SAFETY EVALUATION OF ETHANOL EXTRACT OF *ACTINIOPTERIS RADIATA* IN WISTAR ALBINO RATS
2.0. INTRODUCTION

Plant-based medicaments had served from the outset as the most important therapeutic weapon available to man to fight various human and animal diseases. In recent times there is a renewal and growing interest in the use of plant-derived biologically active compounds as drugs or as leads in the manufacture of more potent medicaments (Houghton and Raman, 1998). Plants, therefore, remain the main source of the active drugs from a natural source and are still indispensable in the traditional medicine for treating a number of diseases. Traditional medicines are used by about 60% of the world population both in the developing countries and developed countries where modern medicines are predominantly used (Mythilypriya et al., 2007). However, many issues related to a lack of scientific evidence about the efficacy and safety of herbal remedies remain unresolved (Malik and Gopalan, 2003; Shekelle et al., 2005). Many reports and warnings have been published, particularly about the potential hepatotoxicity of herbal products, because the liver is a prime target for the toxic effects of general drugs (Stickel et al., 2001; Williams and Iatropoulos, 2002; Ahn, 2004).

For a plant or herbal preparation containing active organic principles to be identified for use in the traditional medicine, a systemic approach is required for the evaluation of efficacy and safety through experiment and clinical findings (Mythilypriya et al., 2007). The general toxicity studies include-acute, subacute and chronic toxicity studies.

**Acute toxicity studies:** In acute toxicity study, the drug effect can be evaluated at single dose level by injecting graded doses (over a wide range) in different groups of animals. The main objective of an acute toxicity test is not only to establish a figure for LD\textsubscript{50} with precision, but also know about the way in which the drug acts as a poison. The studies determine the LD\textsubscript{50}, the ED\textsubscript{50} and the therapeutic index for the drug under investigation (Satoskar et al., 1997).

**Sub-acute toxicity studies:** The toxicity studies of shorter duration i.e., for 14-21 days are called sub-acute toxicity studies.

**Chronic toxicity studies:** These toxicity studies can be carried out for a longer period which usually last for a period of 90 days to over a year.
Despite the varied uses of *A. radiata* in treating infections, inflammations and various other ailments, its toxicological profile have not been reported. This study is therefore aimed at investigating the acute and sub-acute oral toxicity of *A. radiata* in Wistar albino rats using the recommended OECD guidelines for testing of chemicals for safety evaluation.

2.1. EXPERIMENTAL DESIGN

**Acute oral toxicity study**

Acute oral (gavage) toxicity studies were conducted with EEAR in Wistar rats. Healthy Wistar albino rats of either sex weighing 200 ± 50 g were divided into 6 groups of six animals each. Rats were maintained under standard laboratory conditions. After an overnight fast, rats were dosed orally with EEAR in water at doses of 100, 250, 500, 1000 and 2000 mg/kg b. wt. The control group received 1 ml of saline. The animals were observed for a period of 72 hr for any signs of behavioral changes, toxicity and mortality.

**Sub-acute oral toxicity study**

The studies were conducted in accord with Organization for Economic Cooperation Development (OECD) guidelines Wistar albino rats of either sex weighing 200 ± 50 g were divided into 5 groups (group I-V) of six animals each and were housed under standard laboratory conditions. The control animals (group I) received 1ml of saline daily for 30 days. Group II- Group V animals were administered daily with EEAR for 30 days at doses of 50, 100, 250 and 500 mg./kg b. wt, respectively. Rats were observed for mortality and viability twice daily and observed for any toxic manifestations once before treatment and once daily thereafter. Body weight and food consumption were recorded once during the pretest period and weekly thereafter.

On 28th day of the experiment, 24 h urine samples were collected by placing the animals in the metabolic cage with free access to tap water but no feed was given. The urine was free from faecal contamination. Toluene is used as a preservative while collecting the sample. The sediments present in the urine were removed by centrifugation and the collected urine was used for biochemical estimations.

At the end of the study i.e., on 30th day, the animals were fasted for approximately 18 h, then slightly anesthetized with ether and blood samples were
collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters and to separate plasma for biochemical estimations, the other without any anticoagulant and was centrifuged at 4000 rpm at 4°C for 10 minutes to obtain the serum. Both the plasma and serum were stored at 20°C until analyzed for biochemical parameters. Animals were then sacrificed; the organs- liver, kidneys, heart, brain, testis and adrenals were dissected out, washed and transferred to an ice-cold saline solution. The organs were weighed and portions of some of the vital organs-liver, kidneys, brain and heart were fixed in 10 % formalin for histopathological examinations. 10% homogenates of liver and kidney were prepared and the homogenates were analyzed for biochemical parameters.

The hematological parameters assessed included - erythrocyte count, hemoglobin, mean corpuscular volume and total leukocyte count. The clinical biochemistry parameters assessed include - glucose, urea, creatinine, uric acid, bilirubin, total cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and total protein. The urine analysis parameters assessed include - urea, creatinine, uric acid and protein.

**Statistical analysis**

The experimental data has been analysed using one way ANOVA. Dunnet’s test has been employed for multiple comparisons because here the mean value in each group is to be compared with that of control group.

