3. Materials and Methods
3. MATERIALS AND METHODS

To evaluate the phytochemical and pharmacological properties of *Clerodendrum infortunatum*, the material and methods followed in conducting the experiments to accomplish the above are presented hereunder:

Material

3.1. Morphology and distribution of *Clerodendrum infortunatum* L.

*Clerodendrum infortunatum* L. sparsely pubescent shrub 0.9-2.4m. height. It is found in deciduous forests of the Western Ghats of India (Manjunath *et al.*, 2004) and Sri Lanka. In Karnataka it is reported in Bangalore, Belgum, Chikmagalur, Coorg, Hassan, Mysore, North Canara, Shimoga and South Canara (Yoganarasimhan, 1996).

Leaves simple, large, ovate, acuminate, thinly hairy on both sides, entire or denticulate, reticulately veined, base cordate or rounded; quadrangular gregarious branches; petioles 3.8-10 cm; long cylindric hairy (Fig. 7a). Flowers are rather pubescent pedicels, in stalked cymes forming large pubescent panicles, bracts leafy conspicuous, calyx 1.3 cm long in flower, silky pubescent, segments broadly lanceolate, very acute, corolla white tinged with pink; tube 2 cm long, slender; lobes exceeding 1cm, long obtuse, ovary and style glabrous, drupe 8 mm diameter, black, nearly globose, seated on the enlarged pink calyx containing 4-1 pyrenes (Fig. 7b).
3.1.1. Vernacular names

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<tr>
<th>Language</th>
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<tr>
<td>Sanskrit</td>
<td>Barhichuda bhantaka</td>
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<td>Hindi and Bengali</td>
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<td>Kannada</td>
<td>Basavanapaada, Ibbane</td>
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Fig. 7. Clerodendrum infortunatum L.

3.1.2. Medicinal Importance

Leaves and roots employed externally for tumours and certain skin diseases. Fresh juice of leaves are used as tonic for chiretta antiper, febge and in malaria especially of children, snake bite, scorpion cuttings sprouts (Chopra et al., 1956). In ayurveda bark is used for diabetes (Yoganarasimhan, 1996).

3.2. Experimental animals

Wistar albino rats and Swiss albino mice were obtained from Sri Jagadguru Mallikarjuna Murugharajendra College of Pharmacy, Chitradurga, Karnataka, India. Animals were housed six per polypropylene cage.
and given free access to standard laboratory diet (Hindustan Lever Ltd., Bangalore, India) and water during the experiment. The Institutional Ethical Committee (Registration Number 628/2002/CPCSEA) permitted the study under the certification Ref No. SJMCP/IAEC/CLEAR/05/2007-08. Wistar albino rats of either sex, weighing about 240–250 g were used for acute toxicity study and wound healing activity, whereas male rats of the same strain weighing 180–220 g used for in vivo antioxidant and hepatoprotective activity. Adult male Swiss albino mice of 10 to 12 weeks old (22 ± 2 g) were used for acute toxicity, antitumor and antimutagenic studies.

3.3. Ehrlich's Ascites Tumor (EAT) cells

The Ehrlich ascites tumor was initially described as a spontaneous marine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behavior and is able to grow in almost all mice strains (Chen and Watikins, 1970; Segura et al., 2000). It has been used as a transplantable tumor model to investigate the antineoplastic effects of several chemical compounds. The first inoculum of Ehrlich's ascites tumor cells (EAT) was kindly provided by the Manipal Life Sciences Centre, Manipal University, Manipal. EAT cells were thereafter propagated by weekly intraperitoneal injection of 0.3 ml freshly drawn ascitic fluid (diluted 1:5 phosphate buffer saline) from a donor mice-bearing ascites tumor of 6 to 8 day-old into three mice each with a mean body weight of 22 ± 2 g. Transplantation was carried out using a sterile disposable syringe under aseptic conditions.

Methods

3.4. Plant material collection

Leaves and Roots of *C. infortunatum* were collected from Lakkinakoppa Range Forest of Bhadra Wild Life Sanctuary, Karnataka, India. The plant was authenticated by Dr. V. Krishna, Professor of Biotechnology who is also a taxonomist and the same was confirmed from the ‘Flora of Davanagere District’ (Manjunath et al., 2004). The specimen is deposited at Kuvempu University, Shankaraghatta, Karnataka, India. The material was air dried in shade for two weeks, powdered mechanically and stored in airtight containers for further investigations.
3.5. Preparation of Extracts

Extraction of leaves and root parts were performed sequentially with solvents of increasing polarity. This has the advantage of allowing preliminary separation of the metabolites present in the material within distinct extracts and simplifies further isolation. The air-dried and finely ground (1 kg) leaves and root part of plant were extracted in a Soxhlet apparatus successively with petroleum ether, chloroform and ethanol. The extracts were filtered, pooled and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi, Flawil, Switzerland) to yield dried petroleum ether, chloroform and ethanol extracts of leaves and roots. The extracts so obtained from each of solvents were labeled and yield was calculated in terms of grams/weight of the powdered material.

3.6. Preliminary phytochemical screening (Qualitative Analysis)

The crude extracts viz., petroleum ether, chloroform and ethanol extracts of C. infortunatum obtained from each of the solvents were subjected for the following qualitative tests to detect the presence of major chemical groups like alkaloids, flavonoids, glycosides, saponins, tannins and reducing sugars (Harborne, 1984; Trease and Evan, 1989; Kokate, et al., 1996).

3.6.1. Alkaloids

a) Dragendorff’s test

To 2 mg of the ethanolic extract 5 ml of distilled water was added, 2M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff’s reagent was added. Formation of orange or orange red precipitate indicated the presence of alkaloids.

b) Hager’s test

2 mg of the ethanolic extract was taken in a test tube, a few drops of Hager’s reagent were added. Formation of yellow precipitate confirmed the presence of alkaloids.

c) Wagner’s test

2 mg of ethanolic extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner’s reagent were added. A yellow or brown precipitate indicated the presence of alkaloids.
Materials and Methods

d) Mayer’s test

A few drops of the Mayer’s reagent was treated with 2 mg of ethanolic extract. Formation of white or pale yellow precipitate indicated the presence of alkaloids.

