CHAPTER III

MATERIALS AND METHODS

Materials

Animals

Forty adult male and female Wistar rats of body weight between 250-300 g were obtained from the Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed three per cage at the Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health, Bangkok and acclimatized for at least seven days prior to the experiment. All animals were maintained at 25 °C on a 12-hour alternate light-dark cycle in controlled humidity room and allowed freely access to food (C.P. Company) and drinking water.

Instruments

The following instruments were used in the experiment

1. Auto pipettes 20, 100, 200, 1000 and 5000 µl (Gibson, France)
2. Centrifuge (Kokusan, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Metabolic shaker bath (Memert, Japan)
5. pH meter (Beckman Instruments, USA)
6. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessel (Heidolph, Germanny)
7. Refrigerated superspeed centrifuge (Beckman Instruments, USA)
8. Refrigerated ultracentrifuge (Beckman Instruments, USA)
9. Sonicator (Nickel-Electro, England)
10. Spectrophotometer (Jasco, Japan)
11. Surgical equipment
12. Timer
13. Ultra-low temperature freezer (Forma Scientific Inc., USA)
14. Vortex mixer (Clay Adams, USA)

Chemicals

4-aminophenol, acetylacetone, ammonium acetate, aniline hydro chloride, benzyloxyresolufin (BR), bovine serum albumin (BSA), chloroform, cupric sulfate, dimethylsulfoxside (DMSO), ethylenediaminetetraacetic acid (EDTA), ethoxyreso rufin (ER), folin&Ciocalteu's phenol reagent, formaldehyde (37% solution formalin), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), methoxy resorufin (MR), nicotinamide adenine dinucleotide phosphate (NADP), pentoxy resorufin (PR), potassium dihydrogen phosphate (KH_2PO_4), potassium phosphate (K_3PO_4), resorufin, sodium carbonate (Na_2CO_3), sodium citrate, Trisma®base were purchased from Sigma Chemical Co., Ltd., USA

Acetic acid, diethyl ether, hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), methanol HPLC grad (MeOH), phenol, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxyl (NaOH), sulfuric acid (H_2SO_4), trichloroacetic acid (TAC) were purchased from Merck, Germany.

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

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

Glycerol was purchased from Carlo Erba, USA.

Phenobarbital (Gardinal®) was obtained from Siriraj Hospital.

Sodium dithionite was purchased from Fluka Chemical, Japan.

Plant extract

The standardized extract of *C. asiatica* was prepared by Assoc. Prof. Ekarin Saifah and co-worker, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand.

Characteristic of the standardized extract of *C. asiatica*: White to off-white powder containing triterpenoid glycosides not less than 80% and the ratio of madecassoside and asiaticoside content was within 1.5 ± 0.05.

Methods

1. An *in vivo* study: Animal treatment

In each sex group, male and female: Forty rats were randomly divided into 4 groups. Each group comprised 10 rats.

1. Control group: Rats were given distilled water orally at the dosage of 10 ml/kg/day for 90 days.

2. The standardized extract of *C. asiatica* group 1: Rats were given the standardized extract of *C. asiatica* orally at the dosage of 10 mg/kg/day for 90 days.

3. The standardized extract of *C. asiatica* group 2: Rats were given the standardized extract of *C. asiatica* orally at the dosage of 100 mg/kg/day for 90 days.

4. The standardized extract of *C. asiatica* group 3: Rats were given the standardized extract of *C. asiatica* orally at the dosage of 1000 mg/kg/day for 90 days.

The standardized extract of *C. asiatica* for animal administration were prepared by dissolving or suspending 10, 100, 1000 mg of the standardized extract of *C. asiatica* with distilled water to make a concentration of 10, 100, 1000 mg/ml, respectively.

At the end of treatment, rats were anesthetized by diethyl ether inhalation and sacrified by cervical dislocation. Livers were removed from the bodies, for preparation of microsomes.

2. Hepatic microsomal preparation

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

<u>Reagent</u>

1. 0.1 M Phosphate buffer, pH 7.4
   One liter 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 and HCL.
2. 0.1 M Phosphate buffer, pH 7.4, containing 20% glycerol
3. 0.9% w/v NaCl

<u>Procedure</u>

1. After removing from the body, rat livers were perfuse with ice-cold 0.9% w/v NaCl until the entire organ become pale.
2. The livers were rinsed with ice-cold 0.9% w/v NaCl, and blotted dry with gauzes.
3. Each liver was weighed, cut to in pieces and homogenized with 20 ml of 0.1 M phosphate buffer, pH 7.4.
4. The liver homogenates centrifuged at 10,000 *g* for 30 minutes at 4°C,

using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei and mitochondria.

