III. MATERIALS AND METHODS

1. In vitro Studies

The principles involved in plant tissue culture technique are primarily an attempt whereby an explants can be, to some extent, freed from inter organ, inter tissue and inter cellular interaction and subjected to direct experimental control. To ensure the growth and development of explants under in vitro condition it is very essential to maintain the germ free system at all operations. The explants have to be provided with a suitable nutrient medium and proper conditions required for its growth and development. Several techniques have been adopted for in vitro plant cell, tissue and organ culture. Among them the general techniques that are essential in all experiments have to be carried out to regenerate the plantlets from explants.

- Cleaning of glassware
- Preparation of culture media
- Sources of explants
- Disinfectations procedure
- Maintenance of aseptic condition during inoculation
- Culture condition
- Hardening of in vitro plantlets

i) Cleaning of glassware

The glass wares required for the preparation of the media and the maintenance of cultures were soaked over night in 40% chromic acid solution, followed by running under a jet of tap water. Later they were dipped in 5% ‘Tween-20’ solution and again washed with tap water. The glasswares were finally rinsed with distilled water and heat sterilized in an oven at 150°C for
an hour. The contaminated culture vessels prior to washing were autoclaved at 20 lb/in$^2$ for 20 min and the contents were discarded.

**ii) Preparation of culture media**

In the present investigation different nutrient formulations were tried namely, MS medium (Murashige and Skoog medium, 1962), LS medium (Linsemier and Skoog, 1965), B$_5$ medium, Gamborg medium, (Gamborg et al., 1968). Since the response was more in the MS medium, it is used as the basal medium to initiate callus from different explants and regeneration of plantlets from the calli.

All the chemicals used for the preparation of media were of analytical grade (Hi–Media, India). The growth regulators used were procured from Sigma Research Laboratory, America. The macro, micro and organic nutrients were categorized as stocks and were prepared separately. All the nutrient stocks were prepared at 25 X concentrations. The iron stocks were kept in amber color bottle and preserved in darkness. It was made a point that stocks once prepared were not used after three months. The stocks for growth regulators such as 2, 4–D (2, 4 dinitrophenoxy acetic acid), NAA ($\alpha$-naphthalene acetic acid), IAA (Indole 3–acetic acid), IBA (Indole 3–butyric acid), BAP (6–benzyl amino purine) and Kn (6–ferfuryl amino purine) were prepared at 0.1% concentration.

During preparation of the media appropriate quantity of stocks of nutrients and growth regulators were added to the volumetric flask of one-liter capacity. The carbon source, sucrose was added to the solution at the concentration of 3% and made up to desire volume using distilled water. The pH of the medium constituent was adjusted between 56-58 by adding 0.1N hydrochloric acid or 0.1N sodium hydroxide. The media was gelled with 0.8% bacteriological grade agar-agar (Hi- Media, Mumbai) and homogenized in an autoclave at 15 lb/in$^2$ and at a temperature of 120$^0$C for 5 min.
After homogenization about 20 ml of molten medium was dispensed into each culture tube of 25 X 150 mm dimension and 40 ml of media was dispensed into culture bottles and conical flasks of 250 ml capacity respectively. The tubes and the conical flask were plugged with non-absorbent cotton and the bottles were covered with sterilizable polypropylene screw caps.

The culture media were steam sterilized at 15 lb/in\(^2\) and at a temperature of 120\(^\circ\)C for 15 min. Then according to the requirement, media was allowed to solidify with or without slants.

**iii) Source of explants**

The whole plant of *Elephantopus scaber* were collected from the medicinal garden, Kuvempu University, Karnataka, India.

**iv) Disinfectations procedure**

The plant material which is to be cultured were thoroughly washed in running water followed by 5% liquid detergent ‘TWEEN-20’ for few minutes to remove all detritus. The required segmental parts were surface sterilized with 0.1% (W/V) aqueous mercuric chloride (HgCl\(_2\)) for 8-10 min followed by 5-6 rinses with sterilized distilled water and were taken into previously UV radiant sterilized laminar airflow chamber. With the help of a sterilized blade, different parts of the explants were cut into transverse segments of required length and were carefully inoculated onto the required culture medium.

