3. MATERIALS AND METHODS

3.1. Study materials and their sources

For the present study, the two plants *Aegle marmelos* L. belonging to Rutaceae and *Eclipta prostrata* L. to Asteraceae were selected. They were collected from in and around A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur District, Tamil Nadu, India and identified with help of the standard manuals like *The Flora of the Presidency of Madras* (Gamble, 1967) and Indian Medicinal Plants (Kirtikar and Basu, 1994). The identification was confirmed at Rapinat Herbarium, St, Joseph’s College (Autonomous), Tiruchirapalli, Tamil Nadu. Voucher samples were prepared and deposited in the Department of Zoology and Biotechnology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur District, Tamil Nadu, India.


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<th>Kingdom</th>
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A. marmelos is a moderate-sized tree, extensively planted in the Hindu temples which are used for worship. It can withstand various types of soils, climatic conditions and a pH range of 5 to 10. It is present throughout South East Asia as a naturalized species. The plant is a mid-sized, slender, aromatic, armed and gum-bearing tree growing up to 18 metres tall. The tree is leafless or nearly so, for a short time in the hot season. The bael fruit has a smooth, woody shell with a green, gray or yellow peel. It takes about 11 months to ripen on the tree and can reach the size of a large grape fruit and some are even larger. The shell is so hard and it must be cracked with a hammer or machete. The fibrous yellow pulp is very aromatic. It has been described as tasting of marmalade and smelling of roses. Numerous hairy seeds are encapsulated in a slimy mucilage (Fig.1).

3.1.2. Taxonomy of Eclipta prostrata (L)

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It is a perennial herb, distributed in the tropical and sub-tropical regions of the world. It is a common weed in moist situations throughout India, ascending up to 6,000 on the hills. It is very common in clayey moist ground, like bounds of paddy fields. It shows phenotypic plasticity in its habit, leaf morphology and stem colour.

The plant is bitter, hot, sharp and dry in taste and Floral heads 6–8 mm in diameter, solitary; florets white; achene compressed and narrowly winged. In ayurvedic medicine the leaf extract is considered a powerful liver tonic, rejuvenative, and especially good for hair. A black dye obtained from *E. prostrata* is used for dyeing hair and tattooing. *E. prostrata* also has traditional external uses, such as for athlete's foot, eczema and dermatitis. It is reported to improve hair growth and colour (Fig.2).

Tamil: Kayanthara, Hindi: Bhangra, English: Kadimulbirt.

### 3.2. Preparation of leaf powder

The leaves of *A. marmelos* and *E. prostrata* were washed with sterile distilled water and shade dried for two weeks. The dried leaves were powdered separately by using electric grinder.

### 3.3. Preparation of extract (Rao et al., 1995)

The leaf powders of *A. marmelos* and *E. prostrata* were boiled separately in distilled water and filtered through Whatmann no: 40 filter papers. The extracts were evaporated by slow heating (50º C) and
continuous stirring in a water bath. The residues were extracted and it was utilized for phytochemical analysis and pharmacological studies.

3.4. Phytochemical analysis

Phytochemical analysis of the plant extracts was undertaken using standard qualitative methods as described by various authors (Kapoor et al., 1969; Odebiyi and Sofowora, 1990). The plant extracts were screened for the presence of biologically active compounds such as carbohydrates, proteins, alkaloids, phytosterols, tannins, flavonoids, saponins and phenolics.

3.4.1. Carbohydrates

Fehling’s test (Kokate, 1994)

Solution A:

Prepared by dissolving 34.65 g of copper sulphate in distilled water and made upto 500 ml.

Solution B:

Prepared by dissolving 125 g of potassium hydroxide and 173 g of Rochelle’s salt (sodium potassium tartarate) in distilled water and made up to 500 ml. The solutions ‘A’ and ‘B’ were added into the test samples. The contents were boiled for a few minutes. The formation of a red or brick red precipitate indicated the presence of carbohydrates.

Benedict’s test

Prepared by dissolving 173 g of sodium citrate and 100 g of sodium carbonate in 500 ml of distilled water. To this solution 17.3 g of
copper sulphate dissolved in 100 ml of distilled water was added. To 0.5 ml of plant extract, 5 ml of Benedict’s reagent was added and boiled for 5 mins. The formation of a bluish green colour showed the presence of carbohydrates.