5. The supernatants (S9, post mitochondrial fraction) were transferred into ultracentrifuge tube and further centrifuged at 100,000 *g* for 60 minutes at 4°C, using refrigerated ultracentrifuge.
6. The pellets (microsomal subfraction) were resuspended with 5 ml of 0.1 M phosphate buffer, pH 7.4 containing 20% glycerol. The microsomal suspensions aliquoted, kept in microtubes, and stored at –80°C until the time of enzyme assays.

3. Determination of protein concentration in liver microsomes

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 freshly prepared in a sufficient amount for all tubes in the assay (6.5 ml of the solution 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

1. Tubes were labeled in duplicate for 6 standards (0, 100, 200, 300, 400 and 500 μg) and for each unknown sample.
2. The following reagents were added into each standard solution tube:

Standard tube	0	100	200	300	400	500	(μg)
1 mg/ml BSA	0	100	200	300	400	500	(μl)
0.5 M NaOH	500	400	300	250	100	000	(μl)

Each tube was mixed thoroughly, after addition of these reagents.

3. To each unknown sample tube, 490 μl of 0.5 M NaOH and 10 μl of microsome sample were added and then mixed thoroughly.
4. After 6.5 ml of working protein reagent was added to each tube in the assay, the tubes were allowed to stand at room temperature for 10 minutes.
5. While 200 μl of Follin & Ciocalteu's phenol reagent was added to each tube in the assay, the tube was vortexed thoroughly for a minimum of 30 seconds.
6. After the tubes were allowed to stand at room temperature for 30 minutes, the absorbance of the solution was measured by spectrophotometer against the 0 μg standard at 500 nm.

Calculations

1. 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.
2. The protein concentration was expressed in a unit of mg/ml or μg/μl by dividing its amount of protein (from step 1) with the volume of microsome used in the reaction.

4. Determination of total CYP contents in liver microsomes

Rat hepatic microsomal total CYP contents 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. Microsomal sample was diluted to 2 mg/ml with 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol.
2. After a few grains of sodium dithionite was added to the 5 ml diluted sample, the solution was mixed and 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 and 500 nm.

4. Immediately after the sample cuvette was bubbled with carbon monoxide (approximately 1 bubble/second) for 1 minute, the cuvette was placed back to the spectrophotometer 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:

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

5. Analysis of hepatic microsomal CYP activities

5.1 Alkoxyresorufin O-dealkylation assay

Rate of hepatic microsomal alkoxyresorufin O-dealkylation was determined according to the method of Burke and Mayer (1974) and Lubet et al. (1985) with some modifications. Benzyloxyresorufin (BR) and pentoxyresorufin (PR) were used as selective substrates of CYP 2B1/2B2. Ethoxyresorufin (ER) and methoxyresorufin (MR) were used as selective substrates of CYP 1A1 and CYP 1A2, respectively.

Reagents

1. 0.1 M Tris buffer, pH 7.4
2. 20 mM K_3PO_4, pH 7.4
3. Resorufin and Alkoxyresorufins
   a) 0.5 mM MR (MW = 227)
      MR 1.135 mg was dissolved with 10 ml of DMSO.
   b) 0.5 mM BR (MW = 303)
      BR 1.515 mg was dissolved with 10 ml of DMSO.
   c) 0.5 mM ER (MW = 241)
      ER 1.205 mg was dissolved with 10 ml of DMSO.
   d) 0.5 mM PR (MW = 283)
      PR 1.415 mg was dissolved with 10 ml of DMSO.
   e) 0.5 mM Resorufin (MW = 303)

Resorufin 1.175 mg was dissolved with 10 ml of 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 with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contained 1 mmole-of NADP).

   b) 0.5 M Glucose 6-phosphate (G6P), pH7.4

      G6P 1.41 g was dissolved with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contained 5 mmole of G6P).

   c) 0.3 M $MgCl_2$, pH 7.4

      $MgCl_2$ 609.93 mg was dissolved with 20 mM K_3PO_4 and made up to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contained 5 mmole of $MgCl_2$).

   d) Glucose 6-phosphate dehydrogenase (G6PD), pH 7.4

      G6PD was dissolved with 20 mM K_3PO_4 and made up to 10 ml, (10 μl contained 1 unit of G6PD).