**2.2. MATERIALS AND METHODS:**

**Animals**

Experiments were carried out with male albino rats of Wister strain, weighing 250 ± 50 g. They were obtained from Sri Raghavendra suppliers, Bangalore and maintained in standard laboratory conditions, with a 12 hr light/dark cycle and fed with commercial rat feed supplied by Hindustan Lever Ltd., Mumbai under the trade name Gold Mohur rat feed and water *ad libitum*. Experimental animals were handled according to the University and Institutional Legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of social justice and empowerment, Government of India.
Estimation of total protein:
Total protein was estimated by Bradford method (1976).

Reagents:
1. Coomassie brilliant blue G-250
2. 85% Phosphoric acid
3. 95% ethanol.
4. Preparation of Bradford reagent: 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 mL of 95% ethanol. To this 100 mL of 85% phosphoric acid was added. The resulting solution was further diluted to a final volume of one liter.

Procedure:
Bovine serum albumin (BSA) used as standard was pipetted out in the concentrations of 10-100 μg into test tubes. The volume was made up to 0.1 mL with distilled water. 2.5 mL of Bradford reagent was added to the tubes and the contents were mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before one hour in spectrophotometer against a reagent blank. The optical density of the unknown was plotted in the standard curve to obtain the concentration of the protein in the unknown sample. Total protein is expressed in serum and tissues as g/dL and mg/g tissue respectively.

Estimation of bilirubin
Bilirubin content was estimated according to the procedure of Malloy and Evelyn, (1937).

Reagents:
1. Absolute alcohol
2. Hydrochloric acid, 1.5% v/v in distilled water
3. Diazo-reagent, prepared by adding 0.3 mL of solution B to 10 mL of solution A.
4. Solution A: 1g of sulphanilic acid was dissolved in 15 mL of concentrated HCl and made up to 1 litre with distilled water.
5. Solution B: 0.5 g of sodium nitrite was dissolved in water and made up to 100 mL.

6. Standard solution of bilirubin-A solution containing 10 mg bilirubin per 100 mL of chloroform was prepared.

**Procedure:**

Two test tubes were taken and into each 0.2 mL of serum and 1.8 mL of distilled water were added. To the unknown 0.5 mL of diazo reagent was added and to the blank 0.5 mL of 1.5% HCl. Finally to each 2.5 mL of methanol was added, allowed to stand for 30 minutes and the optical density was read in a colorimeter set at 540 nm. Calibration curve was constructed simultaneously with the standards. The amount of bilirubin was calculated using a standard curve.

**Estimation of urea**

Blood urea nitrogen was estimated by the method of Natelson *et al.* (1951) by measuring the coloured complex formed with diacetyl monoxime in acidic medium.

**Reagents:**

1. Sodium tungstate, 10%: 10 g of sodium tungstate was weighed and dissolved in 100 mL of distilled water.

2. 2/3 N sulphuric acid.

3. Diacetyl monoxime (2%) in 2% acetic acid.

4. Sulphuric acid-Phosphoric acid reagent: 140 mL of water was mixed with 150 mL 75% phosphoric acid and then 50 mL of concentrated sulphuric acid was added slowly.

5. Standard: 250 mg of urea was dissolved in 100 mL of distilled water. This solution was diluted 1: 100 to give a solution containing 25µg/mL, which was used as the working standard.

**Procedure:**

0.1 mL of serum/urine sample was mixed with 3.3 mL of distilled water, 0.3 mL of sodium tungstate and sulphuric acid, mixed well and centrifuged. 1 mL of supernatant was mixed with 1 mL of distilled water, 0.4 mL of diacetyl monoxime and 1.6 mL of sulphuric acid-phosphoric acid reagent. The tubes were placed in a boiling water bath.
for 30 min and cooled. The colour developed was read at 480 nm in a colorimeter against water blank. The same procedure was performed with a series of standard. Blood urea level is expressed as mg/dL of serum.

**Estimation of uric acid**

Serum uric acid was estimated by the method of Caraway (1963).

**Reagents:**

1. Phosphotungstic acid: To prepare a stock solution, 50 g of sodium tungstate (Na₂WO₄·2H₂O) was dissolved in 400 mL of distilled water. 40 mL of 85% phosphotungstic acid was refluxed gently for 2 hr, cooled and transferred to a 500 mL flask and made up to the mark with distilled water. This reagent was stored in a brown bottle, diluted 1:10 for use. This can be kept for months in a brown bottle.

2. Sodium carbonate: 15%

3. Standard: 100 mg of uric acid and 60 mg of lithium carbonate were dissolved in about 50 mL of distilled water. This was heated to about 60°C to dissolve the uric acid completely. After cooling, the solution was finally made up to 100 mL with distilled water. 1 mL of stock standard was diluted to 10 mL to give a working standard concentration of 10μg/mL.

**Procedure:**

About 0.5 mL of serum/urine was taken and to this 2.5 mL of distilled water was added followed by 0.6 mL of phosphotungstic acid and 0.6 mL of sodium carbonate. A blank was setup with 3 mL of distilled water. Standard with graded volumes were also setup. After 10 min, the colour developed was read at 640 nm in a colorimeter. Uric acid level is expressed as mg/dL.

**Estimation of creatinine**

Serum creatinine was estimated by the method of Owen *et al.*, (1954).

**Reagents:**

1. Picric acid, 0.04 M: 916mg of picric acid was dissolved in 100 mL of distilled water.

2. Sodium hydroxide, 0.75 N was prepared in distilled water.
3. **Standard:** 100 mg of creatinine was dissolved in 0.1 N Hydrochloric acid and made up to 100 mL. 1 mL of this solution was diluted to 10 mL with distilled water to give a working standard containing 100 µg/mL.