For leaf culture, the surface sterilized leaves were aseptically excised into transverse segments of 5-10 mm each. It includes leaf base with a portion of petiole, the middle lamina portion and the apical segment. In addition the entire leaf material was also used as a source of material for inoculation.
For stem culture the couline stem was defoliated carefully. The stem node was excised from the root part and thoroughly washed with running tap water for 5 min. It is treated with detergent Teepol for 5 min. After rinsed with distilled water, surface sterilization was carried out by treating with 0.1% Hgcl₂ for 5 min. The disinfectant was carefully removed by rinsing with double distilled water for 6-8 times. Then stem nodes were carefully inoculated on the medium placing either vertically or horizontally.

For meristem culture the stem apex was excised to 1 cm length segment. The explants were surface sterilized for 3-4 min. and thoroughly rinsed 4 times in sterile distilled water and transferred each explants to a sterilized petridish. The outer leaves from the each shoot apieces were removed and the ultimate apex measuring approximately 0.5 cm were cut off and inoculated carefully on to the agar medium.

v) Maintenance of aseptic condition during inoculation

In order to initiate aseptic cultures, inoculations were carried out under laminar airflow hood. The transfer area with all the paraphernalia (culture vessels, forceps, scalpel, burner, alcohol, stereomicroscope, etc.) were conveniently arranged and sterilized by exposing to UV radiation for a period of one hour. Before and after inoculation, forceps and scalpel were dipped in alcohol and flamed. The mouth of the tube and conical flasks were opened over the flame and inoculation of the explants and subculture of calli was carried out over the flame. To each flask two to three explants or small calli were inoculated. The cultures were checked daily to verify their aseptic condition. If any contamination found in the culture, they were immediately discarded from the batch and killed by autoclaving.
vi) Culture condition

All the cultures were maintained in a condition providing 22±2°C temperature, 12 hrs. photoperiod [40 μmol m\(^{-2}\) s\(^{-1}\) (Philips cool–white fluorescent tubes)] and 60 to 70% relative humidity. The explants and the calli cultured separately on MS basal medium without growth regulators were considered as control over the hormone-incorporated medium. Each treatment consists of ten replicates. The number of shoots per callus was evaluated at the end of four weeks of incubation and the number of root intact regenerates was evaluated at the end of six weeks of incubation.

The data were statistically evaluated by ANOVA followed by Tukey's Pairwise Comparison Test. The values of \(p<0.01\) were considered as statistically significant.

vii) Hardening of in vitro regenerants

The root intact plantlets recovered were washed with running tap water and the agar sticking to the roots was removed. The plantlets with fully expanded leaves and well-developed roots were first transferred to the small pots containing sterile soil. The regenerated plantlets were hardened for a week by covering with a thin perforated transparent polythene bag to maintain the humidity. Plantlets were watered with 1/10\(^{th}\) strength of MS salt solution for a week and then transferred to the garden soil.

2. Phytochemical Studies

i) Plant material

Fresh whole plant materials of *Elephantopus scaber* Linn were collected from the Joldhal forest ranges of the Western Ghats region of Karnataka. The taxonomic identity was confirmed by comparing with the authenticated
herbarium specimens No. FDD 49, (Manjunatha et al., 2004) deposited at Kuvempu University Herbaria.

**ii) Extraction procedure for *Elephantopus scaber***

**Preparation of aqueous extract**

The materials were shade dried for four days, powdered mechanically and sieved by using mesh size no. 10/44. 500 g of powdered material were pulverized with predetermined volume of distilled water (1/10 w/v). The extract was filtered and evaporated to dryness under reduced pressure using rotary flash evaporator.

**Preparation of ethanol extract**

3 kg of the powder was extracted with 95% ethyl alcohol using soxhlet apparatus, in several batches of 200 g of each. After the effective extraction (40 cycles), the solvent was removed under reduced pressure at 40±5°C, using rotary flash evaporator (Buchi, Switzerland) to get the semi solid mass.

The ethanol extract was subjected for the isolation of the active constituents. It is successively extracted using the solvents methanol and hexane. Each fraction, the solvents were pooled and solvents were removed under reduced pressure at 40±5°C. Finally the methanol and hexane fractions were dried in a dessicator over anhydrous sodium sulphite.

**iii) Qualitative phytochemical investigation**

The aqueous extract, ethanol extract, and the fractions of methanol and hexane of *Elephantopus scaber* were subjected for qualitative phytochemical assay.
Tests for Carbohydrates

a) Molisch’s test: Test solution with few drops of Molisch’s reagent and 2 ml of concentrated sulphuric acid was added slowly through the sides of the test tube. A purple ring is formed at the junction of two liquids.

b) Barfoed’s test: Test solution with Barfoed’s reagent on boiling on a water bath shows brick red precipitation.

c) Benedict’s test: Test solution treated with Benedict’s reagent and boiling on a water bath showed reddish brown precipitation.