<u>Procedures</u>

1. Microsomal sample was diluted with 0.1 M Tris buffer, pH 7.4 to measure out 150 μg of protein (100 μl of microsome was diluted with 0.1 M Tris buffer, pH 7.4 qs to 5000 ml) for the final reaction mixture volume of 1.5 ml.
2. The following reagents were added for the reaction preincubation.
   a) 0.1 M Tris buffer, pH 7.4
   b) 15 μl of 0.5 mM Alkoxyresorufin
   c) 45 μl of the mixture solution of regenerating system containing
      i. 15 μl of 0.1 M NADP
      ii. 15 μl of 0.5 M G6P
      iii. 15 μl of 0.3 M $MgCl_2$
   d) Varied volume of diluted microsomal suspension containing 150 μg of microsomal protein.
3. Three tubes were needed for each microsomal sample (one is the sample blank tube and the others are sample tubes).

4. All tubes were preincubated in a 37 °C shaking water bath for 2 minutes.
5. The reaction was started by an addition of 15 μl of G6PD. For sample blank, add 15 μl of 0.1 M Tris buffer was added instead of G6PD.
6. After 5 minutes incubation, the reaction was stopped by adding 1.5 ml of methanol (HPLC 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 concentration of 0.00625, 0.0125, 0.025, 0.05, 0.075, 0.1, 0.2 nmole/ml of resorufin.
9. Rate of alkoxyresorufin-dealkylation was calculated by dividing the amount of resorufin formed by the time of 5 minutes incubation and amount of microsomal protein (mg) used in reaction. The unit of the enzyme activity was expressed as pmol/mg protein/min.
10. The procedure was verified by performing these following assays.

10.1 Linearity assay

10.1.1 For the EROD and MROD reactions, varying amounts of microsomal protein were used in the reaction (50, 100, 150, 200 and 250 μg of microsomal protein/1 ml of the reaction mixture). Liver microsomes were prepared from β-naphthoflavone (β-NF)-induced rats given β-NF at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Gibson and Skette, 1998; Hammond et al., 1997). The reactions were performed as mentioned above, using ER and MR as selective substrates of CYP1A1 and CYP1A2, respectively. Coefficient of determination (r^2) between amounts of microsomal protein and absorbance of the product (resorufin) were 0.9942 and 0.9924 when ER (Figure A1) and MR (Figure A2) were used as selective substrates, respectively.

10.1.2. For the BROD and PROD varying amounts of microsomal protein were used in the reaction (50, 100, 150 and 200 μg of microsomal protein/1ml of the reaction mixture). Liver microsomes prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Tredger et al., 2002; Soucek and Gut, 1992). The

reaction was performed as mentioned above using BR and PR selective substrates. Coefficient of determination (r^2) between amounts of microsomal protein and the corresponding absorbance of the product (resorufin) were 0.9955 (Figure A3) and 0.9837 (Figure A4) when BR and PR were used as selective substrates, respectively.

10.2 The capacity of the procedure to detect the enzyme induction

10.2.1. For the CYP1A1, CYP1A2 induction, microsomes were prepared from livers of β-NF-induced rats. Four rats for each group were received β-NF at the dose of 80 mg/kg/day intraperitoneally for 3 days or corn oil for the treatment group and the control group, respectively. Liver microsomes were prepared and the reactions were performed using ER and MR as the selective substrates for CYP1A1 and CYP1A2, respectively. EROD and MROD activities were significantly higher in the β-NF-treated group as compared to the control group (Figure A7 and Figure A8, respectively)

10.2.2. For the CYP2B1/2B2 induction, microsomes were prepared from livers of phenobarbital-induced rats. Four rats for each group were treated with phenobarbital at the dosage of 80 mg/kg/day intraperioneally for 3 day or normal saline, for the treatment group and the control group, respectively. The reactions were performed using BR and PR as selective substrates for CYP2B1/2B2. BROD and PROD activities were significantly higher in the phenobarbital-treated group as compared to the control group (Figure A9 and Figure A10, respectively).

5.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP 2E1 was determined based on the rate reaction of aniline 4-hydroxylation according to the method of Schenkman, Remmer and Estabrook (1967). Aniline hydrochloride was used as a selective substrate in this reaction.