**Procedure:**
0.1 mL of serum/urine sample was made up to 2 mL with distilled water. Standards in the range of 10-40 µg/mL were also made up to 2 mL. Blank contained only distilled water. To all the tubes, 1 mL of picric acid followed by 1 mL of sodium hydroxide was added. The resulting colour was read at 540 nm after 15 min. Creatinine level is expressed as mg/dL.

**Estimation of triglycerides**
Triglycerides was estimated as described by Rice (1970) based on the method of Van Handel (1961).

**Reagents:**
2. Sodium chloride-saturated in distilled water.
3. Activated silicic acid: Silicic acid was washed with 4 N HCl and then with distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C overnight prior to use.
4. Alcoholic potassium hydroxide, 0.4%.
5. Sulphuric acid, 0.2 N.
6. Sodium-meta-periodate, 0.1 M: 2.149 g of sodium-meta-periodate was dissolved in 100 mL of distilled water.
7. Sodium meta arsenite, 0.5 M: 6.496 g of sodium meta arsenite was dissolved in 100 mL of distilled water.
8. Chromotropic acid reagent: 1.14 g of chromotropic acid was dissolved in 100 mL of distilled water and stored as a stock solution in a brown bottle. Before use, 10 mL of this solution was mixed with 45 mL sulphuric acid water mixture in the ration of 2:1(v/v).
9. Thiourea, 7%.
10. **Standard:** 100 mg of tripalmitin was dissolved in 100 mL of chloroform.
Procedure:
0.1 mL of serum/Folch-wash tissue lipid extract was mixed with 1 mL of chloroform-methanol mixture and 50 mg of activated silicic acid was added, shaken vigorously and allowed to stand for 30 min. After centrifugation, to 0.5 mL aliquot of supernatant (as well as to standard and blank), 0.5 mL of alcoholic potassium hydroxide was added and the mixture was saponified in a 60-70°C water bath for 20 min. To this, 0.5 mL of 0.2 N sulphuric acid was added and kept in a boiling water bath for 10 min. After cooling the tubes, 0.1 mL of sodium meta periodate was added and allowed to stand for 10 min. The excess periodate was reduced by the addition of 0.1 mL of sodium meta arsenite. Then 5 mL of chromotropic acid was added, mixed thoroughly and kept in a boiling water bath for 30 min. After cooling, 0.5 mL of thiourea solution was added and the colour developed was read at 570 nm. Serum and tissue triglyceride content are expressed as mg/dL and mg/g wet tissue, respectively.

Estimation of cholesterol
Cholesterol content was estimated by the method of Parekh and Jung (1970).

Reagents:
1. Ferric chloride-uranyl acetate reagent: 500 mg of ferric chloride was dissolved in 10 mL of distilled water; 3 mL of concentrated ammonia was added to it and centrifuged. The precipitate was washed several times with distilled water and dissolved in one liter of glacial acetic acid. 100 mg of uranyl acetate was added to the mixture and the contents were shaken well and kept over night. The reagent was also stable for six months.

2. Sulphuric acid-ferrous sulphate reagent: 100 mg of ferrous sulphate was dissolved in 100 mL of glacial acetic acid and 100 mL of sulphuric acid. After cooling to room temperature, the volume was made up to one liter with concentrated sulphuric acid and stored in a brown bottle.

3. Cholesterol standard: 200 mg of cholesterol (recrystallised from ethanol) was dissolved in 100 mL of chloroform. 1 mL of stock cholesterol was diluted to 100 mL to obtain a working standard having 20 μg cholesterol/mL.

Procedure:
To 0.1 mL of serum/Folch-wash tissue lipid extract, 3 mL of ferric chloride-uranyl acetate reagent was added. Then 2 mL of sulphuric acid-ferrous sulphate reagent was
added to all the tubes and the contents were mixed well. After 20 min the colour developed was read against reagent blank at 540 nm. Standards were also treated in a similar manner. Serum and tissue cholesterol content are expressed as mg/dL and mg/g wet tissue, respectively.

**Estimation of blood glucose** (Sasaki *et al.*, 1972)

Blood glucose was estimated by the modified method of Sasaki and Matsui (1972) using O-toluidine reagent.

**Reagents:**

1. Trichloroacetic acid: 10% solution
2. Ortho-toluidine reagent: 12.5 g of thiourea and 12.0 g of boric acid were dissolved in 50 mL of distilled water by heating over a mild flame. 75 mL of redistilled ortho-toluidine and 375 mL of acetic acid were mixed separately. The two solutions were mixed and the total volume was made up to 500 mL with distilled water. The reagent was left in a refrigerator overnight and filtered.
3. Standard Glucose: 10 mg of anhydrous glucose was dissolved in 100 mL of water containing 0.01% benzoic acid as preservative to give a concentration of 100 μg/mL.

**Procedure:**

0.1 mL of blood was immediately mixed with 1.9 mL of trichloroacetic acid solution to precipitate proteins and then centrifuged. 0.1 mL of supernatant was mixed with 4.0 mL of O-toluidine-boric acid reagent and was kept in a boiling water bath for 15 minutes along with blank. The green colour developed was read at 600 nm. A standard curve was obtained using standard glucose solution in the concentration of 20-100 μg/mL. The values were expressed as mg/dL of blood.