Tests for Glycosides

a) Baljet test: The test solution treated with sodium picrate gives yellow to orange color.

b) Keller Killiani test: The test solution was treated with a solution containing few drops of glacial acetic acid and 2 ml of ferric chloride solution. When concentrated sulphuric acid was added, it forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green.

c) Raymond’s test: Test solution treated with dinitrobenzene in hot methanolic alkali gives violet color.

d) Bromine water test: Test solution 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 color.
Tests for Proteins

a) Millions test: Test solution when treated with Millions reagent and heated on a water bath, protein was stained red on warming.

b) Xanthoproteic test: Test solution treated with concentrated nitric acid and on boiling gives yellow precipitate.

c) Biuret test: Test solutions treated with 40% sodium hydroxide and dilute copper sulfate solution gives blue color.

d) Ninhydin test: Test solution treated with ninhydrin reagent gives purple color.

Tests for Tannins

a) Ferric chloride test: Test solution treated with few drops of ferric chloride solution gives dark color.

b) Gelatin test: Test solution treated with gelatin solution gives white precipitate.

Tests for Saponins

a) Foam test: Saponins when mixed with water and shaken showed the formation of froth that was stable at least for 15 minutes.

b) Haemolysis test: 2 ml each of 18% sodium chloride solution was taken in two test tubes. To one test tube 2 ml of distilled water and to the other test tube 2 ml of filtrate was added. Few drops of blood were added to both the test tubes, contents were mixed and tests were observed for haemolysis under microscope.
Tests for Triterpenoids

a) Salkowski test: A few drops of concentrated sulphuric acid were added to the test solution, shaken and allowed to stand, lower layer turns yellow.

b) Liebermann Burchardt test: The test solution treated with acetic anhydride, mixed well and concentrated sulphuric acid was added through the sides of the test-tube, deep red color appeared.

Test for Lactones

a) Legal’s test: Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red color.

b) Feigls test: The compound treated with hydroxylamine hydrochloride (1 ml, 0.5 M solution) and sodium hydroxide (0.2 ml, 6 M) and heat the mixture to boiling. Cool and add hydrochloric acid (2 ml, 1 M). If the solution is cloudy add ethanol (1-2 ml) to make clear and then add ferric chloride solution (1-2 drops, 0.6 M). Add a little more of ferric chloride if necessary to obtain a permanent color. Formation of a magenta color confirms the presence of lactones.

Tests for sterols

a) Salkowski test: A few drops of concentrated sulphuric acid added to the test solution, shaken and allowed to stand, lower layer turn red indicating the presence of sterols.

b) Liebermann-Burchardt test: The test solution was treated with few drops of acetic anhydride and concentrated sulphuric acid was added from the sides of the test tube. A brown ring appears at the junction of the two layers and the upper layer turns green.
Tests for Flavonoids

a) Ferric-chloride test: Test solution with few drops of ferric chloride solution shows blackish red color.

b) Shinoda test: Test solution with few fragments of magnesium ribbon and concentrated hydrochloric acid gives pink to magenta red color.

c) Zinc-hydrochloric acid, reduction test: Test solution with zinc dust and few drops of hydrochloric acid, showed magenta red color.

d) Alkaline reagent test: Test solution when treated with sodium hydroxide solution, showed increase in the intensity of yellow color, which becomes colorless on addition of a few drops of dilute acid.

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

Tests for Alkaloids

a) Mayer’s test: Test solution with Mayer’s reagent (potassium mercuric iodide) gives cream colored precipitate.

b) Wagner’s test: The acidic solution with Wagner’s reagent (iodine in potassium iodide) gives brown precipitate.

c) Hager’s test: The acidic solution with Hager’s reagent (saturated picric acid solution) gives yellow precipitate.

d) Dragendorff’s test: The acidic solution with Dragendorff’s reagent (potassium bismuth iodide) shows reddish brown precipitation.
iv) Isolation of active constituents from ethanol extract of *Elephantopus scaber*:

The method of Paul Pui-Hay But, *et al.*, (1997) has been employed for the isolation of active constituents from the leaves of *Elephantopus scaber*.