3.4.2. Proteins

Million’s test (Walsh and Farrel, 1961)

One part of mercury was digested with 2 parts of concentrated HNO₃ and the resulting solution was diluted with 2 volumes of water. To a small quantity of plant extract, 5-6 drops of Millon’s reagent was added. A white precipitate which turned red on heat indicated the presence of proteins.

3.4.3. Alkaloids (Salehi Surmaghi et al., 1992)

Dragendorff’s test (Kraut reagent – Potassium bismuth iodide)

Prepared by dissolving 8 g of Bi (NO)₃ 5 H₂O in 20 ml of HNO₃ and 2.72 g of potassium iodide in 50 ml of distilled water separately. They were mixed and allowed to stand till KNO₃ got crystallized. The supernatant was decanted and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na₂CO₃ followed by the extraction of the liberated base with ether. To 0.5 ml of plant extract and 2 ml of HCl was added and mixed well. Then 1 ml of reagent was added to this acidic medium. Appearance of orange red precipitate is an indication of the presence of alkaloids.
Wagner’s reagent (Iodine-Potassium iodide solution)

Prepared by dissolving 1.2 g of iodine and 2.0 g of potassium iodide in 5 ml of H₂SO₄ and the solution was diluted to 100 ml. The plant extract (10 ml) was acidified by adding 1.5% HCl and a few drops of Wagner’s reagent. The formation of a yellowish brown precipitate confirmed the presence of alkaloids.

Meyer’s reagent (Potassium mercuric iodide)

Prepared by dissolving 1.36 g of mercuric chloride in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of distilled water separately. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops of the reagent were added to 1 ml of the plant extract. The formation of a pale precipitate showed the presence of alkaloids.

3.4.4. Phytosterols (Malick and Singh, 1980)

About 0.5 ml of test solution was mixed with a minimum quantity of chloroform to which 3- 4 drops of acetic acid and one drop of concentrated H₂SO₄ were added. The formation of a deep blue or green colour showed the presence of steroids.

3.4.5. Tannins (Segelaman et al., 1969)

**Ferric chloride test**

To 2 ml of plant extract, a few drops of 5 % aqueous FeCl₃ solution was added. A bluish black colour formed, which then disappeared. To the
above contents few ml of dilute H₂SO₄ was added. Formation of yellowish brown precipitate, indicated the presence of tannins.

**Lead acetate test**

In a test tube containing about 5 ml of plant extract, a few drops of 1 % solution of lead acetate was added. Formation of yellow or red precipitate indicated the presence of tannins.

**3.4.6. Flavonoids (Somolenski *et al.*, 1972)**

In a test tube containing 0.5 ml of plant extract, 5-10 drops of diluted HCl and a small pinch of zinc or magnesium were added and the solution was boiled for a few minutes. Formation of a reddish pink or dirty brown colour indicated the presence of flavonoids.

**3.4.7. Saponins (Malick and Singh, 1980)**

In a test tube containing about 5 ml of plant extract, a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and kept for 3 min. Formation of a honeycomb like froth showed the presence of saponins.

**3.4.8. Phenols (Malick and Singh, 1980)**

In a test tube one ml of plant extract, 2 ml of distilled water followed by a few drops of 10 % aqueous FeCl₃ solution were added. Formation of a blue or green precipitate indicated the presence of phenols.
3.5. Analysis of Trace elements by Atomic Absorption spectroscopy (AAS) (Sondhi and Agarwal, 1995)

The powdered leaf sample (0.5 g) mixed with 15 ml of triple acid mixture and the contents were mixed gently. The flask with contents were kept overnight on a hot plate set at the lowest temperature. Then the temperature was raised on the hot plate and digested the contents for a few minutes. Then the contents were further digested for 1-2 min with the full heat of the hot plate. The contents of the flask were continuously heated until the refluxing of the H$_2$SO$_4$ starts along the neck of the flask. After the digestion completed, the contents of the flask were cooled by diluting with deionized water and the final volume were made to 50 ml. The digested contents were transferred to a clean polyethylene bottle and stored for further analysis. A blank digestion were also carried out by using all these steps excluding the plant material.