**Estimation of haemoglobin concentration** (Sahli, 1962)

The haemoglobin concentration was estimated by acid haematin method (Sahli, 1962). N/10 hydrochloric acid was taken up to 10.0 mark in a graduated tube. Blood was collected directly into the hemoglobin (Hb) pipette and was transferred into the graduated tube containing N/10 hydrochloric acid. It was allowed to stand for 10 min after thorough mixing. Then N/10 hydrochloric acid was added drop by drop, mixing
between each addition until the blood colour matched with standard colour. Then the amount of Hb was read from the scale on the graduated tube and the Hb concentration was expressed in grams per cent.

**Red blood corpuscle count (Davidson and Henry, 1969)**

RBC count was made with a Neubauer crystalline chamber as described by Davidson and Henry (1969). The blood was drawn up to 0.5 mark in RBC pipette and immediately the diluting fluid was drawn up to 100 mL mark (thus the dilution is 1:200). The solution was mixed well by shaking gently. It was allowed to stand for 2 to 3 min. The counting chamber and cover glass were cleaned and cover slip was placed over the ruled area. Again the solution was mixed well by shaking gently and stemful of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40°. It was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of RBC’s were counted in five small squares of the RBC column under high power and the number of RBC per cubic mm were calculated accordingly.

\[
\text{Number of cells} \times \text{dilution factor} \times \text{depth factor} \\
\text{Area counted}
\]

**White blood corpuscle count (Davidson and Henry, 1969)**

WBC count was made with a Neubauer crystalline chamber as described by Davidson and Henry (1969). Blood is drawn from the vial into WBC pipette up to the mark 0.5 and immediately the diluting fluid was drawn up to the mark 11. The solution is mixed thoroughly by gentle shaking. It was allowed to stand for 2 to 3 minutes. The counting chamber and cover glass were cleansed and cover slip was placed over the ruled area. Again the solution was mixed well by shaking gently and stemful of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40°. It was allowed to stand for 2 to 3 minutes to allow WBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of WBC’s was counted in bigger squares of the chamber. The WBC count was expressed in number of WBC/ cu.mm.
Estimation of packed cell volume (Schalm et al., 1975)

PCV was estimated by microhaematocrit method (Schalm et al., 1975). The blood was drawn into capillary tube containing the anticoagulant, by capillary action to $\frac{3}{4}$ of the length. The tubes were tapped to permit blood flow towards an end and to provide sufficient space to prevent outflow and the opposite ends were sealed. The outside of the capillary tubes were wiped free of blood and the index finger was placed over the moist ends to hold the column of the blood in place as the opposite dry ends were forced into the sealing material to form a tight plug. The capillary tubes were placed in the centrifuge with the sealed ends pointing outward and centrifuged at 12,000g for 2 min. PCV was determined by rolling the capillary tubes on a reader card until the top of the plasma column was aligned with 100% line and the bottom of the packed erythrocyte column represented the PCV in percent.

Mean corpuscular volume

From the estimated values of haemoglobin, RBC count (millions/mm$^3$) and PCV (volumes percent), MCV can be calculated as follows:

$$\text{MCV (}$\mu$m$^3$/red cell) = \frac{\text{PCV}}{\text{RBC count}} \times 10$$

Preparation of the tissue homogenate:

Samples of liver and kidney (100 mg/mL) were homogenized in 50 mM phosphate buffer (pH 7.0), and then centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for assaying the biochemical parameters.

Estimation of Serum glutamate oxaloacetate transaminase/Aspartate amino transferase (SGOT/AST) activity.

SGOT/AST was assayed by the method of King (1956a). The reaction catalyzed by this enzyme involves the formation of glutamate and oxaloacetate from the substrate containing aspartic acid and 2-oxoglutaric acid. The oxaloacetate thus formed was allowed to react with DNPH reagent. The colour developed was measured at 540 nm after the addition of NaOH. A set of standard pyruvate was also run in a similar manner.
Reagents:

1. Phosphate buffer, 0.1 M, pH 7.5.
2. Substrate: 1.33 g weight of aspartic acid and 1.5 mg of 2-oxoglutarate were dissolved in 20.5 mL of 1N NaOH and made up to 100 mL with distilled water.
3. 2, 4-dinitro phenyl hydrazine (DNPH) 0.002%: 20 mg of DNPH was dissolved in 100 mL of 1N HCl.
4. Sodium hydroxide, 0.4 N.
5. Standard: 11 mg of sodium pyruvate was dissolved in 100 mL of phosphate buffer (pH 7.5). This contains 1μmol pyruvate/mL.

Procedure:
1 mL of substrate was incubated at 37°C for 10 min. Then, 0.2 mL of enzyme was added and the mixture was incubated at 37°C for 1 hr. To the control tubes the enzyme was added after the reaction, and it was arrested by the addition of 1 mL of DNPH reagent. The tubes were kept at room temperature for 30 min. Then 5 mL of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The color developed was read against the blank at 540 nm using a colorimeter. The enzyme activity was expressed as units/L (One unit corresponds to enzyme that liberates one micro mole of pyruvate/min).

Estimation of serum glutamate pyruvate transaminase/alanine amino transferase (SGPT/ALT) activity.
SGPT was assayed by the method of King (1965a). This enzyme catalyzes the formation of pyruvate and glutamate from alanine and 2-oxoglutaric acid. The pyruvate formed was made to react with DNPH reagent and the colour developed was measured at 540 nm after the addition of NaOH. A set of standard pyruvate was also run simultaneously.