3.6.2. Carbohydrates

a) Benedict’s test

0.5 ml of aqueous extract, 5 ml of Benedict’s solution was taken and boiled for 5 minutes. Formation of brick red coloured ppt indicated the presence of carbohydrates.

b) Fehling’s test

To 2 ml of aqueous extract, 1 ml mixture of equal parts of Fehling’s solution A and B were added and boiled for few minutes. Formation of red or brick red coloured precipitate indicated the presence of reducing sugar.

c) Molisch’s test

In a test tube containing 2 ml of aqueous extract, 2 drops of freshly prepared 20% alcoholic solution of α-naphthol was added. 2 ml of conc. sulphuric acid was added so as to form a layer below the mixture. Red-violet ring appeared, indicating the presence of carbohydrates which disappeared on the addition of excess of alkali.

d) Barfoed’s test

To the test solution, Barfoed’s reagent was added, boiled on water bath, brick red precipitate was formed.

3.6.3 Flavonoids

a) Shinoda’s test

In a test tube containing 0.5 ml of the ethanolic extract 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicated the presence of flavonoids.
b) Ferric chloride test

Test solution with few drops of ferric chloride solution shows intense green colour.

c) Zinc-Hydrochloric acid reduction test

Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour.

d) Alkaline reagent test

Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.

e) Lead acetate solution test

Test solution with few drops of lead acetate (10%) solution gives yellow precipitate.

3.6.4. Triterpenoids

a) Liebermann - Burchard's test (LB test)

2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a violet coloured ring indicated the presence of triterpenoids.

b) Salkowskii test

When few drops of conc. sulphuric acid was added to the test solution, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

3.6.5. Proteins

a) Biuret's test

To one ml of hot aqueous extract, 5-8 drops of 10% w/v sodium hydroxide solution, followed by 1 or 2 drops of 3% w/v copper sulphate solution were added. Formation of a violet red colour indicated the presence of proteins.
b) **Millon’s test**

One ml of aqueous extract was dissolved in 1 ml of distilled water and 5-6 drops of Millon’s reagent was added. Formation of white precipitate which turns red on heating indicated the presence of proteins.

c) **Xanthoproteic test**

Test solution after treating with concentrated nitric acid and on boiling, gave yellow precipitate.

d) **Ninhydrin test**

Test solution when treated with Ninhydrin reagent gives blue colour.

3.6.6. **Saponins**

a) **Foam test**

In a test tube containing about 5 ml of an ethanolic extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicated the presence of saponins.

b) **Haemolysis test**

2 ml each of 18% sodium chloride solution in two test tubes were taken. To one test tube distilled water was added and to the other 2 ml of filtrate. Few drops of blood were added to both the test tubes. Mixed and observed for haemolysis under microscope.

3.6.7 **Steroids**

a) **Liebermann-Burchard’s test**

2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicated the presence of steroids.
Materials and Methods

b) Salkowski reaction

2 mg of dry extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

c) Sulphur test

Sulphur when added to the test solution, it sinks to the bottom.

3.6.8. Tannins

a) Ferric chloride test

To 1-2 ml of the ethanolic extract, few drops of 5% w/v FeCl₃ solution was added. A green colour indicated the presence of gallotannins, while brown colour indicated the presence of pseudotannins.

b) Gelatin test

Test solution when treated with gelatin solution gives white precipitate.

3.6.9. Starch

0.01g of Iodine and 0.075 g of potassium iodide were dissolved in 5 ml of distilled water and 2-3 ml of an ethanolic extract was added. Formation of blue colour indicated the presence of starch.

3.6.10. Test for quinones

a) Test with potassium iodide

Potassium iodide gives hydrogen iodide on reaction with dilute sulphuric acid. The liberated hydrogen iodide reacts with quinones producing iodine, which can be tested with starch paper, which turns into blue colour.
b) Quenching in UV at 254 nm shows the presence of a naphthoquinone

After spraying with 10% methanolic potassium hydroxide, the test sample showed red fluorescence in UV-365 nm and red to red-brown colour (vis). This confirmed the presence of a naphthoquinone.

3.6.11. Test for glycosides

a. Baljet test

The test solution when treated with sodium picrate gives yellow to orange colour.

b. Keller-Killiani test

The test solution was treated with few drops of ferric chloride solution and mixed. When conc. sulphuric acid containing ferric chloride solution was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.

c. Raymond’s test

The test solution when treated with dinitrobenzene in hot methanolic alkali gives violet colour.

d. Bromine water test

Test solution when dissolved in bromine water gives yellow precipitate.

e. Legal’s test

Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red colour.

3.7. Determination of total phenolics

The concentration of total phenolics in the petroleum ether, chloroform and ethanol extracts of leaves and roots were determined according to the protocol described by Chandler and Dodds (1993). 1 mL of each extract was mixed in a test tube containing
1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% sodium carbonate was added. It was mixed thoroughly and placed in the dark for 1 h, and absorbance was recorded at 725 nm using a UV-VIS spectrophotometer (Shimadzu UV-240 Spectrophotometer, Japan). The total phenolic contents in root extracts of C. infortunatum were expressed as gallic acid equivalents in mg/g of the extract.