The residue obtained from the ethanol extract was taken in a separating funnel, in different batches and it was partitioned between water and chloroform (1:2). The chloroform layer was separated from each batch, pooled and the solvent was removed completely on the water bath to get dark green syrup. The chloroform extract was then partitioned between hexane and 10% aqueous methanol (2:1), in a separating funnel in different batches. The lower, aqueous methanol extract was separated and repeatedly washed with hexane for four to five times.

The upper hexane fractions and the lower methanol fractions were pooled separately; the solvents were removed under reduced pressure and dried over anhydrous sodiumsulphite in a desiccator. The residue obtained from the aqueous methanol extract was chromatographed on silica gel column and eluted successively using hexane and ethyl acetate in the ratio of 1:1, 1:2 and 1:4.

**Chromatographic separations of constituents**

250 g column chromatography grade silica gel H (s.d.fine chem ltd, Mumbai) was taken and activated in hot air oven at 110°C for one hour. The mobile phase consists of hexane and ethyl acetate in the ratio of 1:1, 1:2 and 1:4. Glass wool was plugged at the bottom of the column. Activated silica gel slurry was made by using mobile phase solvent system and charged into the column. The air bubbles in the column were removed by gentle tapping and by the addition of excess of solvent, in order to ensure uniform packing of the adsorbent. The tap of the column is then opened to remove the excess solvent.
and small quantity of the solvent system was allowed to remain on the top of
the column (about 2 cm). The silica gel bed was allowed to stabilize overnight.
Care was taken to prevent the drying of the column by plugging the top end
with the aluminum foil.

The residue obtained from the aqueous methanol extract was dissolved
in small quantity of methanol and carefully introduced into the stabilized
column. The tap at the bottom was operated to run out very small amounts of
solvent in the column in order to bring the sample just below the top of the
medium. The eluting solvent was added gradually to maintain a steady flow.
The rate of flow of the elution was adjusted to 5 to 6 drops/min and allowed the
separation to continue. The eluted fractions were collected at the intervals of
5 ml each. The suitability of the eluted samples was tested by TLC and grouped
in to seven fractions. Fraction seven was recrystallized from chloroform–
hexane to get fine needle crystals of the compound.

The details of the column and TLC of the eluted fractions are given below.

**Details of the column:**

- **Adsorbent**: Silica gel (Hi-Media, Mumbai)
- **Length of the column**: 75 cm
- **Length of the adsorbent**: 60 cm
- **Diameter of the column**: 05 cm
- **Rate of elution**: 5-6 drops/min
- **Volume of elute collected**: 05 ml each

**Thin layer chromatography of eluted fractions of column:**

- **Adsorbent**: Silica gel G (activated)
- **Plate size**: 20 cm x 8 cm
- **Plate thickness**: 3 mm
- **Activation temperature**: 110°C for 1 h
Solvent system: Hexane: Ethyl acetate (1:1, 1:2 and 1:4)
Spraying reagent: Vanillin-sulphuric acid solution followed by heating at 105°C.

The structure and identification of the eluted constituent was confirmed by UV, IR. and MASS spectral studies.

3. Clinical Studies
The clinical parameters carried out are

- Wound healing activity
- Hepatoprotective activity
- Anti-inflammatory activity

I). Wound healing activity

a) Chemicals

Chemicals used in this experiment are of analytical grade obtained from various companies like Sigma Chemical Laboratories, America., E. Merk Co. Mumbai, India., s.d. fine Chemicals Mumbai, India., Ranbaxy Laboratories. Chandighad, India.

b) Extracts, fractions and isolated compound used for wound healing studies

- Aqueous extract of Elephantopus scaber
- Ethanol extract of Elephantopus scaber
- Aqueous methanol fractions of Elephantopus scaber
- Hexane fractions of ethanol extract of Elephantopus scaber
- Deoxyelephantopoin (sesquiterpene lactone) of Elephantopus scaber
c) Drug formulations

Two types of drug formulations were prepared from each of the extracts, fractions, and the isolated compound of *Elephantopus scaber*. For topical administration, 5 g of each of the aqueous and ethanol extracts and methanol and hexane fractions of ethanol extract were separately incorporated with 100 g of 2% sodium alginate (w/w) to get 5% w/w ointment gel. Similarly, 100 mg of deoxyelephantopin was incorporated with 50 g of sodium alginate (w/w) to get 0.2% gel.