Reagents:
1. Phosphate buffer, 0.1 M, pH 7.5.
2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxoglutarate were dissolved in 20 mL of phosphate buffer (pH 7.5). 0.5 mL of 1N NaOH was added and made up to 100 mL with distilled water.
3. 2, 4- dinitro phenyl hydrazine (DNPH) 0.002%: 20 mg of DNPH was dissolved in 100 mL of 1N HCl.

4. Sodium hydroxide, 0.4 N.

5. Standard: 11 mg of sodium pyruvate was dissolved in 100 mL of phosphate buffer (pH 7.5). This contains 1 µmol pyruvate/mL.

Procedure:
1 mL of substrate was incubated at 37°C for 10 min. Then, 0.2 mL of enzyme was added and the mixture was incubated at 37°C for 30 min. To the control tubes the enzyme was added after the reaction, and it was arrested by the addition of 1 mL of DNPH reagent. The tubes were kept at room temperature for 20 min. Then 5 mL of sodium hydroxide was added. A set of standard pyruvate solution were also treated in a similar manner. The color developed was read at 540 nm using a colorimeter. The enzyme activity was expressed as units/L (One unit corresponds to enzyme that liberates one micro mole of pyruvate/min).

Estimation of Alkaline phosphatase (ALP) activity.
The activity of alkaline phosphatase was assayed by the method of King (1965b) using disodium phenyl phosphate as substrate. The colour developed is read at 640 nm.

Reagents:
1. Carbonate-bicarbonate buffer, 0.1 M, pH 9.8.
2. Disodium phenyl phosphate, 0.1 M: 218 mg of disodium phenyl phosphate was dissolved in 100 mL of distilled water.
3. Magnesium chloride, 0.1 M: 952.2 mg of magnesium chloride was dissolved in 100 mL of distilled water.
5. Standard: 10 mg of phenol was dissolved in 100 mL of distilled water. The standard solution contained 100 µg phenol/mL.

Procedure:
The incubation mixture containing 1.5 mL buffer, 1 mL substrate, 0.1 mL magnesium chloride were preincubated at 37°C for 10 min. Then 0.1 mL of enzyme was added and incubated at 37°C for the 15 min. The reaction was arrested by the addition of 1 mL of Folin-phenol reagent. The control tubes received enzyme after the addition of Folin-phenol reagent. Then 1 mL of sodium carbonate was added and the tubes were
incubated at 37°C for 10 min. The colour developed was read at 640 nm in a colorimeter. Standards and blank were treated in a similar manner. The activity is expressed in terms of units/L (One unit corresponds to enzyme that liberates one micro mole of phenol/min/mg protein under incubation conditions).

**Histopathological studies** (Raphael, 1976)

The tissues (liver, kidney, brain and heart) preserved in neutral buffered formalin were used for the study of histopathological changes. Tissues were processed, which involves dehydration, clearing and infiltration of the tissue with paraffin and then the tissues were sectioned. The sections are mounted on glass slides and smeared with a drop of Mayer's egg albumin. The slides are dried on a hot plate at about 50°C for 30 min. The sections are then stained with Mayer’s Hematoxylin-Eosin stain and observed under light microscope.

### 2.3. RESULTS AND DISCUSSION

**Acute toxicity study**

In the acute toxicity study, EEAR up to the dose level of 2000 mg/kg of body weight did not exhibit any lethality or toxic symptoms and behavioural changes. Further dosing to estimate the LD$_{50}$ of the drug was not per formed. According to Organization for Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, LD$_{50}$ dose of 2000 mg/kg and above is categorized as unclassified and hence the drug is found to be safe. As 50, 100, 250 and 500 mg/kg of body weight was well tolerated by the animals without any behavioral changes during the treatment, further studies were carried out with 50, 100, 250 and 500 mg/kg of body weight of EEAR.

**Sub-acute toxicity study**

**Effect of EEAR on mortality and body and organ weights**

In the subacute toxicity study, the EEAR treated groups did not show any significant changes in body weight increment compared to the control group (Table 2.1). The organ weights in the test drug treated groups remained normal, indicating that EEAR was not toxic in these vital organs. Moreover, no signs of observable toxicity and mortality were detected during the experimental period. The changes in body and organ weights have been used as an indicator of adverse effects of drugs and chemicals (Raza et al., 2002; Teo et al., 2002) and to assess the response of therapy to
drugs (Joshi et al., 2007). Since no death was recorded in the acute toxicity study, and no changes in body and organs weights were observed at all doses used, the EEAR can be claimed to be non-toxic.

**Effect of EEAR on haematological parameters**
The haematological status after the oral administration of EEAR during sub-acute toxicity studies was assessed and shown in Table 2.2. There was no significant variation \((p > 0.05)\) was observed for hemoglobin, RBC, WBC, MCV and PCV were observed in the drug treated groups compared to the control group. The little increase in the hemoglobin levels might be due to the increased absorption of iron. The slight increase in hemoglobin and WBC levels might be speculated to the immunopotentiating effect of the plant extract. The extract did neither improve nor produced any deleterious effects on the hematological parameters, which indicates that EEAR may not be toxic and does not affect circulating red cells, hematopoiesis, or leukopoiesis.