3.6. Gas Chromatography-Mass Spectroscopy (GC-MS) analysis (Ivanova et al., 2002)

Sample Preparation

About 0.4 g of samples such as *A. marmelos* and *E. prostrata* were soaked separately in 50 ml of methanol overnight. The extracted samples were filtered through Whatmann no.1 filter paper and the filtrates were concentrated to dryness. The dried extracts were diluted with methanol and 1 µl was injected into GC-MS.
**GC-MS analysis**

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column elite-5 ms fused silica capillary column (30 × 0.25 mm ID × 0.25 µm film thickness, composed of 5% phenyl 95% Dimethyl polysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 1.0µl was employed (split ratio of 10:1) injector temperature 290°C; ion-source temperature 150°C. The oven temperature was programmed from 50°C, with an increase of 7°C/min, to 150°C.

The oven temperature were programmed from 50°C, with an increase of 7°C/min, to 150°C hold for 5 min, then 7°C/min to 280°C hold for 10 min. Mass spectra were taken at 70 eV (electron ionization technique); a scan interval of 0.2 seconds and fragments were scanned from 40 to 600 Da.

**Identification of Components**

Interpretation on mass spectrum of GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The spectrums of the separated components were needed for identification of the components.
3.7. Pharmacological Studies

3.7.1 Selection of Animals

In these experiments, thirty healthy male albino rats of Wistar strains, 4 months of age, weighing 160-200 g were used. The animals were obtained from the animal house of Sri Venkateshwara Enterprises, Bangalore, India. On arrival, the animals were placed in well ventilated stainless steel cages (40×25×15 cm) with six animals per cage, under standard laboratory conditions (temperature 25 ± 20°C) with dark and light cycle (12/12 h). The animals were fed with standard pellet diet supplied by M/s. Rayans Biotechnologies Pvt. Ltd., Hyderabad and water ad libitum. All the animals were acclimatized to laboratory condition for a week before the commencement of the experiment. All the experimental processes and protocols used in this study were reviewed by the Institutional Animal Ethical Committee according to the Indian National Science Academy guidelines for the use and care of experimental animals (CPCSEA/265).

3.7.2. Taxonomy of Albino rat

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</table>
3.7.3. Hepatotoxic agents (Rajakrishnan et al., 1997)

The hepatotoxic dose were standardized as 20 % alcohol (7.9 g/kg body weight) orally, using an intragastric tube for 21 days.

3.7.4. Groupings and Experimental Design (Fig. 5)

The animals were divided into five groups. Each group contained six animals.

**Group I** : Normal control (n = 6, the animals were given normal saline only).

**Group II** : Hepatotoxic control (n = 6, the animals were given 1 ml of 20% alcohol for 21 days).

**Group III** : Treatment group (n = 6, the animals were given 1 ml of 20% alcohol for 21 days and from 22\textsuperscript{nd} to 42\textsuperscript{nd} days the extract of *A. marmelos* was given to animals through the intragastric tube (7.9 g/Kg b. wt)

**Group IV** : Treatment group (n = 6, the animals were given 1 ml of 20% alcohol for 21 days and 22\textsuperscript{nd} to 42\textsuperscript{nd} days the extract of *E. prostrata* was given to animals through intragastric tube (7.9 g/kg. b.wt).

**Group V** : Treatment group ( n = 6, the animals were given 1 ml of 20% alcohol for 21 days and from 22\textsuperscript{nd} to 42\textsuperscript{nd} days the animals were given silymarin drug through intragastric tube (1g/kg.b.wt).

At the end of the drug treatment period, all the animals were anaesthetized by application of light chloroform and blood samples were
collected from a group of animals from dorsal aorta by heparinized syringe in vacutainer tubes. Plasma was separated from the collected blood by centrifugation of 3000 rpm for 5 minutes. Separate blood samples were collected from another group of anaesthetized animals in glass test tubes and allowed to coagulate for 30 mins. Serum was separated by centrifugation at 3000 rpm for 2 min. Plasma and serum samples were kept at -20°C for biochemical analysis.

Finally the animals were sacrificed by cervical decapitation, the perfused liver of each animal was dissected out and washed with isotonic solution and then homogenized to get 11 per cent solution of tissue homogenate in Tris Hcl buffer (10 mm, pH 8.0). This homogenate was used for the determination of various parameters.