3.8.1. Chemicals and Instruments

All chemicals and solvents used in the study were of analytical grade. Diphenyl 2-picryl hydrazyl radical (DPPH), phenazine methosulfate (PMS) and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Nitroblue tetrazolium (NBT), butylated hydroxy toluene (BHT), nicotinamide adenine dinucleotide (NADH) and ascorbic acid were obtained from Lobo Chemicals, Mumbai. Trichloroacetic acid (TCA) and potassium chloride were obtained from Ranbaxy Fine Chemicals Ltd., India. UV spectrophotometer (Shimadzu 2450), homogenizer, centrifuge (Remi, India) and pH meter (Elico Ltd., India) instruments were used for the study.

3.8.2. Preparation of stock solution of extracts

The stock solution of extracts were prepared by dissolving 100 mg of dried extracts in 10 ml of methanol to make a stock solution of 10 mg/ml. Aliquots from this stock solution were further diluted with methanol to get the final concentrations viz. 20, 40, 60, 80, 100 and 120 µg/ml.

3.8.3. Reagents

i. DPPH

Stock solution of DPPH was prepared by dissolving 32.5 mg in 10 ml of methanol and then the total volume was made up to 25 ml with methanol in volumetric flask.
Materials and Methods

ii. Test solution

Samples of various concentrations were prepared by dissolving in methanol as described above.

3.8.4. Free radical scavenging activity

Free-radical scavenging activity of all extracts at different concentrations was tested in the following models.

3.8.4.1. DPPH radical Activity

The DPPH* free radical scavenging potential of the extracts was determined by using the modified method of Brand-Williams, (1995). Different concentrations of leaves extracts and standard BHT and α-tocopherol were taken in different test tubes and the volume was adjusted to 1ml using MeOH. Freshly prepared 2 ml of 0.1 mM DPPH solution was mixed and vortexed thoroughly and left in dark for 30 min. The absorbance of stable DPPH* was measured at 517 nm. The DPPH control (containing no sample) was prepared using the same procedure. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following equation: DPPH radical scavenging activity.

\[
(\% \text{ inhibition}) = \left( \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \right) \times 100
\]

Where Abs Control is the absorbance of DPPH radical + methanol; Abs Sample is the absorbance of DPPH radical + sample extract/standard. Butylated hydroxytoluene (BHT), a well-known antioxidant was used as a positive control.

3.8.4.2 Hydroxyl radical (HO*) scavenging activity

The hydroxyl radical scavenging activity was determined according to the modified method of Chung et al., (1997). The Fenton reaction mixture containing 200 μL of 10 mM FeSO₄·7H₂O, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose
was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) containing 500 µL of different concentration of leaf and root ethanol extract. Freshly prepared 200 µL of 10 mM H₂O₂ was added to the mixture and incubated for 4 h at 37 °C. Later, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and placed in boiling water bath for 10 min. The mixture was brought to room temperature and centrifuged at 2000 rpm for 5 min and absorbance was measured at 532 nm. The percentage of hydroxyl scavenging activity was calculated by employing the following formula. And compared with the standard BHT and α-tocopherol.

\[
\text{Absorbance of sample} \times 100 \div \text{Absorbance of blank} \times 100
\]

3.8.4.3. Superoxide anion (O₂⁻) scavenging activity

Using the method of Nishimiki et al. (1972) the superoxide anion scavenging activity was determined, wherein a mixture of 1 ml of nitroblue tetrazolium (NBT) (156 µM NBT in 100 mM phosphate buffer, pH 7.4) 1 ml NADH (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml leaf and root ethanol extract was prepared in water. To this mixture 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) was added to start the reaction. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was measured at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. BHT and α-tocopherol were used as standard.

3.8.4.4. Nitric oxide radical (NO) scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined by using the Griess Illosvoy reaction of (Garrat, 1964). Griess Illosvoy reagent was slightly modified using naphthylethylenediamine dihydrochloride (0.1% w/v) instead of 1- naphthylamine (5%). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 mL different concentrations of leaf and root ethanol extract was incubated for 150 min.
at 25°C. The reaction mixture (0.5 mL) was mixed with 1 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for the completion of diazotisation reaction. The resultant mixture was then added with 1 mL of naphthyl–ethylenediamine dihydrochloride (0.1%) and allowed to stand for 30 min in diffused light. The absorbance of the pink coloured chromophore was measured at 540 nm against the corresponding blank solution. Scavenging capacity of the extract was compared with standard drug BHT and α-tocophferol.

3.8.4.5. Measurement of reducing power

The reducing power was determined by the method of Oyaizu (1986). In brief, 2.5 mL fraction of leaf and root of C. infortunatum was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then cooled rapidly, subsequently added with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3,000 rpm for 10 min. 5.0 mL supernatant was mixed with 5 mL of distilled water and 1 mL of FeCl₃ (1%) and the absorbance was measured at 700 nm. Increase in the absorbance of the reaction mixture was interpreted as increase in the reducing power of the extracts and the results were compared with BHT that was used as a positive control.

3.9. Pharmacological Investigations

3.9.1. Drug formulations

Suspension of petroleum ether, chloroform and ethanol extracts of leaves and roots were prepared in 1% Tween 80 or in carboxymethyl cellulose so as to obtain the dosage range in different concentrations. The intraperitoneal suspensions of the isolated constituents were prepared in 0.1% DMSO. For topical application, 4% (w/w) ointment cream (cream base + extract) of petroleum ether, or chloroform or ethanol extracts was prepared by the fusion method (melting ingredients method) as described by Bharath (1996). Briefly, for the preparation of 100 g of cream base 2 g of white Bee wax, 3 g of hard paraffin, 90 g of white soft paraffin and 5 g of cetyl alcohol were mixed and warmed to get a homogenous cream base. To study the dead space model animals were administered orally with the extracts. To dissolve the extracts 1% gum tragacanth was used.
3.9.2. Acute toxicity studies

The acute toxicity study for petroleum ether, chloroform and ethanol extracts of leaves and roots of *C. infortunatum* were performed using the Wistar albino rats of either sex and Swiss albino mice on the other hand ethyl acetate fraction obtained from ethanol extract of root which had significant *in vitro* cytotoxicity was also tested for acute toxicity study in Swiss albino mice. In the pilot toxicity experiments the three extracts of leaves and roots and ethyl acetate fraction were administrated orally in increasing doses starting from 500, 1000, 1500, 2000 and 2500 mg/kg b.w. The rats and mice were observed for 24 h for such behavioural signs as nervousness, excitement, dullness, ataxia or death and the signs of toxicity were also observed up to 14 days after the oral administration.

