CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Forty adult female Wistar rats of body weight between 180-230 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed two per cage at the Faculty of Medicine, Srinakharinwirot University, Bangkok and acclimatized for at least a week prior to the experiment. All animals were in a controlled humidify room at a constant temperature of 25 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed free access to food (C.P. company, Thailand) and drinking water. During the time of experimention, food and water consumption were recorded every five days, and body weight of each rat were recorded every week.

2. Instruments

The following instruments were used in the experimentation.

1. Autopipettes 20, 100, 200, 1000 and 5000 µl (Gibson, France)
2. Buchi Rotavapor R-200
3. Buchi heating bath B-490
4. Centrifuge (Hettich Roto Magna, Japan)
5. Circulating aspirator WJ-20
6. Cooling bath
7. Fluorescence spectrophotometer (Jasco, Japan)
8. High performance liquid chromatography (HPLC) (Shimazu, Japan)
9. Metabolic shaker bath (Heto, Denmark)
10. pH meter (Beckman Instruments, USA)
11. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
12. Refrigerated superspeed centrifuge (Beckman Instruments, USA)

13. Refrigerated ultracentrifuge (Beckman Instruments, USA)
14. Sonicator (Elma, Germany)
15. Spectrophotometer (Jasco, Japan)
16. Surgical equipments
17. Timer
18. Ultra-low temperature freezer (Forma Scientific Inc., USA)
19. Vortex mixer (Clay Adams, USA)

3. Chemicals

These following chemicals were used in the experimentation:

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 from T.I.G., Thailand.

Glycerol was purchased from Carlo Erba, USA.

Hydrochloric acid (HCl), diethyl ether , magnesium chloride ($MgCl_2$), methanol (HPLC 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.

Except indicated, water used in this study was ultrapure water which was prepared by ELGASTAT MAXIMA UF® (ELGA Ltd, England).

Methods

1. Preparation of *C. comosa* ethanolic extract

C. comosa rhizomes were acquired from a traditional drug store in Bangkok. The rhizomes weighing 50 kg were washed, dried, and cut into pieces before drying at 60 °C for 12 hour and ground into fine powder. The 9 kg herbal powder was extracted with 95% ethanol by keeping in an extraction percolater for 24 hours. The ethanolic fraction was dried under vacuum in rotary evaporator and dried again in vacuum desiccator. The *C. comosa* ethanolic extract was kept in tightly closed and light protected container, and stored in a refrigerator until use.

2. Chemical identification test

2.1 *C. comosa* sample preparation

Five milligram of *C. comosa* ethanolic extract was mixed with 50 ml of methanol. Twenty microlitre of the solution was injected into the HPLC.

2.2 HPLC chromatographic systems

Apparatus: Shimazu® LC-10AD HPLC pump, a communication bus module (CBM-10A), an autoinjector (SIL-10A), a column oven (CTO-10A), a spectro UV-VIS detector (SPD-10A) and computerized integrator.

Column: Prevail (C_{18}) 5μ, stainless steel column, 250x4.6 mm (Waters Associates Pty-Ltd., Molford, MA, USA)

UV detector: 254 nm

Mobile phase: Methanol: H_2O (8:2)

Flow rate: 1.0 ml/min.

2.3 Standard calibration curve

Reference standards (0.5-2.5 mg), 1,7 diphenyl-4,6-heptadiene-3-ol were accurately weighed, transferred into 50-ml volumetric flasks and dissolved in methanol yielding five concentrations of the standard solutions of 0.5, 1, 1.5, 2 and 2.5 mg/50ml,

respectively. Twenty microlitre of each individual standard solution of 1,7 diphenyl-4,6-heptadiene-3-ol was injected into the HPLC system. The standard curve of 1,7 diphenyl-4,6-heptadiene-3-ol was constructed between the concentration of 1,7 diphenyl-4,6-heptadiene-3-ol (mg/50ml) and the corresponding area under the chromatogram.