For oral administration, suspensions of 60 mg/ml w/v of each of the aqueous and ethanol extracts and 20 mg/ml w/v of each of the methanol and hexane fractions of ethanol extract and 4 mg/ml w/v of the isolated compound deoxyelephantopin were incorporated with 1% w/v of gum tragacanth. The drug formulations were prepared on every fourth day. The drugs were administered by using oral feeding tube.

d) Animal

Swiss Wistar strain rats of either sex weighing 150-200 g were procured from the National College of Pharmacy, Shimoga, Karnataka State and were maintained at standard housing conditions. The animals were fed with commercial diet (Hindustan Lever Ltd., Bangalore) and watered with *ad libitum* during the experiment. The Institutional Ethical Committee (Reg. No.144/1999/CPCSEA/SMG) permitted the study.

Acute toxicity studies

For the detection of acute toxicity of extracts, fractions and isolated compound of *Elephantopus scaber*, “up and down” or “staircase” method was adopted (Ghosh, 1984).
The animals were given in different dose levels viz. 1000, 2000, 3000, and 4000 mg/kg body weight of each of the extracts and 500, 1000, 1500 and 2000 mg/kg.b.w. of each of the fractions and 20, 30, 40 and 50 mg/kg.b.w. of the isolated compound to determine the safer dose. Gum tragacanth (1% w/v) was used as vehicle to suspend the various extracts, fractions and isolated constituent 1/10 of these doses (300 mg/kg b.w. of each of aqueous and ethanol extract, 100 mg/kg b.w. of each of methanol and hexane fractions of ethanol extract and 4 mg/kg b.w. of the isolated compound deoxyelephantopin) were selected for the evaluation of wound healing efficacy.

Seven groups containing six animals of each were used for each of the excision and incision wound models. The group I was considered as control, which received only vehicle 1% tragacanth, the group II served as reference standard that received 0.2% w/w nitrofurazone ointment. (Pulok K Mukhergee, 2002) The animals of the group III were treated with aqueous extract and the group IV was treated with ethanol extract of Elephantopus scaber. The animals of the group V and VI were received methanol and hexane fractions of ethanol extract respectively, while the animals of the group VII were received the isolated compound deoxyelephantopin.

e) Wound healing models studied
   - Excision wound model
   - Incision wound model
   - Dead space wound model

**Excision wound model**

The rats inflicted with excision wounds as described by Morton and Malone (1972) and Ehrlich (1969). The animals of all the groups were anaesthetized by open mask method with anaesthetic ether. The rats were
depilated on the back. Excision wound was inflicted by cutting away 500 mm$^2$ full thickness of skin of a predetermined area. Rats were left undressed to the open environment. The experiment consists of sodium alginate gel as control and nitrofurazone as reference standard drug. The formulated test drug ointment gels consists of crude extracts, fractions and isolated compounds respectively were applied topically once in a day starting on the day of the operation, till the wound was completely healed.

Epithelization time was noted in number of days till the complete fall off the scar. The contraction of wound was calculated in percent of reduction in wound area. The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper in every alternate day. The changes of wound area at different stages of healing is expressed as mm$^2$ according to the methods of Udupa, et al., (1994) and Saha, et al., (1997).

**Incision wound model**

The animals of each group used for incision model were anaesthetized and two paravertebral long incisions of 6 cm length were made through the skin and cutaneous muscles at distance of about 1.5 cm from midline on each side of the depilated back of rat. The animals were caged individually at laboratory condition without using any systemic antimicrobials. After the incision, the parted skin was stitched with black silk by 0.5 cm apart using surgical thread (No.000) and curved needle (No.11). For good adoption of the wound both wound edges were tightened with the continuous thread. The wound was left undressed. The reference standard drug and the formulated drug samples of *Elephantopus scaber* were applied to the wound twice a day until complete recovery of wound.
Determination of tensile strength