3.9.3. Evaluation of hepatoprotective and *in vivo* antioxidant activity of ethanol extracts of *C. infortunatum* leaves and roots

Drug induced liver injury is one of the serious complications since liver is central metabolic deposition virtually for all drugs and foreign substances (Zimmerman *et al.*, 1993; Kaplowitz, 1991; Farrel, 1994). The liver is involved in a wide variety of metabolic activities such as carbohydrate and lipid metabolism, secretion of bile as well as synthesis of proteins, although it is often considered as only a detoxification organ. The drugs are usually metabolized without injury to the liver even though many fatal and near-fatal drug reactions occur. Some compounds are known to produce metabolites that cause liver injury in an uniform, dose dependent fashion (Mitchell *et al.*, 1973; Baerg *et al.*, 1970; Klein, 1989). The most widely accepted experimental model for hepatotoxicity is the rat/carbon tetrachloride model, which is remarkably similar to human alcoholic cirrhosis (Sundari *et al.*, 1997) the reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical have been implicated in the pathophysiology of various clinical disorders. The inhibition of free radical generation can serve as facile model for evaluating activity of hepatoprotective agents.

Among the extracts of leaves and roots of *C. infortunatum* tested for their *in vitro* antioxidant activity, ethanolic extract was found to possess significantly higher
antioxidant activity. Hence, the ethanol extract was selected for evaluation of hepatoprotective activity of *C. infortunatum*. Since the antioxidant defense play a major role in heptoprotection, the studies envisage to evaluate the *in vivo* antioxidant activity of leaf and root ethanol extract of *C. infortunatum*.

### 3.9.3.1. Leaf ethanol extract

Extracts of leaves of *C. infortunatum* were prepared as described in section 3.5.

### 3.9.3.2. Treatment Protocol

Male albino rats of the Wistar strain, weighing 180-220 g, were employed in the present studies. The animals were divided into five groups of six animals in each group.

- **Group I:** Served as normal control and received saline (1 ml/kg, p.o.) daily for 7 days and received paraffin (1 ml/kg, p.o.) on 7th day.

- **Group II:** Served as CCU control and received saline (1 ml/kg, p.o.) daily for 7 days and received CCU: Paraffin (1:1, 2 ml/kg, p.o.) on 7th day.

- **Group III:** Treated with ethanol extract (50mg/kg, b.w/day) and received CCU: paraffin (1:1, 2 ml/kg) on 7th day.

- **Group IV:** Treated with ethanol extract (100mg/kg/day) and received CCU: paraffin (1:1, 2 ml/kg, p.o) on 7th day.

- **Group V:** Treated with ethanol extract (200mg/kg/day) and received CCU: paraffin (1:1, 2 ml/kg, b.w) on 7th day.

The ethanol extract was suspended in 0.5% sodium carboxymethylcellulose and was fed to Group III, IV and V rats via oral route at 50, 100 and 200 mg/kg body weight for 7 days. Groups I and II were simultaneously administered with saline until 7th day. Groups II, III, IV and V were given a single oral dose of carbon tetrachloride (1:1 in liquid paraffin) at 1.25 mL/kg body weight at an interval of 6 h after the administration of last dose of extract/saline on the 7th day. Animals were sacrificed after 24 h of CCl4 administration. Blood and liver samples were collected for following studies.
3.9.3.3. Assessment of Serum Non-specific marker enzymes

a) Determination of Serum aspartate transaminase

Aspartate aminotransferase (AST) also known as Serum glutamic oxaloacetic transaminase (SGOT) activity was determined according to the method of Rietman and Frankel (1957).

Procedure

0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mm of α-ketoglutarate, and 100 mm L-aspartate in 100 ml phosphate buffer (0.1M, pH 7.4) at 37°C and incubated for 60 min. After incubation 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and absorbance was read after 10 min at 505 nm using reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37°C was followed in the same manner. The enzyme activity was calculated from standard (sodium pyruvate, 2 mM) calibration curve, the enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.

b) Determination of Serum Alanine transaminase (ALT) activity

Alanine transaminase (ALT) also known as Serum glutamate pyruvate transaminase (SGPT) activity was determined according to the method of Rietman and Frankel (1957).

Procedure

0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 ml of phosphate buffer 0.1 M, pH 7.4) at 37°C and incubated for 30 min. After incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl was added, mixed well and kept at room temperature for 20 min. at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after incubation at 37°C was also followed in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate, 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.
c) **Determination of Serum Alkaline Phosphatase (ALP) activity**

Serum ALP activity was determined according to the method of King and King (1954).

**Procedure**

ALP in serum reacts with disodium phenyl phosphate under alkaline pH 10 release phenol. Phenol reacts with 4-aminoantipyrene in the presence of alkaline oxidizing agent to give a red coloured complex, absorbance is measured at 510 nm against reagent blank. 0.05ml of serum was incubated with 0.5 ml of the buffered substrate (1 ml of 0.254 g of disodium phenyl phosphate dehydrate /dl water mixed with 1 ml of carbonate buffer pH 10) and 1.54 ml of distilled water at 37°C for 15 min. After the incubation, 2 ml chromogen (1 ml of 0.6 g 4-aminoantipyrene /dl water and 1 ml of ferricyanide 2.4 g /dl water) reagent was added and absorbance measured at 510 nm, Phenol (10 mg%) was used as the standard for the calibration curve. The activity (KA/dl) is converted to IU/l by multiplying with 7.1.