2.4 Quantitation of 1,7 diphenyl-4,6-heptadiene-3-ol in *C. comosa* ethanolic extract

Appropriate dilution of *C. comosa* ethanolic extract was prepared in methanol for injection into the HPLC system. The injection volume was 20 µl. Area under the peaks obtained from the chromatogram were used to calculate amount of 1,7 diphenyl-4,6-heptadiene-3-ol in the extract using the standard curve mentioned above. Amount of 1,7 diphenyl-4,6-heptadiene-3-ol in *C. comosa* ethanolic extract was expressed as total 1,7 diphenyl-4,6-heptadiene-3-ol.

3. Animal treatment

Forty rats were randomly divided into 4 treatment groups. Each treatment group comprised 10 rats as followings:

1. Control group: Rats were given orally with 1 ml/kg/day of corn oil for 30 days.
2. *C. comosa* group I: Rats were given orally at dosage of 100 mg/kg/day of *C. comosa* ethanolic extract for 30 days.
3. *C. comosa* group II: Rats were given orally at dosage of 250 mg/kg/day of *C. comosa* ethanolic extract for 30 days.
4. *C. comosa* group III: Rats were given orally at dosage of 500 mg/kg/day of *C. comosa* ethanolic extract for 30 days.

C. comosa for animal administration was prepared daily by dissolving 500 mg of *C. comosa* ethanolic extract with 1 ml of corn oil to make a concentration of 500 mg/ml of *C. comosa* suspension and was votex-mixed before feeding to experimental animals.

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

At the end of the treatment, animals were fasted for 12 hours before anesthesized with diethyl ether by inhalation. Blood samples were collected by heart puncture on 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 sample was transferred to another tube. Whole blood and serum samples were investigated for hematology and clinical blood chemistry, respectively.

4.1 Clinical blood chemistry

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total protein, albumin, globulin, blood urea nitrogen (BUN), serum creatinine (SCr), glucose, total cholesterol, triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), sodium, potassium, calcium, chloride, estrogen, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in serum samples were analyzed by Professional Laboratories Management Corp. Co., Ltd., Bangkok.

4.2 Hematology

Hematocrit (Hct), hemoglobin (Hb), red blood cell (RBC) count, RBC indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), RBC morphology, platelet count, white blood cell (WBC) count and % differential WBCs in whole blood samples were determined by Professional Laboratories Management Corp. Co., Ltd., Bangkok.

5. Hepatic microsomal 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

Procedures

1. After collecting blood sample, liver was perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale, then the liver was immediately removed from the body.
2. The liver was rinsed with ice-cold 0.9% w/v NaCl, and blotted dry with gauze.
3. The whole liver was weighed, cut into pieces and homogenized with 3 volume of 0.1 M phosphate buffer, pH 7.4.
4. The liver homogenates was 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 supernatant (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.

6. Determination of protein concentrations 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. 16x125 mm. Tubes were labeled in duplicate for 7 standards (0, 50, 100, 150, 200, 250 and 300 μg) and for each unknown sample.
2. The following reagents were added into each standard solution 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)

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 Folin & 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 a minimum of 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.

7. Spectral determination of total CYP contents in liver microsomes

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. A few grains of sodium dithionite was added to the 5 ml diluted sample with gentle mixing, the solution was then transferred to the sample and reference cuvettes in 2.5 ml. for each 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 approximately one 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:

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

8. Analysis of hepatic microsomal CYP activities

8.1 Alkoxyresorufin O-dealkylation assay

Rate of hepatic microsomal alkoxyresorufin O-dealkylation were 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 specific substrates of CYP 2B1 and CYP 2B2. Ethoxyresorufin (ER) and Methoxyresorufin (MR) were used as specific substrates of CYP 1A1 and CYP1A2, respectively.

Reagents

1. 0.1 M Tris buffer, pH7.4
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 = 235)

      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), pH 7.4

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 3 mmole of $MgCl_2$).