3.8. Haematological analysis

3.8.1. Estimation of Blood Haemoglobin

Haemoglobin were measured by the method of Drabkin and Austin (1932). The collected blood (0.02 ml) were diluted with 5 ml of the reagent. The diluted blood was mixed well and allowed to stand for 10 minutes, to ensure the completion of the reaction. The solution was read at 540 nm together with the standard solution of cyanmethemoglobin. Blood haemoglobin levels were expressed as g/dl.

3.8.2. Enumeration of red blood corpuscles

The total erythrocyte count was determined accurately by the method of Huxtable (1990). Blood was sucked exactly upto the 0.5 ml
mark in the RBC pipette and the diluent fluid was drawn immediately
upto the mark and mixed thoroughly. It was left for 2-3 min for proper
mixing. The Neubauer counting chamber was placed along with its cover
glass in position.

The capillary stem of the pipette was emptied which contains only
the diluting fluid. This was done by discarding the first 3-5 drops. One
drop of diluted blood was released into the groove of the Neubauer
counting chamber. It was left for cells to settle for 2-3 minutes. The
counting chamber was placed under the microscope and the ruled area was
located. Erythrocytes were counted in the 5 squares of the counting areas
of 1 mm square. The number of cells in the 4 corner squares and one
central square was counted. The results were expressed as number of
cells/mm³ of blood.

3.8.3. Red Cell Indices (Wintrobe, 1981)

Red cell indices namely MCV, MCH and MCHC were calculated
by following formula

MCV - Mean Corpuscular Volume

The volume of the average RBC was calculated by:

\[ \frac{Hct}{RBC} \times 10 = MCV \]

MCH - Mean Corpuscular Haemoglobin: (Weight of haemoglobin in each
cell) calculated by:

\[ \frac{Hb}{RBC} \times 10 = MCH \]
MCHC - Mean Corpuscular Haemoglobin Concentration in the average RBC was calculated by:

\[
\frac{Hb}{Hct} \times 10 = MCHC
\]

3.8.4. **Enumeration of white blood corpuscles** (Raghuramulu *et al.*, 1983)

In a clean test tube, 0.38 ml of WBC diluent fluid and 0.02 ml of blood were mixed well with the help of Hbshahli pipette. Then, the WBC were counted as described for RBC using Neubauer counting chamber. The results were expressed as number of cells/mm³ of blood.

3.8.5. **Determination of Packed cell volume**

Packed cell volume was determined by centrifugation using Wintrobe tubes, by the method of Wintrobe (1932).

Blood sample (0.6 ml) was taken in a Windrode tube and centrifuged for 30 minutes in a relative centrifugal field (RCF) from 2000 to 2300 xg. Then the volumes of the packed cells were noted.

3.9. **Estimation of enzymatic antioxidants**

3.9.1. **Estimation of superoxide dismutase (SOD)**

Superoxide dismutase activity were assayed by the method of Kakkar *et al.* (1984). In this method, 0.5 ml of the plasma were diluted to 1.0 ml with ice cold water, followed by 2.5 ml ethanol and 1.5 ml chloroform (chilled reagents). This mixture were shaken for 60 seconds at 4°C and then centrifuged. The assay mixture contained 1.2 ml of sodium
pyrophosphate buffer, 0.1 ml of PMS and 0.3 ml of NBT and appropriately diluted for enzyme preparation in a total volume of 3 ml. These actions were started by the addition of 0.2 ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by an addition of 1 ml glacial acetic acid. The reaction mixture were stirred vigorously and shaken with 4 ml n-butanol. The mixture were allowed to stand for 10 minutes, centrifuged and the butanol layer was separated. The colour intensity of the chromogenin butanol layer was measured in a calorimeter at 520 nm. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50% inhibition of NBT reduction in one minute under assay conditions.

Superoxide dismutase activity was expressed as U/ml for plasma, U/mg Hb for erythrocyte lysate and U/mg protein for tissues (U-The amount of enzyme required to inhibit % nitro blue tetrazolium (NBT) reduction).

