CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Fifty adult male Wistar rats of body weight between 250-300 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 ± 2 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to food (C.P. company, Thailand) and drinking water.

2. Instruments

The following instruments were used in the experimentation.

1. Autopipettes 20, 100, 200, 1000 and 5000 µl (Gibson, France)
2. Centrifuge (Hettich Roto Magna, Japan)
3. Fluorescence spectrophotometer (Jasco, Japan)
4. Metabolic shaker bath (Memmert Germany)
5. pH meter (Beckman Instruments, USA)
6. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
7. Refrigerated superspeed centrifuge (Beckman Instruments, USA)
8. Refrigerated ultracentrifuge (Beckman Instruments, 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

The following chemicals were purchased from Sigma Chemical Co., USA: Acetylacetone, 4-aminophenol, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), cupric sulfate, dimethylsulfoxide (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.

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 Chemical Corp, 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* extracts

C. comosa hexane and ethanolic extracts were kindly provided by Professor Apichart Suksamram, Faculty of Sciences, Ramkamhaeng University.

The rhizomes of plant were collected from Nakhon Pathom, Thailand. The dried rhizome powder (30 kg) was extracted with n-hexane in a Soxhlet extractor to give a pale browish viscous oil (1.01 kg). The marc was subsequently extracted with 95% ethanol, the ethanolic fraction was dried under vacuum in rotary evaporator and dried again with high vacuum pump to give a dark reddish-brown viscous oil (1.30 kg). For

administration to animals, both hexane and ethanolic extracts were dissolved in corn oil.

Chemical identification test

Hexane and ethanolic extracts of *C. comosa* were characterized by Suksamrarn et al. (unpublished data) and the TLC chromatogram were shown in the appendix F. The major constituent of hexane extract was 1,7-diphenyl-5-hydroxy-(1*E*)-1-heptene (1). The major constituents of ethanolic extract were 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene (6) and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene (7).

2. Animal treatment

Fifty rats were randomly divided into 5 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 1: Rats were given *C. comosa* hexane extract orally at the dosage of 250 mg/kg/day for 30 days.
3. *C. comosa* group 2: Rats were given *C. comosa* hexane extract orally at the dosage of 500 mg/kg/day for 30 days.
4. *C. comosa* group 3: Rats were given *C. comosa* ethanolic extract orally at the dosage of 250 mg/kg/day for 30 days.
5. *C. comosa* group 4: Rats were given *C. comosa* ethanolic extract orally at the dosage of 500 mg/kg/day for 30 days.

C. comosa extracts for animal administration were prepared daily by dissolving 250 and 500 mg of *C. comosa* hexane extract or ethanolic extract with 1 ml of corn oil to make a concentration of 250 and 500 mg/ml of *C. comosa* suspension and were vortex-mixed before feeding to experimental animals. During the time of experiment, rat body weight, food and water consumption were recorded every week.

3. 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 microlitres 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 and centrifuged for collecting serum. Whole blood and serum samples were investigated for hematology and clinical blood chemistry, respectively.

4. Clinical blood chemistry

The following clinical blood chemistry parameters in serum samples were analyzed by Professional Laboratories Management Corp. Co. Ltd., Bangkok: aspatate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total protein, albumin, globulin, blood urea nitrogen (BUN), serum creatinine (SCr), sugar, total cholesterol, triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), sodium, potassium, calcium and chloride.

5. 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.

6. 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 centrifuged, to pellet intact cells, cell debris, nuclei and mitichondria.
5. The supernatant (S9, post mitochondria 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.

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

Procedures

1. 16×125 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 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 tube 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.

8. 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 containning 20% v/v glycerol.
2. A few grains of sodium dithionite was added to the 5 ml diluted sample, the solution 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 and 500 nm.
4. Immediately after the sample cuvette was bubble 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 diferrence 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:

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

9. Analysis of hepatic microsomal CYP activities

9.1 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), 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 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, adjusting pH to 7.4 with HCl or NaOH (10 µl contained 1 unit of G6PD).

Procedures

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 ul of the mixture solution of regenerating system containing
- 15 µl of 0.1 M NADP
- 15 µl of 0.5 M G6P
- 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 sample blank and the others are samples).
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 0.1 M Tris buffer 15 µl 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 0.0125, 0.025, 0,05, 0.075, 0.1, 0.2, 0.4 nmole/ml
9. Rate of alkoxyresorufin O-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/ mgprotein/ 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, respectively. Correlation coefficient (r^2) between amounts of microsomal protein and absorbance were 0.9915 and

0.998 when ER and MR were used as selective substrates, respectively (Figure B1 and B2, Appendix B, page 81, 82, respectively).

10.1.2. For the BROD reaction, varying amounts of microsomal protein were used in the reaction (50, 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 selective substrate. Correlation coefficient (r^2) between amounts of microsomal protein and the corresponding absorbance was 0.9836 (Figure B3, Appendix B, page 83).

10.2. The capacity of the procedure to detect the enzyme induction

10.2.1. For the CYP1A1, CYP1A2 induction, microsomes were prepared from 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 or 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 (23 folds and 18 folds, respectively) in the β-NF –treated group as compared to the control group (Figure B4, Appendix B, page 84 and Figure B5, Appendix B, page 85, respectively).

10.2.2. For the CYP2B1/2B2 induction, microsomes were prepared from phenobarbital-induced rats. Four rats for each group were treated with phenobarbital at the dosage of 80 mg/kg/day intraperitoneally 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 activities. BROD and PROD activities were significantly higher (41 folds and 42 folds, respectively) in the phenobarbital –treated group as compared to the control group (Figure B6, Appendix B;page 86 and Figure B7; Appendix B; page 87, respectively).

9.2. 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 aniline hydrochloride, 30 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 of 1% 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 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 step 6 in the same manner as sample.
9. The procedure was verified by varying amounts of microsomal protein (prepared from the normal rat) used in the reaction (1, 4 and 6 mg of microsomal protein/ 1 ml of the reaction mixture). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amounts of microsomal protein and the corresponding absorbance was 0.9988 (Figure B8).

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.

9.3. 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 (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 9.1)
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 microsomes in 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 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 ul of ice-cold 12.5% w/v TCA after 10 minutes incubation.
5. All tube were centrifuged at 3,000 r.p.m. for 10 minutes.
6. One milliliter of the supernatant was transferred to another new tube. One milliliter 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 a wavelength of 412 nm.
9. 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.
10. The procedure was verified by varying amounts of microsomal protein used in the reaction (1, 2, 4 and 5 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 amounts of microsomal protein and the corresponding absorbance was 0.9981 (Figure B9).

Statistical analysis

All numeric quantitative data were presented as mean $\pm$ 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 $p<0.05$.