CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Thirty male Wistar rats weighing between 150-200 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornprathom, Thailand. Animals were housed one 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°C, respectively. During the time of experimentation, body weight of each rat, food consumption, volume of drinking water were recorded every 5 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), ethylenediaminetetraacetic acid (EDTA), 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.

Glycerol was 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

Dextran was obtained from Thai Otsuka Pharmaceutical Co. Ltd., [S. Charoen Bhaesaj], Thailand

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

Methods

Preparation of *M. citrifolia*

1. Two kilograms of fresh *M. citrifolia* fruits were harvested from Phanomsarakam, Chachoengsao Province, Thailand. All fruits used in this study were collected during June – July 2002.
   (Approximately twenty fresh fruits of *M. citrifolia* weighed two kilograms.)
2. The fruits were rinsed with water, drained and sliced into small pieces. After added with 1000 ml of distilled water, crushed fruits were blended to obtain *M. citrifolia* fruit juice by using a blender.
3. The mixture was centrifuged at 2400 r.p.m. for 10 minutes.
4. The supernatant was filtered through 3 layers of whatman® filter papers No.1.
5. The filtrate was lyophilized to dryness using Lyophilizer.
6. The yield from step 5 was ground to powder using motar and pestle. The powder of *M. citrifolia* was kept in tight container at 2-8 °C until the time of extract characterization and animal treatment.

According to the method mentioned above, two kilograms of fresh *M. citrifolia* fruits resulted in the freeze-dry powder of 60 g. Therefore, fresh *M. citrifolia* fruit yielded approximately 3% w/w of lyophilized *M. citrifolia* powder. The freeze-dry powder appeared dark-brown color, unpleasant taste and foul odor.

Partial characterization of *M. citrifolia*

Phenol-sulphuric acid (PSA) test was used to quantify carbohydrate content in *M. citrifolia* fruit extract. The method was modified from the method of Keleti and Lederer (1974).

Reagents

1. 1 mg/ml Dextran
2. Concentrated sulphuric acid
3. 5% v/v Liquid phenol

Procedure

Each concentration of standard and unknown sample was prepared in duplicate.

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

Standard tube	0	10	20	30	40	μg
1 mg/ml Dextran	0	10	20	30	40	(μl)
H_2O	200	190	180	170	160	(μl)

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

2. Each unknown sample tube contained 200 μl of *M. citrifolia* (0.1 - 0.25 mg/ml in H_2O) or (20, 25, 40 and 50 μg of *M. citrifolia* powder in H_2O).
3. After 200 μl of 5% v/v of liquid phenol was added, each tube was mixed thoroughly.
4. Concentrated H_2SO_4 (1 ml) was rapidly added and the mixture was incubated for 10 minutes at room temperature.
5. Each tube was incubated in a 37°C shaking water bath for 15 minutes. The absorbance of the mixture was measured spectrophotometrically at 490 nm.

Calculations

The average absorbance of each standard was plotted against its amount of carbohydrate. The best-fit regression line was drawn through the points. The amount of carbohydrate in each unknown sample was obtained by comparing its absorbance against the standard curve. The carbohydrate content was expressed in a unit of mg of carbohydrate/mg of lyophilized powder of *M. citrifolia*.

Results from the PSA test showed the *M. citrifolia* fruit extract contained 0.47 ± 0.01 mg (mean ± SEM; n = 4) of carbohydrate per mg of the extract using dextran as a standard. A representative linear relationship between carbohydrate contents in *M. citrifolia* and amount of *M. citrifolia* fruit extract was shown in Figure 3.1.

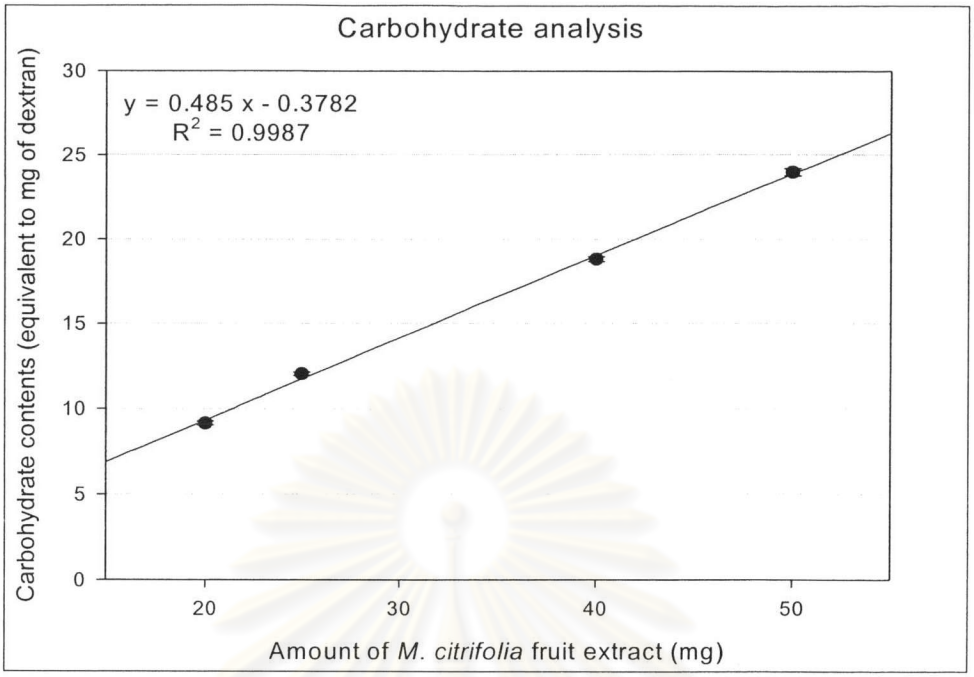

Figure 3.1 A representative linear relationship between carbohydrate contents in *M. citrifolia* and amount of *M. citrifolia* fruit extract; y = 0.485x – 0.3782 (R^2 = 0.9987) whereas Y = carbohydrate contents (equivalent to mg of dextran), X = amount of *M. citrifolia* fruit extract (mg). The individual mark represented the value of mean ± SD of n = 2.