Reagents

1. 10 mM Aniline hydrochloride

   Aniline hydrochloride 129.6 mg was dissolved and made up to 100 ml with double distilled water. The solution was stored in a light-protection bottle.

2. 6% w/v Trichloroacetic acid

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

3. 20% w/v Trichloroacetic acid

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

4. 1% w/v Phenol

   Phenol 20 g and 40 g of NaOH 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 of 4-aminophenol

   4-aminophenol 0.0365 g were dissolved in methanol 1 ml, then made up to 10 ml with water. Then 0.1 ml of this solution was added to 15 g of trichloroacetic acid and made up to 250 ml with double distilled water.

7. NADPH regenerating system

Procedures

1. Each 2 ml of the reaction mixture comprised microsome containing 5 mg of protein, 500 μl of 10mM aniline hydrochloride, 45 μl of NADPH regenerating system and Tris buffer, pH 7.4 qs to 1980 μl.
2. All tube were preincubated in a shaker water bath at 37 °C for 2 minutes.
3. The catalytic reaction was initiated by an addition of 20 μl of G6PD.
4. After 30 minutes of incubation the reaction was terminated by adding 1 ml of ice-cold 20% w/v trichloroacetic acid. The reaction tubes were placed on ice for at least 5 minutes.
5. The solution was then centrifuged at 3,000 r.p.m. for 10 minutes.
6. After 1 ml of the supernatant was transferred to a new tube, 1 ml 10% phenol and 1 ml of 1 M Na_2CO_3 were added to each tube. The solution was mixed and kept at room temperature for 30 minutes.
7. The absorbance was measured by spectrophotometer at a wavelength of 630 nm.

8. A standard curve was carried out using 5 concentrations of 4- aminophenol standard solution (0, 2, 4, 6, 8 and 10 μM) following the procedure from step 6 in the same manner as sample.
9. The procedure was verified by varying amounts of microsomal protein (prepared from a normal rat) used in the reaction (1, 2, and 6 mg of microsomal protein/ 1 ml of the reaction mixture). The reaction was performed as mentioned above. Coefficient of determination (r^2) between amounts of microsomal protein and the corresponding absorbance of the product (4-aminophenol) was 0.9920 (Figure A5).

Calculations

Rate of aniline 4-hydroxylation was calculated by dividing amount of product the formed (4-aminophenol) in a unit of nmole by the time of incubation (30 minutes) and amount of microsomal protein (mg) used in the reaction. The unit was expressed as nmole/mg protein/min.

5.3 Erythromycin N-demethylation assay

Rate of hepatic microsomal erythromycin N-demethylation was determined using the method of Nash et al. (1953) and Friedli (1992). Erythromycin stearate was used as a selective substrate of CYP3A.

Reagents

1. Formaldehyde (MW = 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 made up to 200 ml with water, then adjusting pH to 7.4 with HCl or NaOH.
4. 10 nM Erythromycin stearate

   Erythromycin stearate 0.1018 g was dissolved with ultrapure water and made up to 10 ml.
5. NADPH regenerating system
6. 12.5% w/v TCA

   TCA 12.5 g was made up with double distilled water to 100 ml.
7. 100 units/ml G6PD
8. 0.02 M NaOH

9. Nash reagent

   Nash reagent was freshly prepared, the solution comprised 30 g of ammonium acetate, 0.4 ml of acetylacetone, 0.6 ml of glacial acetic acid and water qs. to 100 ml.

Procedures

Each unknown sample as performed in duplicate.

1. To make a final volume of 1 ml microsomal incubation reaction, the preincubation mixture was composed of microsome containing 4 mg of microsomal 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 in sample tubes, whereas 840 µl of 20 mM phosphate buffer, pH 7.4 was substituted for microsomes in each sample blank tube.
2. All tubes were preincubated in a shaker water bath at 37°C for 3 minutes.
3. The reaction was initiated by an addition of 15 µl of G6pD. For the sample blank, 15 µl of 20 mM phosphate buffer, pH 7.4 was added instead of G6PD.
4. The reaction was stopped by adding 500 µl of ice-cold 12.5% w/v TCA after 10 minutes of the incubation.
5. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes.
6. One millilitre of the supernatant was transferred to another new tube. One millilitre of Nash reagent was added to each tube and mixed well by vortex mixer
7. All tubes were warmed in a shaker bath at 50°C for 30 minutes.
8. Absorbance of the mixture was measured by spectrophotometer at the wavelength of 412 nm.
9. A formaldehyde standard curve was constructed by adding 1 ml of formaldehyde solution at concentrations of 0.00468, 0.00937, 0.01875, 0.0375, 0.750 and 0.150 µmol/ml with 1 ml of Nash reagent and performed the procedure in the same manner as the sample tubes described above.
10. The procedure was verified by varying amounts microsomal protein used in the reaction (1, 2, 4 and 6 mg of microsomal protein/1 ml of the reaction mixture). Liver microsomes were prepared from phenobarbital-induced rats given phenobarbital at dosage of 80 mg/kg/day