In incision wound model the wounds closed with interrupted sutures were removed on 9th day of wounding and the tensile strength is measured on the day 10 by the method of Lee, et al., (1968). The rats were anaesthetized and animals were secured to operation table in its natural position and with the help of a marker pen equidistant lines were drawn on either side of the incision wound. (2 mm away from the wound margin on adjacent normal skin, leaving about 5 mm wound towards both the ends). Two allies forceps were firmly applied on one side of the incision wound on left and right side. The forceps applied on the left side was fixed to a metal rod, which fixed firmly to the operation table. The forceps applied on right side was connected to a light polythene container by means of a thread that passes over a pulley (Fig. 20) Water was allowed to flow at constant rate into the polythene container so as to build a gradual pulling force that was necessary to disrupt the wound. As soon as the gaping of the wound observed, the flow of water was stopped in the rubber tube by means of an occlusion clamp. Further opening of the wound was avoided by releasing the pulling force on the wound immediately, which was achieved by lifting up the polythene container. The volume of water in the polythene container was weighed. The tensile strength is expressed as the minimum weight of water necessary to bring about the gaping of the wound. Three such readings were recorded for a given incision wound. The procedure was also repeated on the incision wound model on other side, thus obtaining six readings for each animal. The mean tensile strength was calculated by taking six readings in six animals of each group.

The tensile strength of the animals treated with the different extracts, fractions, isolated constituent of Elephantopus scaber and the standard drug were compared with control.
Dead space wound model

Six groups of animals containing six in each group were anesthetized by open mask method with anesthetic ether. Dead space wounds were created by subcutaneous implantation by means of sterilized cylindrical grass piths (2.5 cm x 0.3 cm) in the region of groin.

The animals of group I was served as control, which received only the vehicle 1% tragacanth. Oral suspensions of Elephantopus scaber of aqueous and ethanol extracts were administered to the animals of group II and III in the dose of 300 mg/kg b.w. The groups of animals IV and V were administered with the methanol and hexane fractions of ethanol extract in the oral dose of 100 mg/kg b.w. While group VI is treated with deoxyelephantopin in the dose of 4 mg/kg b.w. respectively.

The granulation tissues formed on the grass piths were harvested on 10th post wounding day. The fresh and dry weights of the granulation tissues were noted. Simultaneously the buffer extract of the wet granulation tissues were used for the determination of tensile strength and for the histological examination. A part of the dried granulation tissue was used for the determination of hydroxyproline content by the method of Wossner (1961).

Statistical analysis

The results of these experiments are expressed as mean ± SE of six animals in each group. The data were statistically evaluated by ANOVA followed by Tukey's Pairwise Comparison Test. The values of p<0.01 were considered as statistically significant.
Estimation of hydroxy proline contents in granulation tissue by colorimetric method. Wossner (1961)

Materials Required:

Glass homogenizer viz., glass mortar and pestle reagent bottles (narrow mouth or neck) 250 ml (six in numbers), dropping bottles for reagents, graduated measuring cylinders: 50 ml and 100 ml capacity, beakers: 250 ml (four in numbers), test tubes (twenty in numbers), pipette: 1ml, 2 ml, 10 ml and 25 ml, reagents bottles with stoppers, water bath with temperature regulators, watch glasses (ten numbers), sterile glass ampoules (25 ml capacity), burrets with end pill tiles, photochem colorimeter, blotting papers, tissue papers and graph papers.

Preparation of chemical reagents:

i) Hydroxyproline standard solution: A stock solution was prepared by dissolving 25 mg of vacuum dried hydroxyproline in 250 ml of 0.001N hydrochloric acid. The working standard solutions were prepared by diluting the stock solution with water to obtain the concentration of 1 to 10 µg/2 ml.

ii) Citrate buffer: 50 g citric acid monohydrate, 12 ml glacial acetic acid, 120 g sodium acetate trihydrate and 34 g of sodium hydroxide were dissolved in about 600 ml of distilled water and the final volume was made up to one liter. The pH was adjusted to 6.0 and stored in the refrigerator.

iii) Chloramin-T: Chloramine-T of 0.05 N solutions was prepared freshly by dissolving 1.14 g of chloramine-T in 20 ml of water to which 30 ml of methyl cellusolve (ethylene glycol mono methyl ether) and 50 ml of citrate buffer was added.
iv) **Perchloric acid**: Solution of 3.15 M was prepared by diluting 27 ml of 70% perchloric acid to 100 ml with water.

v) **P-dimethyl aminobenzaldehyde**: Solution of 20% was freshly prepared before the use by adding 20 g P-dimethyl aminobenzaldehyde to 100 ml of methyl cellusolve. (ethylene glycol mono methyl ether).