3.9.3.4. **Preparation of liver homogenate**

Liver homogenate (10%) was prepared with 0.15 M KCl and centrifuged at 8000 rpm for 10 min. The cell-free supernatant was used for following assays;

3.9.3.5. **Assay of hepatic antioxidant enzymes**

a) **Superoxide dismutase (SOD)**

SOD activity was estimated by Beauchamp and Fridovich (1971) method. The reaction mixture consisted of 0.5 ml of hepatic PMS, 1 ml 50 mM sodium carbonate, 0.4 ml of 25 μM NBT and 0.2 ml 0.1 mM EDTA. The reaction was initiated by the addition of 0.4 ml of 1 mM hydroxylamine hydrochloride. The change in absorbance was recorded at 560 nm. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%.

73
b) **Catalase activity (CAT)**

Catalase activity was estimated by the method of Claiborne (1985). The assay mixture consisted of 1.95 ml of phosphate buffer (0.005 M, pH 7.0), 1.0 ml H₂O₂ (0.019 M), 0.005 ml of liver homogenate (10%, w/v). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of nmol of H₂O₂ consumed/min/mg of protein.

c) **Peroxidase**

The peroxidase assay was carried out as per the method of Nicholas (1962). Briefly, to the 0.5 mL of liver homogenate 1 mL each of KI (10 mM) and sodium acetate (40 mM) solutions were added and the absorbance was read at 353 nm. Twenty micro litre of hydrogen peroxide (15 mM) was added and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit per minute. The specific activity was expressed in terms of units per milligram of protein.

3.9.3.6. Assay of hepatic antioxidant molecules

a) **Glutathione (GSH)**

GSH was assayed by the method of Jollow *et al.* (1974). An aliquot of 1.0 ml of liver homogenate (10%, w/v) was precipitated with 1.0 ml of sulphosalicylic acid (4%, w/v). The samples were kept at 4°C for 1 h and then centrifuged at 3500 rpm for 15 min. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow colour developed was measured at 412 nm.

b) **Estimation of lipid peroxidation (LPO)**

Buege and Aust (1978) method was followed to estimate the lipid peroxidation. In brief, the peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride to the mixture of 0.5 mL liver homogenate and 0.15 M KCl at 37°C for 30 min and stopped by
Materials and Methods

adding 2 ml of ice cold mixture of 0.25 N HCl containing 15% TCA, 0.30% TBA and 0.05% butylatedhydroxy toluene (BHT). The mixture was heated at 80 °C for 60 min., the samples were cooled and centrifuged and the absorbance of the supernatant was measured at 532 nm. The results were expressed as MDA equivalents, which were calculated by using an extinction coefficient of 1.56. Lipid peroxidation was expressed as MDA equivalents in nannomoles per milligram of protein.

3.9.3.7. Histopathological Studies

The liver tissue was dissected out and fixed immediately in 10% formalin solution, dehydrated in gradient ethanol (50–100%), cleared in xyline and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (H–E) dye for microscopic observation.

Dehydration: The tissues were treated with 80 % alcohol for 2 h. followed by the treatment with 90 % alcohol for 1 h. Finally the tissues were treated with absolute alcohol for 3 h.

Clearing: The tissues were then treated with xylol for 2 h to clear the alcohol and to make room for paraffin.

Infiltration and impregnation: After clearing, the tissues were transferred to a bath of molten paraffin wax in an embedding oven for infiltration and impregnation for 2 h. The final paraffin wax bath ensures the replacement of residual xylol with pure wax.

Embedding: Embedding was carried out by placing the infiltrated tissues in mould containing warm liquid paraffin. This enabled the tissue to take sections by using the technique of microtomy (Galighter). The sections were taken at 5-μ thickness, attached to microscopic slides using Mayer’s glycerol-albumin mixture. Prior to staining the slides were placed in xylene for 5 min. to remove the paraffin from the sections.

Hydration: Hydration was carried out by passing the tissues through decreasing grades of alcohol and finally in distilled water.
Materials and Methods

**Staining:** This was followed by the staining with Harris haematoxylin for 30 minutes, washed in running tap water and the slides were quickly dipped in and out of 0.5 % HCl. Again washed in water for 30 to 60 seconds and dipped in dilute ammonia water. Once again washed in water, rinsed in 95% alcohol and stained with eosin for 10 to 60 seconds.

The slides were placed for 30 to 60 seconds in 70 %, 95 %, absolute alcohol and finally in xylene. Excess of xylene was drained and the sections were mounted on Canada balsam with a cover slip. The sections were examined microscopically, for the evaluation of histopathological changes.

3.9.3.8. Root ethanol extract

To determine the hepatoprotective effect of *C. infortunatum* root ethanol extract, all the experimental protocols described above were repeated using ethanol extract of roots, only few changes were made in the concentration of extracts and grouping of animals which are mentioned as follows.

The animals were divided into four groups of six animals in each group.

Group I: Served as normal control and received distilled water (1 ml/kg, p.o.) daily for 7 days and received paraffin (1 ml/kg, p.o.) on 7th day.

Group II: Served as CCl4 control and received saline (1 ml/kg, p.o.) daily for 7 days and received CCl4: paraffin (1:1, 2 ml/kg, p.o.) on 7th day.

Group III: Treated with ethanol extract (125mg/kg. b.w/day) and received CCl4: paraffin (1:1, 2 ml/kg ) on 7th day.

Group IV: Treated with ethanol extract (250mg/kg/day) and received CCl4: paraffin (1:1, 2 ml/kg, p.o) on 7th day.

