CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Thirty male Wistar rats weighing between 200-300 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornprathom, Thailand. Animal 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 was recorded every week whereas food consumption and 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. Metabolic shaker bath (Heto, Denmark)
5. pH meter (Beckman, USA)
6. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
7. Refrigerated superspeed centrifuge (Beckman, USA)
8. Refrigerated ultracentrifuge (Beckman, USA)
9. Sonicator (Elma, Germany)
10. Spectrophotometer (Jasco, Japan)
11. Surgical equipments
12. Timer

13. Ultra-low temperature freezer (Forma Scientific Inc., USA)

14. Vortex mixer (Clay Adams, USA)

3. Chemicals

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

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_2HPO_4) and Trisma® base were purchased from Sigma Chemical Co., USA.

Ammonium acetate was purchased from APS Finechem, Australia.

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

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

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

Hydrochloric acid (HCl), diethyi 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.

Potassium permanganate ($KMnO_4$) and ferric chloride ($FeCl_3$) were purchased from Merck, Germany.

Rosmarinic acid, caffeic acid and ursolic acid were obtained from Medicinal Plant Research Institute, Department of Medical Sciences, Thailand.

Sodium dithionite was purchased from Fluka Chemic, Japan.

Methods

Preparation of *O. grandiflorus* aqueous extract (ยุวดี วงษ์กระจ่าง, 2533 และ นาถฤดี สิทธิสมวงศ์, 2542)

1. Fifteen kilograms of dry powder of *O. grandiflorus* leaves and tips of the stems which were gathered shortly before the flowering period were purchased from Jaophaya Arpaipubate hospital, Prajinburi province, Thailand.
2. Each one hundred gram of the powder was extracted two times with 800 ml of boiled water followed by 400 ml of boiled water for 15 minutes each.
3. The combined solutions were filtered and evaporated on water bath until dry.
4. The *O. grandiflorus* aqueous extract was ground into fine powder and kept in a tightly closed and moisture protected container at 2-8^{O}C.

Chemical identifications (ดวงเพ็ญ ปัทมดิลก, 2545)

1. **Preliminary test with color reaction test**

1.1 Test with potassium permanganate TS

1.1.1 Test solution:

Two grams of dry powder of *O. grandiflorus* was refluxed with 50 ml of water for 30 minutes, filtered and then evaporated the filtrate to 25 ml.

1.1.2 Reagent: potassium permanganate TS

Potassium permanganate (3.3 grams) was dissolved in 1000 ml of water. The solution was boiled for 15 minutes and allowed it to stand at room temperature for at least 2 days before filtering.

1.1.3 Procedure:

Two milliliter of the test solution was added with a few drops of potassium permanganate TS. The solution was shaked well and the color of the mixture was noted.

1.2 Test with ferric chloride TS

1.2.1 Test solution:

The solution of *O. grandiflorus* was prepared in the same way as in 1.1.1

1.2.2 Reagent: ferric chloride TS

One gram of ferric chloride hexahydrate was dissolved in water to produce 100 ml of the solution.

1.2.3 Procedure:

One milliliter of the test solution was added with a few drops of ferric chloride TS. The color of the solution was then observed.

2. Confirmatory test with thin layer chromatographic analysis

System of thin layer chromatography

1. Adsorbent: Silica gel GF_{254} precoated plate
2. Developing solvent system: Toluene : ethyl acetate : formic acid (9:9:1)
3. Detection: UV 254 nm and vanillin-phosphoric acid reagent
4. *O. grandiflorus* methanol extract of aqueous extract: 0.25 g of the aqueous extract was refluxed with 25 ml of methanol for 10 minutes, filtered and evaporated the filtrate until dryness.
5. Test solution: 9.55 mg of *O. grandiflorus* methanol extract of aqueous extract was dissolved with 1 ml of methanol. Five microliter of the solution was spotted on TLC plate.
6. Reference solutions included
    1. Two microliter of the solution of 2.72 mg/ml caffeic acid
    2. Two microliter of the solution of 0.12 mg/ml rosmarinic acid
    3. Two microliter of the solution of 0.10 mg/ml ursolic acid

3. Determination of potassium content in *O. grandiflorus* aqueous extract

Potassium content in *O. grandiflorus* aqueous extract was determined using atomic absorption spectrophotometer.

1. Test solution: 0.3003 g of *O. grandiflorus* aqueous extract was dissolved with double distilled water. The solution was added with 2.0 ml of nitric acid and made up to 25 ml with double distilled water.
2. Wavelength: 766.5 nm
3. Flame: air-acetylene
4. Air flow: 13.5 L/min
5. Acetylene flow: 2.00 L/min

Effects of *O. grandiflorus* aqueous extract on hepatic CYP and clinical blood chemistry

1. Animal treatment

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

1. Control group: Rats were given orally with 1 ml/kg/day of distilled water for 30 days.
2. *O. grandiflorus* group I: Rats were given orally with 0.96 g/kg/day of *O. grandiflorus* aqueous extract for 30 days.
3. *O. grandiflorus* group II: Rats were given orally with 4.8 g/kg/day of *O. grandiflorus* aqueous extract for 30 days.

Note:

- The lower oral dosage (0.96 g/kg/day) of *O. grandiflorus* aqueous extract used in this study was the dosage that caused no abnormalities in a chronic toxicity study (นาถฤดี สิทธิสมวงศ์ และคณะ, 2542). This dosage regimen of *O. grandiflorus* was approximately closed to the dosage (750 mg/kg, p.o.) that was found to possess diuretic effect in rats (Englert, J. and Harnischfeger, G., 1991). This dosage was equivalent estimated to 7 folds of the dosage recommended for traditionally used in human.

