples were prepared in duplicate.

1. To make a final volume of 1 ml microsomal incubation, each preincubation mixture was composed of microsome containing 4 mg of protein, 100 μl of 10 mM erythromycin stearate, 45 μl of NADPH regenerating system and 20 mM phosphate buffer, pH 7.4 qs. to 985 μl.
2. All tubes were preincubated in a shaker bath at 37°C for 3 minutes.
3. The reaction was started by adding 15 μl of G6PD. Sample blank was added 20 mM phosphate buffer, pH 7.4 instead of G6PD.
4. After 10 minute incubation, the reaction was stopped with 500 μl of ice-cold 12.5% w/v TCA.
5. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes. One milliliter of the supernatant was transferred to another new tube. One milliliter of freshly prepared Nash reagent was added to each tube and mixed homogeneously.
6. All tubes were warmed in a shaker bath at 50°C for 30 minutes.
7. The absorbance of the mixture was measured spectrophotometrically at 412 nm.
8. The formaldehyde standard curve was constructed by adding 1 ml of formaldehyde standard at concentrations of 0.0156, 0.0313, 0.0625, 0.125 and 0.25 μmol/ml with 1 ml of Nash reagent and mixed homogeneously. The absorbance of the mixture was measured spectrophotometrically at 412 nm.
9. The procedure was verified by varying amount of microsomal protein used in the reaction (2, 4, 8 mg of microsomal protein/ml of the reaction mixture). The liver microsome was prepared from phenobarbital-incuced rats given phenobarbital at the

dosage of 80 mg/kg/day intraperitoneally for 3 days (Soucek and Gut, 1992). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 1 (Appendices, page 114).

- Calculations

Rate of erythromycin N-demethylation was calculated by determining the amount of formaldehyde formed, divided by mg of protein used and 10 minutes of total reaction period. The unit was expressed as nmole/mg protein/min.

7. Data analysis

All numeric data were presented as mean ± standard error of the mean (SEM). A one way analysis of variance (ANOVA) and Student-Newman-Keuls test were used for statistical comparison at a significant level of $p < 0.05$.