**Procedure:**

Six test tubes were taken and labeled as H¹, H², H³, H⁴, H⁵ and H⁶. To the test tube H¹, (taken as blank) 2 ml of distilled water was added. From the freshly prepared stock solution of hydroxyproline aliquot concentrations containing 2 μg/2 ml, 4 μg/2 ml, 6 μg/2 ml, 8 μg/2 ml and 10 μg/2 ml were prepared with distilled water and were added to the test tubes H², H³, H⁴, H⁵ and H⁶ respectively. The pH of the solution was adjusted to 6-7.

To each of the test tubes (H¹ to H⁶), 1ml of chloramin T solution was added in a sequential order. The contents were mixed and allowed to stand for 20 minutes at room temperature. Then 1 ml of perchloric acid was added to each of the test tubes in the same order, contents were mixed and allowed to stand for 5 minutes.

Finally 1 ml of P-dimethyl-aminobenzaldehyde solution was added to each of the test tubes and shaken until no schlieren color appeared. The tubes were then placed in a 60°C water bath for 20 min; cooled then optical density was read at 570 nm. The optical densities were plotted against the aliquots to get the standard graph.
A sample of granulation tissue of the corresponding group of animals weighing around 300 mg was homogenized separately in a glass homogenizer. The homogenate were taken separately in 25-ml glass ampoules in to which 10 ml of 6 N hydrochloric acid was added. The ampoules were sealed and hydrolyzed at 130°C for 3 hrs. The ampoules were cooled and opened. The contents were transferred to the six corresponding test tubes labeled as G₁, G₂, G₃, G₄, G₅ and G₆ respectively. The pH adjustment, incubation and reading of OD were carried out in a manner similar to that of obtaining the standard graph.

**Histological studies of the wound** (Kanai L. Mukhergee, 2000)

The buffer extracts of the wet granulation tissues were used for the histological studies, by fixing in 10% neutral formalin solution. Granulation tissues were dehydrated with a sequence of ethanol-xylene solutions, infiltrated and embedded with paraffin. The sections were taken at 5 μm thicknesses, stained with haematoxylin-eosin dye (H&E stain) and then observed under a microscope for morphological changes that includes tissue regeneration, fibrosis, granulations, epithelization, collagenation etc.

II). Hepatoprotective activity

a) Chemicals

Chemicals used in this experiment are of analytical grade obtained from various companies like Sigma Chemical Laboratory. America., E.Merk Co. Mumbai., s.d. fine chemicals, Mumbai., Ranbaxy Laboratories. Chandighad, India.

b) Extracts and compounds used for screening

- Aqueous extract of *Elephantopus scaber*
- Ethanol extract of *Elephantopus scaber*
- Deoxyelephantopin (sesquiterpene lactone) of *Elephantopus scaber*
c) Drug formulations

For oral administration sterilized distilled water suspensions of 60 mg/ml of aqueous extract, 60 mg/ml of ethanol extract and 10 mg/ml of the isolated compound deoxyelephantopin were prepared and incorporated separately with 1% w/v of gum tragacanth.

d) Antihepato toxic studies

For the assessment of the antihepatotoxic effects of *Elephantopus scaber*, the animals were divided into six groups of six rats in each. The animals of group-I served as control, which received with 1 ml/kg b.w.of 1% gum tragacanth orally for 14 days. Liver damage was induced in the animals of groups II, III, IV V and VI, of *Elephantopus scaber* by intra-peritonial injection of carbon tetrachloride (E-Merk Co. Mumbai, India) in the dose of 0.1 ml/kg.b.w. /day, for 14 days. The animals of the group- III were received with the standard drug silymarine (Ranbaxy lab, Chandhighad) orally in the dose of 50 mg/kg.b.w. /day, (Venkatesan, et al., 2003) for 14 days.

The animals of the group, IV and V were administered with oral suspensions of aqueous and ethanol extracts of *Elephantopus scaber* in the dose of 300 mg/kg.b.w. /day respectively for 14 days. While the animals of group VI were administered with isolated compound deoxyelephantopin in the dose of 10 mg/kg.b.w. /day (higher dose) for 14 days.

Assessment of hepatoprotective activity

The animals of all the groups were sacrificed on day 14, under light ether anesthesia. Blood samples were drawn directly from carotid artery in to the dry centrifuge tube and allowed to coagulate for 30 min at 37°C. Serum was separated by centrifugation at 2500 rpm for 10 min. and the clear serum was subjected for different biochemical assays viz., total bilirubin, total
protein, serum alanine amino transaminase, serum aspartate amino transaminase, and serum alkaline phosphatase activities.