- The higher oral dosage (4.8 g/kg/day) of *O. grandiflorus* aqueous extract used in this study was the highest dose used in a chronic toxicity study. Given orally to rats for 6 months, this dosage of the extract was not shown to affect growth, food consumption as well as did not cause any severe toxicity in rats (นาถฤดี สิทธิสมวงศ์ และคณะ, 2542). This dosage was equivalent estimated to 35 folds of the dosage recommended for traditionally used in human.

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

At the end of the treatment, animals were fasted for at least 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

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

2.2 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), electrolytes (sodium, potassium, chloride and calcium), uric acid, total cholesterol, triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), and glucose in serum samples were analyzed by Professional Laboratory Management Corp. Co., Ltd.

3. Liver microsome preparation

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

Reagents:

1. 0.9% w/v Sodium chloride
2. 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 or HCl
3. 0.1 M Phosphate buffer, pH 7.4, containing 20% v/v glycerol

Procedure:

1. Before removing from the body, rat livers were quickly perfused *in situ* 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, O.H., *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 Bovine serum albumin (BSA) in 0.5 M NaOH
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent

   Working protein reagent 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, T. and Sato, R. (1964).

Reagents:

1. 0.1 M Tris buffer, pH 7.4 containing 20% v/v glycerol
2. 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 4 ml diluted samples, a few grains of sodium dithionite were added with gentle mixing, then 2 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 and CYP2B1/2 were determined by measuring the rate of *O*-dealkylation of ER, MR and BR & PR, respectively, using the method of Burke, M.D. and Mayer, R.T. (1974) and Lubet, R.A., *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

3.1 0.5 mM MR (MW = 227)

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

3.2 0.5 mM BR (MW = 303)

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

3.3 0.5 mM ER (MW = 241)

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

3.4 0.5 mM PR (MW = 283)

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

3.5 0.5 mM Resorufin (MW = 235)

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

4. NADPH regenerating system

NADPH regenerating system comprised the solution as following:

4.1 0.1 M NADP, pH 7.4

0.765 g of NADP 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)

4.2 0.5 M G6P, pH 7.4

1.41 g of G6P 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)

4.3 0.3 M $MgCl_2$, pH 7.4

609.93 mg of $MgCl_2$ 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 $MgCl_2$)

4.4 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 2 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
   2.1 0.1 M Tris buffer, pH 7.4
   2.2 0.5 mM Alkoxyresorufin 20 μl
   2.3 The mixture of NADPH regenerating system 60 μl containing
      - 0.1 M NADP 20 μl
      - 0.5 M G6P 20 μl
      - 0.3 M $MgCl_2$ 20 μl
   2.4 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 20 μl of G6PD. Sample blank was added with 20 μl of 0.1 M Tris buffer instead of G6PD.
6. After 5 minute incubation, the reaction was stopped with methanol 2 ml (gradient grade).
7. All tubes were centrifuged at 3,000 rpm for 10 minutes. The supernatant was transferred to another tube.
8. The fluorescence intensity of the supernatant 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 constructed using the duplicated resorufin concentrations of 0.002, 0.005, 0.010, 0.050, 0.200, 0.625 and 1.250 nmol/ml.

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.

Verification of alkoxyresorufin *O*-dealkylation:

The procedure was verified by varying amount of rat 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 (Soccek, P and Gut, I., 1992). The reaction was performed as mentioned above using benzyloxyresorufin as a substrate. Correlation coefficient (r^2) between amount of microsomal protein and fluorescence intensity was 0.9934 (Figure 35).

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., *et al.* (1967). Aniline hydrochloride was used as a specific substrate of CYP2E1.

Reagents:

1. 10 mM Aniline hydrochloride

   129.6 mg of aniline hydrochloride 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

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

3. 20% w/v TCA

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

4. 1% w/v Phenol

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

5. 1M Na_2CO_3

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

6. 10 μM 4-aminophenol

0.0365 g of 4-aminophenol was 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 solution for reaction incubation, each preincubation mixture composed of microsome containing 5 mg 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 bath at $37^{\circ}C$ 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 1 ml of 20% TCA. The tubes were placed on ice for 5 minutes.
5. The solution was centrifuged at 3,000 rpm for 10 minutes. One milliliter of supernatant was transferred to another tube, then 1 ml of 1% w/v phenol and 1 ml of 1 M 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. The units were expressed as nmol /mg protein/ min.

Verification of aniline 4-hydroxylation:

The procedure was verified by varying amount of rat microsomal protein used in the reaction (2.5, 5.0 and 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 36).

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, T. *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)

   0.1018 g of erythromycin stearate 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

   12.5 g of TCA 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 minutes incubation, the reaction was stopped with 500 μl of ice-cold 12.5% w/v TCA.
5. All tubes were centrifuged at 3,000 rpm 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, 0.1250 and 0.2500 μmol/ml with 1 ml of Nash reagent and mixed

homogeneously. The absorbance of the mixture was measured spectrophotometrically at 412 nm.

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.

Verification of erythromycin *N*-demethylation:

The procedure was verified by varying amount of rat 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 dose of 80 mg/kg/day intraperitoneally for 3 days (Soccek, P and Gut, I., 1992). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 0.9835 (Figure 37).

7 Data analysis

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