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

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

For each assay, the mixture solution of NADPH regenerating system was freshly prepared in a 1:1:1 ratio of 0.1 M NADP, 0.5 M G6P and 0.3 M $MgCl_2$, 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. Microsomal sample was diluted with 0.1 M Tris buffer, pH 7.4 to measure out 300 µg of protein for the final reaction mixture volume of 2 ml.
2. The following reagents were added for the reaction preincubation.
   a) 0.1 M Tris buffer, pH 7.4
   b) 20 µl of 0.5 mM Alkoxyresorufin
   c) 60 µl of the mixture solution of NADPH regenerating system containing
      - 20 µl of 0.1 M NADP
      - 20 µl of 0.5 M G6P
      - 20 µl of 0.3 M $MgCl_2$

d) Varied volume of diluted microsomal suspension containing 300 μg of microsomal protein

3. Three tubes were prepared for each microsomal sample. One tube was a sample blank and the others were 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 20 μl of G6PD (1 unit of G6PD/1ml of reaction mixture volume). For the sample blank tube, 20 μl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
6. After 5-minute incubation, the reaction was stopped by adding 2 ml of methanol (HPLC graed).
7. All tubes were centrifuged at 3,000 r.p.m. for 10 minutes.
8. The absorbance was measured by fluorescence spectrophotometer, using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.
9. A resorufin standard curve was carried out using the duplicated resorufin concentrations of 0.002, 0.005, 0.01, 0.05, 0.2, 0.625 and 1.25 nmole/ml.
10. Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of 5-minute incubation and amount of microsomal protein (mg) used in the reaction.
11. The procedure was verified by varying amount of microsomal protein used in the reaction (50,100, 150 and 200 μg of microsomal protein/ 1 ml of the reaction mixture). Liver microsomes were 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 as a substrate. Correlation coefficient (r^2) between amount of microsomal protein and fluorometric absorbance was 0.9943 (Figure B1).

8.2 Aniline 4-hydroxylation assay

The catalytic activity of CYP2E1 was determined based on the rate of hepatic microsomal aniline 4-hydroxylation, according to the method of Schenkman, Remmer, and Estabrook (1967). Aniline hydrochloride was used as a specific 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 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 (The preparation was described in 8.1)

Procedures

1. Each 2 ml of the reaction mixture comprised microsome containing 5 mg of protein, 500 μl of aniline hydrochloride, 30 μl of NADPH regenerating system and Tris buffer, pH 7.4 qs to 1980 μl.

2. All tubes 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. For the sample blank tube, 20 μl of Tris buffer, pH 7.4 was used instead 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 of 1% phenol and 1 ml of 1M Na_2CO_3 were added to each tube. The solution was mixed homogeneously and kept at room temperature for 30 minutes.
7. The absorbance was measured by spectrophotometer at a wavelength 630 nm.
8. A standard curve was carried out using 5 concentrations of 4-aminophenol standard solutions (0, 2, 4, 6, 8 and 10 μM) following the procedure from sted 6 in the same manner as sample.
9. 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 0.9948 (Figure B2).

Calculations

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

8.3 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 specific substrate of CYP3A.

Reagents

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

   Erythromycin stearate 0.1018 g was dissolved with double distilled water and made up to 10 ml.
5. NADPH regenerating system (The preparation was described in 8.1)
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, 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 microsome in sample blank tube.
2. All tubes were preincubated in a shaker bath at 37°C for 3 minutes.
3. The reaction was initiated by an addition of 15 µl of G6PD. For 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 incubation.

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 well by vortex mixer.
6. All tubes were warmed in a shaker bath at 50°C for 30 minutes.
7. Absorbance of the mixture was measured by spectrophotometer at a wavelength of 412 nm .
8. A formaldehyde standard curve was constructed by adding 1 ml of formaldehyde 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 manner as the sample tubes described above.
9. The procedure was verified by varying amount of microsomal protein used in the reaction (2, 4, 6 and 8 mg of microsomal protein/ 1 ml of the reaction mixture). Liver microsomes were 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. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 0.9861 (Figure B3).

Calculations

Rate of erythromycin N-demethylation was calculated by determining amount of formaldehyde formed, dividing by mg of microsomal protein used and time of incubation (10 minutes). The unit was expressed as nmole/mg protein/min.

9. Data 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 $p < 0.05$.