The ethanol extract was suspended in 0.5% sodium carboxymethylcellulose and was fed to Group III and IV rats via oral route at 125 and 250 mg/kg body weight for 7 days. Groups I and II were simultaneously administered with saline until 7th day. Groups
II, III and IV were given a single oral dose of carbon tetrachloride (1:1 in liquid paraffin) at 1.25 mL/kg body weight at an interval of 6 h after the administration of last dose of extract/saline on the 7th day. Animals were sacrificed after 24 h of CCl₄ administration. Blood and liver samples were collected and experiments were conducted according to the protocols described in the sections 6.3.3- 6.4.3.

3.9.4. Evaluation of wound healing activity of Clerodendrum infortunatum leaf and root extracts

Validation of the ethnotherapeutic claim of C. infortunatum L. was investigated to evaluate its wound-healing potency in experimental rats. Three models, namely excision, incision and dead space wounds were used in this study.

3.9.4.1. Leaf extracts

Extracts of leaves of C. infortunatum were prepared as described in section 3.5.

3.9.4.2. Drug formulation

To study the incision and excision wound model a cream base was used for topical application. 4% (w/w) ointment cream (cream base + extract) of petroleum ether, or chloroform or ethanol extracts was prepared by the fusion method (melting ingredients method) as described by Bharath (1996). Briefly, for the preparation of 100g of cream base 2 g of white Bee wax, 3 g of hard paraffin, 90 g of white soft paraffin and 5g of cetyl alcohol were mixed and warmed to get a homogenous cream base. To study the dead space model animals were administered orally with the extracts. To dissolve the extracts 1% gum tragacanth was used.

3.9.4.3. Excision wound model

A circular wound of about 500 mm² was made on the depilated, ethanol-sterilized dorsal thoracic region of rats under light ether anesthesia (Leite et al., 2002). The animals were divided into five groups of six animals. Group I was untreated (control); Group II was treated with 1% (w/w) nitrofurazone (Ranbaxy Pvt. Ltd., Mumbai, India) ointment and served as a reference standard (positive control); Groups III, IV and V were treated
topically with the cream base prepared from petroleum ether, chloroform and ethanol extract root of *C. infortunatum*, respectively, on a wound created on the dorsal back of rats daily until the wounds healed completely (Chatterjee and Chakravority, 1993). The percentage of wound closure was recorded on day 4, 8, 12 and 16 and the wound area was traced and measured planometrically. The actual value was converted into a percent value taking the size of the wound at the time of wounding as 100%.

3.9.4.4. Incision wound model

As explained above, rats were anaesthetized prior to and during creation of the wound. The dorsal fur of the animals was shaved with a clipper. A longitudinal paravertebral incision, six centimeters in length was made through the skin and coetaneous muscle on either side of the vertebral column of the rat as described by Ehrlich and Hunt (1969). Care was taken to see that incision was at least 1 cm lateral to vertebral column. After the incision, surgical sutures were applied to the parted skin at intervals of one centimeter. The wounds were left undressed. The sutures were removed on the 8th post wound day and the treatment was continued. The skin-breaking strength was measured on the 10th day by the method described by Lee and Tong (1968).

3.9.4.5. Dead space wound model

For the dead space wound model the animals were divided into five groups containing six each. Group I served as the control and was orally treated with 1 ml/kg of 1% gum tragacanth readily available (Merck Pvt. Ltd., Mumbai, India). Groups II, III and IV were treated with an oral dose of petroleum ether, chloroform and ethanol extract (250 mg/kg b.w) respectively. The animals were anaesthetized with light ether anesthesia and the dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths measuring 2.5 cm×0.3 cm (Sisco, Bangalore, India) one on either side of the dorsal paravertebral surface of the rats. The granulation tissue formed on the grass piths were removed on 10th post wounding day and subjected to breaking strength and histological study.
3.9.4.6. Root extracts

To determine the wound-healing potency of root extracts of *C. infortunatum* all the experiments described above were repeated, few changes were made in concentration of extracts and grouping of animals.

3.9.5. Evaluation of antitumor activity of *Clerodendrum infortunatum* leaf and root extracts

3.9.5.1. Preparation of extracts

The air-dried and finely ground leaves and root part of plant were extracted in a Soxhlet apparatus successively with petroleum ether, chloroform and ethanol. The extracts were filtered, pooled and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi, Flawil, Switzerland) to yield dried petroleum ether, chloroform and ethanol extracts (as described in section 3.5). The last ethanol extract (25 gm) of leaves and roots were further partitioned between water and ethyl acetate to obtain ethyl acetate soluble fractions of leaf and root amounting to 4 and 6 gm respectively.

3.9.5.2. Culture of EAT cells *in vivo*

The Ehrlich Ascitic Tumor (EAT), derived from a spontaneous murine mammary adenocarcinoma was maintained in the ascitic form by sequential passages in Swiss albino mice by means of weekly i.p. transplantations of $5 \times 10^5$ tumor cells. After intraperitoneal inoculation of Ehrlich tumor cells, the ascitic volume and cells number increase drastically. This has been associated to an increase in peritoneal vascular permeability (Fastajia and Dumont, 1976).

3.9.5.3. *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity of *C. infortunatum* leaves and roots extracts was assayed using EAT cell lines. Briefly, $1 \times 10^6$ cells of EAT cells were suspended in 0.1 ml of phosphate buffered saline (PBS) (0.2M, pH 7.4), various concentrations of extract (10$\mu$g/ml to 1mg/ml) and phosphate buffer in final volume of 1 ml were incubated at 37°C for 3 h. After incubation the viability of the cells was determined by trypan blue exclusion method (Yalwar, 1974).
The percentage cytotoxicity was calculated using the formula,

\[ \text{Percentage cytotoxicity} = 100 - \frac{Tc - Dc}{Tc} \times 100, \]

where \( Tc \) = total EAT cells, and \( Dc \) = dead EAT cells.