**Effect of EEAR on biochemical parameters**
The serum urea, uric acid, creatinine, protein, bilirubin, glucose, triglycerides, cholesterol and glucose levels of EEAR treated rat groups and control group are depicted in Table 2.2. The non-protein nitrogen compounds include urea, uric acid and creatinine. These are end products of protein metabolism and must be removed continually to ensure continued protein metabolism in the cells (Guyton, 1981). The elevation in the plasma creatinine concentration indirectly suggests kidney damage specifically renal filtration mechanism (Wasan et al., 2001). In this study, there was no significant alteration \((p>0.005)\) of blood urea, serum creatinine and uric acid in the EEAR treated groups even at 500 mg/kg b.wt indicate that the test drug did not interfere with renal function and that renal integrity was preserved (Kaneko, 1989).

Serum bilirubin levels on the other hand, are related to the function of hepatic cell (Moss and Butterworth, 1974). Increase in the serum protein levels may be a sign of impaired renal function. There was no significant change \((p>0.05)\) observed in the serum bilirubin and protein levels of the rats treated with EEAR when compared with control group (Table 2.2). The serum glucose level of rats receiving EEAR at the dose of 500 mg/kg b.wt. was found to be significantly \((p<0.05)\) decreased than that of the control group. Since this change did not occur in other EEAR treated groups, it was
not a dose-dependent change and should not be due to the plant extract. The change in the levels of plasma cholesterol is an indirect indicator of liver function (Hilaly et al., 2004). There was no significant changes were observed in total cholesterol and triglycerides levels in serum of the EEAR treated groups (p > 0.01) when compared to control group.

Urinary protein, urea, uric acid and creatinine levels in the EEAR treated rats when compared to the control group and are represented in Table 2.2. Creatinine level in urine was found to be significantly (p< 0.01) increased in EEAR treated (500mg/kg bw.t) rats when compared to the control group. All other parameters were found to be non significantly varying when compared with that of control rats. The insignificant alterations in the non-protein nitrogenous substances in serum and urine indirectly manifests the non-hazardous nature of EEAR in maintaining the homeostasis of protein and non-protein nitrogen in the body fluids of treated and control groups.

SGPT, SGOT and ALP are good indices of liver and kidney damage, respectively and increase in these parameters indicate hepatic (Aniagu et al., 2005) and renal damage (Martin et al., 1981). There was slight elevations but no deleterious changes in the levels of AST, ALT and ALP were found in serum, liver and kidney of the EEAR treated groups when compared to the control groups (Table 2.3). Hence, from the above out comes it is evident that EEAR did not provoke any deleterious effects on liver and kidney tissues in treated groups. The organ protective efficacy of the drug is further confirmed by the histopathological examination of these organs.