3.9.2. Estimation of Lipid Peroxidase (LPO)

Lipid peroxidase was estimated by the method of Okhawa (1970). According to this method, 0.1 ml of tissue homogenate were mixed with 4 ml of 0.85 N H₂SO₄. Then, 0.5 ml of phosphortungsitc acid were added and stirred well. The contents were centrifuged for 10 minutes.

The supernatant were discarded and the sediment mixed with 2.0 ml of N/12 H₂SO₄ and 0.3 ml of 10% phosphotungstic acid. The mixture
were centrifuged for 10 min. The sediment were suspended in 4.0 ml of distilled water and 1 ml of TBA reagent. The tubes were kept in a boiling water bath of 41 hrs. After cooling, 5 ml of butanol were added to each tube and the colour extracted in the butanol phase were read at 532 nm. The lipid peroxide content were expressed as nanomoles of TBA reactants/mg protein.

3.9.3. Assay of Catalase (CAT)

The activity of catalase was measured by the method of Sinha (1970). To 0.05 ml of the tissue homogenate, 1.2 ml of phosphate buffer and 1.0 ml of hydrogen peroxide were added to commence the enzyme reaction. The decrease in absorbance were measured at 240 nm at 30 secs interval for 3 min. The blank were run simultaneously with 1.0 ml of distilled water. The activity of catalase were expressed as n moles of H$_2$SO$_4$ utilised/min/mg of protein.

3.9.4. Estimation of Reduced Glutathione (GSH)

Reduced Glutathione was estimated using the method of Beutler et al., (1963). One ml of homogenate/blood were precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 5 ml of supernatant, 2 ml of DTNB were added and the total volume were made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm. The concentration of glutathione were expressed as µg/mg protein.
3.9.5. Estimation of Glutathione Peroxidase (GPₓ)

The assay of glutathione peroxidase were carried out using the method of Rotruck (1973). The reaction mixture consisted of 0.2 ml each of EDTA, sodium azide, H₂O₂. 0.4 ml phosphate buffer and 0.1 ml tissue homogenate.

The mixture was incubated at 37°C at different time intervals. The reaction was arrested by adding 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed was read at 420 nm immediately. The level of GPₓ was expressed as µmoles of glutathione oxidized/min/mg protein.

3.9.6. Estimation of Glutathione-S-Transferase (GST)

The assay of glutathione-S-Transferase was carried out using the method of Habig et al. (1974).

According to this method, 1 ml of the homogenate, 1 ml of phosphate buffer, 1.7 ml of H₂O and 0.1 ml of CDNB were added. After incubation at 37°C for 15 min 0.1 ml of GSH were added and the change in OD were read at 340 nm. The results were expressed as µmoles of CDNB conjugated/min/mg protein.

3.10. Estimation of non-enzymatic Antioxidants

3.10.1. Estimation of Vitamin E

Vitamin E was estimated in plasma and erythrocyte membrane by the method of Desai (1984).
To 0.2 ml of the sample (plasma) taken in a glass stoppered centrifuged tube, 1.8 ml of redistilled methanol were added and thoroughly mixed. 3.0 ml of petroleum ether were then added and tubes were shaken rapidly in a mechanical shaker for 3 minutes. The tubes were centrifuged and 2.0 ml of the ether layer transferred to fresh tubes and evaporated to dryness. To the lipid residue, carefully redissolved in 3.0 ml of absolute methanol, 0.2 ml of 0.2% bathophenanthroline reagent was added and mixed. The tubes were protected from exposure to direct light and the assay was carried out rapidly from this point. Then 0.2 ml of ferric chloride reagent was added and mixed in a vortex mixture.

After one minute, 0.2 ml of orthophosphoric acid reagent was added and shaken well. Tubes containing standard α-tocopherol, 2-10 μg were treated in a similar manner along with a blank containing 3.0 ml of methanol. The α-tocopherol values were expressed as mg/dl for plasma and µg/mg protein for erythrocyte membranes. The values were expressed as mg/dl for plasma and µg/mg protein for erythrocyte membranes.

3.10.2. Estimation of Vitamin C

The level of plasma vitamin C was determined by the method of Omaye et al. (1979).