intraperitoneally for 3 days (Tredger et al., 2002; Soucek and Gut, 1992). The reaction was performed as mentioned above. Coefficient of determination (r^2) between amounts of microsomal protein and the corresponding absorbance of the product was 0.9939 (Figure A6).

6. An *In vitro* study

Inhibition effects of the standardized extract of *C. asiatica* on CYP catalytic activities were investigated *in vitro* by performing the co-incubation of the standardized extract of *C. asiatica* and a selective substrate of each CYP isoform.

6.1 Inhibitory effect of the standardized extract of *C. asiatica* on CYP1A1, CYP1A2 and CYP2B1/2B.

Procedure

1. Various concentrations of the standardized extract of *C. asiatica* were prepared in such a way that equal volume (150 µl) of the solutions were used in the reaction. The final concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0, 100, 250, 500, 1000, 2000 µg/ml.
2. Fifteen microliter of BR and PR was used as a selective substrate of CYP 2B1/2B2 for 1.5 ml reaction mixture. ER and MR were use as selective substrate of CYP1A1 and CYP1A2, respectively.
3. Liver microsomes prepared from rats in the phenobarbital treatment group were used in the reaction of BROD and PROD whereas β-NF induced rat microsomes were used in the reactions of EROD and MROD.
4. The reactions were performed in the same manner as described earlier for alkoxyresorufin o-dealkylation assay except that various concentrations of the standardized extract of *C. asiatica* were added concomitantly with the selective substrate.
5. Enzymes activities were plotted against the corresponding concentrations of the standardized extract of *C. asiatica* added in to the reaction and the median inhibitory concentrations (IC_{50}) were calculated.

6.2 Inhibitory effect of the standardized extract of *C. asiatica* on CYP2E1

Procedure

1. Various concentrations of the standardized extract of *C. asiatica* were Prepared in such a way that equal volume(200) µl of the solutions were used in the reaction. The final concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0, 100, 250, 500, 1000, 2000 µg/ml.
2. Five hundred microliter of aniline hydrochloride was used as a selective substrate for 2 ml reaction mixture.
3. Liver microsomes prepared from normal rats group were used in the reaction of aniline 4-hydroxylation.
4. The reactions were performed in the same manner as described earlier for aniline 4-hydroxylation assay except that various concentrations of the standardized extract of *C. asiatica* were added concomitantly with the selective substrate.
5. Enzymes activities were plotted against the corresponding concentrations of the standardized extract of *C. asiatica* added into the reaction and the median inhibitory concentration (IC_{50}) was calculated.

6.3 Inhibitory effect of the standardized extract of *C. asiatica* on CYP3A

Procedure

1. Various concentrations of the standardized extract of *C. asiatica* were prepared in such a way that equal volume (200) µl of the solutions were used in the reaction. The final concentrations of the standardized extract of *C. asiatica* in the reaction mixture were 0, 100, 250, 500, 1000, 2000 µg/ml.
2. One hundred microliter of erythromycin stearate was used as a selective substrate for 1 ml reaction mixture.
3. Liver microsomes prepared from normal rats group were used in the reaction of erythromycin N-demethylation.
4. The reactions were performed in the same manner as described earlier for Erythromycin N-demethylation except that various concentrations of the standardized extract of *C. asiatica* were added concomitantly with the c selective substrate.

5. Enzymes activities were plotted against the corresponding concentrations of the standardized extract of *C. asiatica* added into the reaction and the median inhibitory concentration (IC_{50}) was calculated.

Statistical analysis

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

For calculation of IC_{50}, the % of enzyme inhibition was transformed to probit unit. The linear regression method was used to fit a curve between probit unit and log dose by using computer program. The IC_{50} was calculated from the linear regression equation.