**Effect of EEAR on histopathology of internal organs**

According to OECD guidelines for testing acute toxicity LD_{50} dose of 2000 mg/kg b.wt and above is categorized as unclassified and considered to be safe. In acute toxicity study the oral administration of EEAR up to the dosage of 2000 mg/kg b. wt did not cause any lethal changes and morality and in sub-acute toxicity studies, there were no significant changes in haematological, biochemical parameters and urinary constituents and were within the normal range which confirmed the non-toxic nature of the extract. To further prove that EEAR is safe at higher dose for long term usage, histopathology of internal organs of rats treated with 500 mg /kg b. wt was examined.
Table 2.1: Body weight (g) and relative organ weight (g/kg) of experimental animals receiving ethanol extract of *A. radiata* during sub-acute toxicity.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Group I (Control)</th>
<th>Group II (EEAR treated-50 mg/kg b.wt.)</th>
<th>Group III (EEAR treated-100 mg/kg b.wt.)</th>
<th>Group IV (EEAR treated-250 mg/kg b.wt.)</th>
<th>Group V (EEAR treated-500 mg/kg b.wt.)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight</td>
<td>215.03 ± 0.974</td>
<td>218.44 ± 0.247*</td>
<td>219.38 ± 0.307*</td>
<td>219.38 ± 0.343*</td>
<td>220.11 ± 0.380*</td>
<td>14.831</td>
</tr>
<tr>
<td>Final body weight</td>
<td>220.37 ± 0.661</td>
<td>219.79 ± 0.365</td>
<td>221.06 ± 0.520</td>
<td>220.27 ± 0.0262</td>
<td>221.44 ± 0.348</td>
<td>2.105</td>
</tr>
<tr>
<td>Liver</td>
<td>26.170 ± 0.041</td>
<td>26.148 ± 0.261</td>
<td>26.47 ± 0.305</td>
<td>26.95 ± 0.430*</td>
<td>26.64 ± 0.362</td>
<td>0.779</td>
</tr>
<tr>
<td>Right Kidney</td>
<td>3.17 ± 0.027</td>
<td>3.15 ± 0.027*</td>
<td>3.161 ± 0.025*</td>
<td>3.165 ± 0.039</td>
<td>3.17 ± 0.042</td>
<td>0.064</td>
</tr>
<tr>
<td>Left Kidney</td>
<td>2.732 ± 0.050</td>
<td>2.740 ± 0.018</td>
<td>2.762 ± 0.017</td>
<td>2.753 ± 0.06</td>
<td>2.747 ± 0.016</td>
<td>0.064</td>
</tr>
<tr>
<td>Brain</td>
<td>3.69 ± 0.016</td>
<td>3.68 ± 0.011</td>
<td>3.69 ± 0.006</td>
<td>3.70 ± 0.017</td>
<td>3.69 ± 0.013</td>
<td>0.195</td>
</tr>
<tr>
<td>Heart</td>
<td>2.42 ± 0.009</td>
<td>2.417 ± 0.015</td>
<td>2.41 ± 0.017</td>
<td>2.412 ± 0.013</td>
<td>2.425 ± 0.010</td>
<td>0.258</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.812 ± 0.015</td>
<td>1.825 ± 0.011</td>
<td>1.810 ± 0.011</td>
<td>1.81 ± 0.014</td>
<td>1.818 ± 0.007</td>
<td>0.303</td>
</tr>
<tr>
<td>Right Testis</td>
<td>5.642 ± 0.016</td>
<td>5.728 ± 0.008*</td>
<td>5.743 ± 0.014*</td>
<td>5.632 ± 0.012</td>
<td>5.637 ± 0.012</td>
<td>0.254</td>
</tr>
<tr>
<td>Left Testis</td>
<td>5.590 ± 0.009</td>
<td>5.598 ± 0.011</td>
<td>5.577 ± 0.008</td>
<td>5.583 ± 0.008</td>
<td>5.598 ± 0.014</td>
<td>0.861</td>
</tr>
<tr>
<td>Right adrenal</td>
<td>0.076 ± 0.002</td>
<td>0.076 ± 0.01</td>
<td>0.07 ± 0.001</td>
<td>0.075 ± 0.001</td>
<td>0.075 ± 0.002</td>
<td>0.988</td>
</tr>
<tr>
<td>Left adrenal</td>
<td>0.082 ± 0.01</td>
<td>0.083 ± 0.01</td>
<td>0.082 ± 0.002</td>
<td>0.082 ± 0.001</td>
<td>0.081 ± 0.001</td>
<td>0.336</td>
</tr>
<tr>
<td>Mortality</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. * indicates significantly (P<0.05) different from control at 5% level. Data was analyzed by one way ANOVA followed by Dunnet’s test.
Table 2.2: Hematological, biochemical and urinary parameters of rats receiving ethanol extract of *A. radiata* during sub-acute toxicity studies.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (EEAR treated-50 mg/kg b.wt.)</th>
<th>Group III (EEAR treated-100 mg/kg b.wt.)</th>
<th>Group IV (EEAR treated-250 mg/kg b.wt.)</th>
<th>Group V (EEAR treated-500 mg/kg b.wt.)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/dl)</td>
<td>6.74 ± 0.012</td>
<td>7.78 ± 0.012</td>
<td>6.76 ± 0.015</td>
<td>6.73 ± 0.06</td>
<td>6.74 ± 0.005</td>
<td>56.941</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.072 ± 0.018</td>
<td>0.077 ± 0.013*</td>
<td>0.075 ± 0.016*</td>
<td>0.073 ± 0.006</td>
<td>0.073 ± 0.006</td>
<td>16.504</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>14.81 ± 0.011</td>
<td>14.79 ± 0.014</td>
<td>14.75 ± 0.015</td>
<td>14.77 ± 0.009</td>
<td>14.80 ± 0.010</td>
<td>484.212</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>4.66 ± 0.001</td>
<td>4.12 ± 0.011*</td>
<td>4.55 ± 0.002*</td>
<td>4.59 ± 0.001*</td>
<td>4.64 ± 0.013</td>
<td>82.408</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.72 ± 0.007</td>
<td>0.68 ± 0.004*</td>
<td>0.77 ± 0.007</td>
<td>0.74 ± 0.012</td>
<td>0.73 ± 0.008</td>
<td>17.083</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>115.41 ± 0.012</td>
<td>114.99 ± 0.007*</td>
<td>114.93 ± 0.012*</td>
<td>115.38 ± 0.019</td>
<td>115.40 ± 0.007</td>
<td>18322.23</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>85.43 ± 0.008</td>
<td>86.22 ± 0.002*</td>
<td>86.15 ± 0.007*</td>
<td>85.39 ± 0.009</td>
<td>85.41 ± 0.009</td>
<td>895.288</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>112.21 ± 0.010</td>
<td>115.61 ± 0.007*</td>
<td>113.48 ± 0.010*</td>
<td>112.38 ± 0.010</td>
<td>112.22 ± 0.005</td>
<td>199.989</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>15.45 ± 0.011</td>
<td>15.46 ± 0.025</td>