One ml of the plasma was mixed thoroughly with 1.0 ml of ice cold 10% TCA and centrifuged for 20 minutes at 3500 g. To 0.5 ml of the supernatant, 0.1 ml of DTC reagent was added and mixed well. The tubes incubated at 37°C for three hours. 0.75 ml of ice cold 65% sulphuric acid were added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standard containing 10 – 50 μg of
ascorbic acid was processed similarly along with a blank containing 0.5 ml of 10% TCA. The colour developed were read at 520 nm. Vitamin C values were expressed as mg/dl for plasma.

3.11. Assay of Hepatic enzymes

3.11.1. Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT)

The SGOT level were assayed using the method of King (1965). The assay mixture contains 1 ml of substrate and 0.2 ml of serum was incubated for 1 hr at 37°C. To the control tubes serum was added after arresting the reaction by adding 1 ml of DNPH. The tubes were kept at room temperature for 30 min. Added 0.5 ml of NaOH and the colour developed was read at 540 nm. The activity of SGOT was expressed as µmoles of pyruvate formed/min/mg of protein.

3.11.2 Estimation of Serum Glutamate Pyruvate Transaminase (SGPT)

The SGPT level was assayed using the method of King (1965). The assay mixture contains 1 ml of substrate and 0.2 ml of serum were incubated for 1 hr at 37°C. After that, added 1 ml of DNPH and kept at room temperature for 20 min. Serum was added to control tubes after the reaction was arrested by an addition of 1 ml of DNPH. Added 5 ml of NaOH and the colour developed was read at 540 nm. The activity of SGPT was expressed as µmoles of pyruvate formed/min/mg of protein.
3.11.3 Assay of Gamma Glutamyl Transferase (GGT)

The serum GGT levels were assayed using the method of Rosaki and Rau (1972). The reaction mixture contained 0.5 ml of substrate, 1 ml of Tris HCl, 2.2 ml of Glycyl glycine and 0.2 ml homogenate. The total volume was made up to 4 ml with water. After an incubation for 30 min at 37°C the samples were heated at 100°C for 5 min and centrifuged. The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of GGT was expressed at µmoles of p-nitroaniline formed/min/mg of protein.

3.11.4. Estimation of Serum Alkaline Phosphatase (ALP)

The serum ALP levels were assayed using the method of King (1965). The reaction mixture containing 1.5 ml carbonate buffer, 1 ml Disodium phenyl phosphate, 0.1 ml magnesium chloride and 0.1 ml of serum was incubated at 37°C for 15 min. The reaction was arrested by the addition of Folin’s phenol reagent. Control tubes were also treated similarly but serum was added after the reaction was arrested with Folin’s phenol reagent and then add 1 ml of Sodium Carbonate. The colour developed was read after 10 min at 640 nm. The activity of ALP was expressed as µmoles of phenol liberated/min/mg of protein.

3.11.5. Estimation of Serum Bilirubin

The serum bilirubin levels were assayed by Malloy and Evlyn (1937). 0.2 ml serum were taken and made up to 2 ml of Diazo reagent, 2.5 ml of methanol. The blank 0.2 ml serum were added and made up to 2 ml with water and added 0.5 ml of diazo blank and 2.5 ml methanol. The
colour developed were read at 540 nm. The values were expressed as mg/dl.

3.11.6. Estimation of Serum protein

Serum protein content was estimated by the method of Lowery et al. (1951). Aliquot sizes of the standard were taken in different tubes and made up to the total volume of 1.0 ml. 4.5 ml of alkaline copper reagent were added to all the tubes and incubated at room temperature for 10 min.

0.5 ml of Folin’s reagent were added to all tubes and incubated at room temperature for 20 min. The serum and blank were also treated similarly. The blue colour developed were read at 640 nm. The protein content were expressed as g/dl.

3.12. Histological Studies

Histology of the liver tissues was performed by the method of Sujai Suneetha (1993). On the 42nd day, liver tissues were taken from animals which were fasted overnight under ether anesthesia. The whole liver from each animal was removed after killing the animals, was placed in 10 per cent formalin solution and immediately processed by the paraffin technique section of 5 μm thickness were cut and stained by haematoxylin and eosin for histological examination. The photomicrographs of histological studies were taken.
3.13. Statistical analysis

The data were statistically analyzed and all values were expressed as mean ± SEM. The data were also analyzed by One Way ANOVA using SPSS Software. P<0.05 was considered significant.