Effects of *M. citrifolia* on hepatic CYP and clinical blood chemistry

1. Animal treatment

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

1. Control group: Rats were given orally with 1 ml/kg/day distilled water for 30 days.
2. *M. citrifolia* treated group I: Rats were given orally with 600 mg/kg/day of *M. citrifolia* for 30 days.
3. *M. citrifolia* treated group II: Rats were given orally with 1200 mg/kg/day of *M. citrifolia* for 30 days.

Note
- The oral dosages of *M. citrifolia* (600 mg/kg/day & 1200 mg/kg/day) used in this study were estimated corresponding to the dosage of *M. citrifolia* that demonstrated a cancer preventive effect (Wang and Su, 2000).
- *M. citrifolia* was prepared as solutions containing 10, 20 and 40 % w/v of *M. citrifolia* in H_2O.
- During the treatment period, body weight, food consumption and volume of drinking water were recorded at every five days.

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

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 for an approximate volume of 5 ml. Five hundred microliters of whole blood was transferred to a microtube containing a few grains of EDTA sodium and mixed thoroughly. The remaining blood was transferred to another tube, allowed to stand in a slope posture in order to collect the highest amount of serum. Whole blood and serum were investigated for the hematology and clinical blood chemistry, respectively.

2.1 Clinical blood chemistry

Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, blood urea nitrogen (BUN), serum creatinine (SCr), total cholesterol, triglyceride (TG),

high density lipoprotein cholesterol (HDL-C), glucose, sodium, potassium and chloride in serum samples were analyzed by the Faculty of Topical Medicine, Mahidol University, Bangkok.

2.2 Hematology

Hemoglobin (Hb), hematocrit (Hct), platelet count, white blood cell (WBC) count, % differential WBCs and RBC morphology were determined by the Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok.

3. Liver microsome preparation

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

After collecting blood sample, liver was immediately removed and perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale. Then, the liver was perfused with ice-cold 0.9% w/v NaCl and blotted dry with gauze. The whole liver was weighed, cut into pieces and homogenized with 3 times of its weight by phosphate buffer, pH 7.4. The liver homogenate was centrifuged at 10,000 *g* for 30 minutes at 4°C using refrigerated superspeed centrifuge. The supernatant (S9, post mitochondrial fraction) was transferred into ultracentrifuge tubes and centrifuged at 100,000 *g* for 60 minutes at 4°C using refrigerated ultracentrifuge. The pellets (microsomal subfraction) were resuspended with 5 ml of phosphate buffer, pH 7.4 containing 20% glycerol. Microsomal subfractions (or microsomes) were aliquoted to microtubes and stored at -80°C until the time of enzyme 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 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:

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

6. Determination of CYP activities

6.1 Alkoxyresorufin O-dealkylation assays

The catalytic activities of CYP1A1, CYP1A2, CYP2B1/2B2 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, pH7.4
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 μl 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 μl 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 μl 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 1225 μl
   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) Diluted microsomal suspension 200 μl (contained 300 μg of 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.
7. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes.
8. Using an autopipette, clear supernatant of each tube was transferred to a cuvette.

9. The absorbance was read on the fluorescence spectrophotometer by using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.
10. A resorufin standard curve was constructed using the duplicated resorufin concentrations of 0.002, 0.005, 0.010, 0.050 and 0.200 nmol/ml.
11. Rate of dealkylation was calculated by dividing the amount of resorufin formed, with the mg of microsomal protein used and 5 minutes of the incubation period.

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, J.B., Remmer, H., and Estabrook, R.W. (1967).

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 2 L with double distilled water.
6. 10 μM 4-aminophenol

   4-aminophenol 0.0365 g were dissolved in methanol 1 ml, then made up to 10 ml with double distilled water. This aminophenol solution 0.1 ml was taken and then added into TCA 15 g. Finally, 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 5 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.

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.

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).

Reagents

1. Formaldehyde standard (M.W. 30, 37% solution formalin)
2. 20 mM KH_2PO_4

3. 20 mM Potassium phosphate buffer, pH 7.4

   Fifty milliliters of 20 mM KH_2PO_4 was added with 39.1 ml of 0.02 M NaOH and double distilled water qs. to 200 ml. The solution was adjusted to pH 7.4 with HCl and NaOH.

4. 10 mM Erythromycin stearate

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

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

6. 12.5% w/v TCA

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

7. 0.02 M NaOH

8. 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 8 mg of protein, 100 µl of 10 mM erythromycin stearate, 30 µl of NADPH regenerating system and 20 mM phosphate buffer, pH 7.4 qs. to 1000 µl.
2. All tubes were preincubated in a shaker bath at 37°C for 3 minutes.
3. The reaction was started by adding 10 µl of G6PD. Sample blank was added with10 µl of 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 performed the procedure in the same maner as the sample tubes as described in 5.

Calculations

Rate of erythromycin N-demethylation was calculated by determining the amount of formaldehyde formed, extrapolating to mg of protein used and dividing by 10 minutes of total reaction period.

7. Data analysis

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