<td>15.49 ± 0.015</td>
<td>15.43 ± 0.027</td>
<td>15.46 ± 0.014</td>
<td>50.38</td>
</tr>
<tr>
<td>RBC (x10^6/mm³)</td>
<td>7.06 ± 0.028</td>
<td>7.24 ± 0.016</td>
<td>7.21 ± 0.015</td>
<td>7.18 ± 0.018</td>
<td>7.09 ± 0.033</td>
<td>1.412</td>
</tr>
<tr>
<td>WBC (x10^7/mm³)</td>
<td>6.32 ± 0.009</td>
<td>6.48 ± 0.015</td>
<td>6.44 ± 0.017</td>
<td>6.38 ± 0.007</td>
<td>6.31 ± 0.004</td>
<td>14.001</td>
</tr>
<tr>
<td>MCV (µm³/red cell)</td>
<td>50.08 ± 0.007</td>
<td>51.41 ± 0.003*</td>
<td>50.16 ± 0.016</td>
<td>50.12 ± 0.008</td>
<td>50.06 ± 0.010</td>
<td>740.074</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>37.44 ± 0.015</td>
<td>38.22 ± 0.009*</td>
<td>37.48 ± 0.020</td>
<td>37.46 ± 0.015</td>
<td>37.43 ± 0.012</td>
<td>212.762</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>Values</th>
<th>Values</th>
<th>Values</th>
<th>Values</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/dl)</td>
<td>7.73 ± 0.019</td>
<td>7.705 ± 0.014</td>
<td>7.697 ± 0.017</td>
<td>7.687 ± 0.043</td>
<td>7.683 ± 0.014</td>
<td>0.794</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>2.378 ± 0.015</td>
<td>2.405 ± 0.06</td>
<td>2.442 ± 0.011*</td>
<td>2.42 ± 0.005</td>
<td>2.4 ± 0.007</td>
<td>5.852</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>0.098 ± 0.001</td>
<td>0.096 ± 0.001</td>
<td>0.097 ± 0.003</td>
<td>0.096 ± 0.001</td>
<td>0.097 ± 0.001</td>
<td>0.768</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.793 ± 0.004</td>
<td>0.813 ± 0.007*</td>
<td>0.790 ± 0.004</td>
<td>0.822 ± 0.006*</td>
<td>0.785 ± 0.003</td>
<td>9.278</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. * indicates significantly (P<0.05) different from control at 5% level. Data was analyzed by one way ANOVA followed by Dunnet’s test.
Table 2.3: Effect of ethanol extract of *A. radiata* on marker enzymes in serum, liver and kidney of control and treated rats during sub-acute toxicity studies.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (EEAR treated-50 mg/kg b.wt.)</th>
<th>Group III (EEAR treated-100mg/kg b.wt.)</th>
<th>Group IV (EEAR treated-250 mg/kg b.wt.)</th>
<th>Group V (EEAR treated-500 mg/kg b.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (IU/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>40.263 ± 0.565</td>
<td>41.160 ± 0.544</td>
<td>39.958 ± 0.112</td>
<td>39.653 ± 0.448</td>
<td>39.110 ± 0.288</td>
</tr>
<tr>
<td>ALT</td>
<td>22.883 ± 0.489</td>
<td>24.667 ± 0.791*</td>
<td>23.687 ± 0.181</td>
<td>24.065 ± 0.169</td>
<td>22.942 ± 0.086</td>
</tr>
<tr>
<td>ALP</td>
<td>0.019 ± 0.001</td>
<td>0.015 ± 0.001*</td>
<td>0.022 ± 0.002</td>
<td>0.017 ± 0.002</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>F-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (IU/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>31.722 ± 0.283</td>
<td>32.077 ± 0.306</td>
<td>31.760 ± 0.011</td>
<td>32.085 ± 0.322</td>
<td>31.060 ± 0.309</td>
</tr>
<tr>
<td>ALT</td>
<td>95.542 ± 0.306</td>
<td>95.307 ± 0.023</td>
<td>95.753 ± 0.185</td>
<td>95.020 ± 0.015</td>
<td>95.858 ± 0.134</td>
</tr>
<tr>
<td>ALP</td>
<td>0.028 ± 0.001</td>
<td>0.027 ± 0.001</td>
<td>0.031 ± 0.001*</td>
<td>0.027 ± 0.002</td>
<td>0.028 ± 0.001</td>
</tr>
<tr>
<td>F-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney (IU/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>27.125 ± 0.015</td>
<td>27.147 ± 0.156</td>
<td>27.192 ± 0.007</td>
<td>28.220 ± 0.288*</td>
<td>27.522 ± 0.112</td>
</tr>
<tr>
<td>ALT</td>
<td>34.610 ± 0.07</td>
<td>34.443 ± 0.309</td>
<td>33.212 ± 0.219*</td>
<td>34.120 ± 0.078</td>
<td>34.810 ± 0.318</td>
</tr>
<tr>
<td>ALP</td>
<td>0.185 ± 0.004</td>
<td>0.186 ± 0.01</td>
<td>0.187 ± 0.02</td>
<td>0.186 ± 0.02</td>
<td>0.188 ± 0.001</td>
</tr>
<tr>
<td>F-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. * indicates significantly (P<0.05) different from control at 5% level. Data was analyzed by one way ANOVA followed by Dunnet's test.
No abnormal signs of internal organs were observed by gross examination. Histopathological examinations of some selected vital organs (liver, kidney, brain and heart) from EEAR treated animals when compared to control groups, show normal architecture (Fig. 2.1a, 2.1b, 2.2a, 2.2b, 2.3a, 2.3b, 2.4a and 2.4b).

In conclusion, EEAR can be considered safe, as it did not cause any lethality or adverse changes in the general behaviour in acute toxicity studies up to the dose of 2000 mg/kg b.wt and also there was no observed detrimental effects caused by EEAR (up to 500 mg/kg b.wt) in haematological, biochemical and histopathological changes in sub-acute toxicity study in rat model. This shows the non toxic nature of the EEAR and thus can be safely administered for further in vivo studies to study its antioxidant activities (short-term and long-term treatment).
Figure 2.1a. Liver of control rats. H & E, 10X.

Figure 2.1b. Liver of EEAR treated (500mg/kg b.wt) rats. H & E, 10X.

Figure 2.2a. Kidney of control rats. H & E, 10X.

Figure 2.2b. Kidney of EEAR treated (500mg/kg b.wt) rats. H & E, 40X.
Figure 2.3a. Brain of control rats. H & E, 10X.

Figure 2.3b. Brain of EEAR treated (500mg/kg b.wt) rats. H & E, 10X.

Figure 2.4a. Heart of control rats. H & E, 10X.

Figure 2.4b. Heart of EEAR treated (500mg/kg b.wt) rats. H & E, 10X.