3.9.5.4. \textit{In vivo} antitumor activity

i) Treatment and drug administration

Extracts, which had significant \textit{in-vitro} antitumor activity (Petroleum ether extract, ethyl acetate soluble fraction obtained from ethanol extract of root and ethanol extract of leaf and root) were further selected for screening \textit{in-vivo} antitumor activity. The animals were divided into six groups consisting of eight animals in each group. The EAT cells containing \( 5 \times 10^6 \) cells/0.5 mL of PBS were injected into the peritoneal cavity of all the animals except normal control group.

Group 1: Normal (received 0.5 ml of 0.1% of DMSO)

Group 2: EAT control (received 0.5 ml of 0.1% of DMSO)

Group 3: EAT-bearing mice (received 0.5 ml of 250mg/kg b.w of petroleum ether extract of root suspended in 01% DMSO)

Group 4: EAT-bearing mice (received 0.5 ml of 150mg/kg b.w of Ethyl acetate fraction of root suspended in 01% DMSO)

Group 5: EAT-bearing mice (received 0.5 ml 250mg/kg b.w of Ethanol extract of leaf suspended in 01% DMSO)

Group 6: EAT-induced mice (received 0.5 ml 250mg/kg b.w of Ethanol extract of root suspended in 01% DMSO)

Treatment was started 24 h after inoculation of tumor cells, (once daily as a single dose) for 10 days. On 10\textsuperscript{th} day after last dose and 6 h fasting 5 mice from each group were sacrificed for the study antitumor activity. Rest of the animal of each group were
kept to check the mean survival time and percentage increase in life span of the tumor bearing mice. Antitumor activity was screened by determining different parameters like body weight analysis, total ascitic fluid volume, total cell number. Samples of blood used for several measurements are described below.

ii) **Body weight**

Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment (day 0) and sequentially on every alternative day during the treatment period.

\[
\text{Increase in body weight (\%)} = \left( \frac{\text{Wt. of each animal on 14th day} - \text{Wt. of each animal on 0 day}}{\text{Wt. of each animal on 0 day}} \right) \times 100
\]

iii) **Tumour volume**

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min.

iv) **Tumour cell count**

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares was counted.

v) **Viable / non-viable tumour cell count**

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and nonviable cells were counted.

vi) **Percentage increase life span (% ILS)**

The effect of MEMP on tumour growth was monitored by recording the mortality on daily basis for a period of 6 weeks and percentage increase in life span (%ILS) was calculated by using the formula
Materials and Methods

\[
\text{MST} = \frac{\text{Day of first death} + \text{date of last death}}{2}
\]

\[
\text{ILS (\%)} = \frac{\text{Mean survival of treated group}}{\text{Mean survival of control group-1}} \times 100
\]

3.9.6. Evaluation of In vivo Antimutagenic activity

3.9.6.1. Selection of Experimental Drug Dose

Petroleum ether extract and ethyl acetate fraction of roots of \textit{C. infortunatum} were used to investigate their antimutagenic activity. Experimental drug dose was standardized by detecting the lethal dose. Behavior of the animals was carefully studied.

3.9.6.2. Selection of experimental mutagen dose

Ethyl methane sulfonate (EMS) a nonfunctional alkylating agent a well known mutagen was employed to induce, chromosomal aberrations. A concentration of 80mg/kg b.w. is the conditioning dose for inducing minimum frequency chromosomal aberrations in bone narrow cells of mouse was selected based on the earlier reports of Riaz Mahmood and Vasudev (2003). Required dose of mutagen was obtained by dissolving EMS in 0.7% NaCl solution.

3.9.6.3. Treatment Protocol

Two sets of animals were maintained for treatment. First set comprised the control animals and second set the experimental. First set consisted of two groups (A/B) of six animals each. Control A animals were treated daily with 0.5 ml of 0.1 % DMSO for five days, control B were treated with 0.5ml of 0.1 % DMSO along with EMS (80mg /kg b.w) alone on 5th day. Second set of experimental animal were divided into 4 groups (C/D/E/F) and petroleum ether extract and ethyl acetate fraction alone were administered orally to C/D and E/F groups at the concentrations 250 and 150 mg /kg b.w respectively. Drug treatment lasted for 5 d with an interval of 24 h was maintained between every treatment. On fifth day experimental set group (E/F) animals have
Materials and Methods

received 80mg EMS/kg body weight of mice. On sixth day all the animals were
sacrificed by cervical dislocation, 90 min. before sacrifice, 0.5 ml of 0.05% of colchicine
in 0.9% saline was administered intraperitoneally.


The routine air dry technique of Evans et al. (1964) was followed for preparation
of slides from bone marrow cells of each mouse. Five slides were prepared from each
animal. Slides were then coded and stained with Giemsa: distilled water solution (1:6 v/v)
stained. Slides were subjected to microscopic analysis for the presence of chromosome
aberrations. Non-overlapping and well spread metaphase plates were selected.
Chromosome aberrations such as chromatic breaks, intra-chromatic deletions, minutes,
achromatic lesions, chromosome breaks chromatid exchanges and RB complexes were
scored.

3.9.7. Statistical analysis

The data obtained from each of experiment were subjected to one-way ANOVA
followed by Tukey’s Multiple Comparison Test. The F values, df values and P values
were analyzed and recorded in the respective tables.

3.10. Isolation and Characterization of the bioactive constituents from leaf
petroleum ether and ethanol extract of C. infortunatum

From the interesting results of above pharmacological activity and cytotoxic
activity, the crude petroleum ether and ethanol extract of leaf and petroleum ether and
ethyl acetate fraction obtained from ethanol extract of root part was chosen to be the
main subject for isolation.

Thin layer chromatography (TLC) was carried out using Silica gel (Merck) and
commercially available readymade aluminum foiled sheets with silver nitrate
impregnated silica gel (Merck). Column chromatography was carried out using Silica
gel (Merck, 70-230 mesh) or neutral alumina (S.D. Fine Chemicals Pvt. Ltd., Bombay).
All the chemicals and reagents used were obtained in high purity either from S.D. fine
chemicals Pvt. Ltd., Bombay, India or E.Merck Pvt. Ltd., Bombay, India.
3.10.1. Leaf petroleum ether extract

The air-dried and finely ground (1kg) leaf material of the plant was extracted in a Soxhlet apparatus successively with petroleum ether, chloroform and ethanol. The first petroleum ether extract was found to be active after screening for wound healing activity, considering this, petroleum ether extract was subjected to column chromatography. After complete evaporation of solvent, the extract/residue (20 gm) was chromatographed over silica gel 60 (230-400 mesh) and eluted sequentially with hexane-EtOAc (20:1), hexane-EtOAc (20:5), and hexane- EtOAc (20:7) each 1000 ml to give three sub fractions I, II, and III, each fractions were evaporated separately under reduced pressure, and kept for 24 hours. In the III sub fraction containing watch glass we found white powder along the edges. By giving wash with hexane removed the colour impurities and white amorphous powder (200mg) was collected and coded as LP1, Further more compound was tested for TLC( Thin Layer chromatography), we found single spot. The concentrates of the other eluates gave trace quantity of yellow resinous mass, which were not processed further.

3.10.2. Leaf Ethanol Extract

The ethanolic extract (5g) gm was chromatographed over silica gel and eluted with a stepwise gradient of hexane-methanol (1:0, 9:1, 4:1, 1:1, 0:1). hexane-methanol (4:1) fraction was collected and concentrated to get a light brown residue (500mg). This residue was subjected to preparative TLC using the solvent system chloroform-methanol (9:1) to yield a light yellow amorphous powder (40mg). The recovered compound was washed with cold methanol and filtered. This compound was designated LE1.

3.10.3. Root petroleum ether extract

One kilogram of the powdered root material was refluxed with 1/10 (w/v) petroleum ether, in a Soxhlet apparatus for 48 h in batches of 250 g each. The petroleum ether extract was allowed to stand for 24 hours which showed white precipitation in the bottom of round bottom flask. The precipitation was collected by filtering the supernatant, the precipitated compound was dried and washed with petroleum ether to remove color impurities and allowed to dry. The dried compound again dissolved in
warm chloroform filtered and kept it for 48 hours to evaporate the solvent content and washed with methanol to remove the impurities finally the white crystalline compound was collected and checked for TLC for further separation in different solvent system, this showed the single spot which confirmed the presence of single compound. The compound collected and coded as RPl and the yield of compound was found to be (500mg/ 250gm dry material).

3.10.4. Root ethyl acetate fraction

The ethanolic extract (25g) was suspended in H₂O (500 mL) and then partitioned using Ethyl acetate (500 mL x 3). The ethyl acetate fraction 5 gm was chromatographed over silica gel and eluted with a stepwise gradient of hexane-ethyl acetate (1:0, 9:1, 8:2, 7:3, 0:1). Hexane-ethyl acetate (9:1) fraction was collected and concentrated to get a light brown residue (500mg). This residue was subjected to preparative TLC using the solvent system hexane: ethyl acetate (9:1) to yield a light yellow amorphous powder (42mg). The recovered compound was washed with heaxane and the residue was checked for Thin Layer Chromatography (TLC) we found single spot. This compound was designated RE1. The remaining 7:3 fraction was kept for evaporation in room temp, after 24 hours white precipitate formation was found in the bottom of beaker, after complete solvent evaporation, the residue was washed with methanol to remove the colour impurities finally white cryastalline compound 80 mg was collected and coded as RE2. The concentrates of the other elutes gave trace quantity of yellow resinous mass, which were not processed further.

3.10.5. Characterization

The characterization requires the identification of molecular framework, the nature of the functional groups, which are present, their location with in the skeletal structure and finally the establishment of any stereo chemical relationship, which might exist.

The characterization of the compounds has been revolutionized by the progressive adoption of the wide range of spectroscopic techniques which are now available. These
have been applied extensively in the preparative section to confirm the structure of the expected compounds. The same was applied extensively in the preparative section to confirm the structure of newly isolated compounds.

Melting points of all the isolated compounds were recorded in a Toshniwal melting point apparatus. The IR Spectra were recorded with KBr pellets on a Perkin-Elmer 1710 FT-IR spectrophotometer. \(^1\)HNMR spectra of the compound were taken on Brukur (400 MHz) Spectrometer using \(\text{CDCl}_3\) and methanol as solvents. Mass spectra were recorded on a MAT 312 spectrophotometer and FAB-MS (positive) data on JEOL SX 102/DA–600.

3.11 Pharmacological evaluation of isolated constituents from petroleum ether, ethanol extract of leaf and petroleum ether, ethyl acetate fraction of roots of *Clerodendrum infortunatum* L.

The intraperitoneal suspensions of the isolated constituents were prepared by using 0.1% DMSO. For topical administration, 1 % w/w ointment cream bases of all test isolated compounds were prepared by fusion method by following the method as described for extracts.

The selection of petroleum ether and ethanol extract of leaf, similarly petroleum ether extract and ethyl acetate fraction obtained from ethanol extract of root for the isolation and characterization of active principles was mainly based on its performance in all the pharmacological studies carried out in the present investigations. After confirming the antioxidant activities and other such abilities the above said extracts were subjected for further micro phytochemical analysis. As a result, a few very important bioactive compounds were obtained. Subsequently, these bioactive compounds viz., clerosterol, quercetin, betulinic acid cleroamine and lupeol acetate were also subjected for few of the pharmacological evaluation with an intention to get a clear picture about the beneficial properties of *Clerodendrum infortunatum* L. Therefore, the protocols employed for this study were similar to those which have been used in the evaluation of crude extracts and hence, no details of any of those protocols are being mentioned for the isolated compounds to avoid repetition of the information.