Biochemical Assays

a) Determination of total bilirubin in serum (Mallory, et al., 1937)

Reagents

i) Diazo-reagent: 100 g of sulphanillic acid was dissolved in 1.5 ml of concentrated hydrochloric acid and the solution was made up to 100 ml with distilled water (Diazo-A). 50 mg of sodium nitrite was dissolved in 100 ml of distilled water (Diazo-B). 10 ml of Diazo-A was mixed with 0.3 ml of Diazo-B just before use.

ii) Diazo-blank: 1.5% hydrochloric acid (v/v)

iii) Methanol

iv) Standard bilirubin: 10 mg of bilirubin was dissolved in 100 ml of chloroform (0.1 μg/ml)

Procedure

0.2 ml of serum of corresponding group of animals (normal, CCl₄ treated, CCl₄ + aqueous/ethanol extract treated and CCl₄ + isolated constituent treated), 1.8 ml of distilled water and 2.5 ml of methanol was taken in each two test tubes labeled as T and T¹. To the test tube T, 0.5 ml of Diazo reagent and to the test tube T¹, 0.5 ml of Diazo blank reagent was added. The solutions in the test tubes were mixed and were kept in dark at room temperature for 30 min. Optical density was read at 540 nm against distilled water. Two more test tubes of each were taken and marked as ‘S’ and ‘B’. 1.8 ml of distilled water, 2.5 ml of methanol and 0.5 ml of Diazo reagent was added to both the tubes. Then to the marked tubes ‘S’, 0.2 ml of standard bilirubin and to the tube
‘B’, 0.2 ml of distilled water was added. The tubes were allowed to stand for 30 min. and optical density was measured at 540 nm against distilled water.

Total bilirubin was calculated by using the following formula:

\[
\text{Total bilirubin (mg/100 ml of serum)} = \frac{\text{O.D. of } T - \text{O.D. of } T'}{\text{O.D. of } S - \text{O.D. of } B} \times 10
\]

b) Determination of total serum protein and albumin-globulin ratio

(Kingsley, et al., 1939).

Reagents

Biuret reagent: 45 mg of sodium potassium tartrate was dissolved in about 400 ml of 0.2 N sodium hydroxide solutions. 15 g of copper sulfate crystals was added with constant stirring. After dissolving the copper sulfate, 5 g of potassium iodide was added and the solution was made up to one liter with 0.2 N sodium hydroxide solutions.

28% sodium sulfite: 28 g of sodium sulfite was dissolved in 100 ml of distilled water.

0.9% sodium chloride: 900 mg of sodium chloride was dissolved in 100 ml of distilled water.

Standard protein solution: 200 mg of standard bovine serum albumin was dissolved in 100 ml of distilled water.
Procedure

Separation of albumin from serum protein: 0.2 ml of serum and 5.8 ml of sodium sulfite solution were mixed and allowed to stand for 5 min. The content was filtered through a Whatmann no. 44 dry filter paper and the clear filtrate was used for the estimation of albumin.

Development of color: Four test tubes were labeled as B (Blank), ‘S’ (standard), ‘T.P.’ (Total protein) and ’A’ (albumin) for the serum of each group. To the test tube B, 3 ml of distilled water, to the test tube ‘S’, 3 ml of standard protein solution, to the test tube ‘T.P.’ 0.1 ml of serum respective animals and 2.9 ml of 0.9% sodium chloride and to the test tube ‘A’ 3 ml of above filtrate were added. The contents were mixed by gentle shaking and 3 ml of biuret reagent was added to all the tubes. The test tubes were allowed to stand for 10 min. after mixing thoroughly, optical density was measured at 540 nm. Total protein of the serum and albumin globulin ratio was calculated by using the following formula.

Calculation

\[ \text{Total protein (gm/100ml)} = \frac{\text{O.D. of T.P. - O.D of B}}{\text{O.D. of S - O.D. of B}} \times \text{concentration of standard} \]

\[ = \frac{\text{O.D. of T.P. - O.D. of B}}{\text{O.D. of S - O.D. of B}} \times \frac{\text{100}}{\text{1}} \times \frac{\text{X}}{\text{X}} \times \frac{\text{X}}{\text{6}} \]

\[ = \frac{\text{O.D. of T.P. - O.D. of B}}{\text{O.D. of S - O.D. of B}} \times \frac{\text{0.1}}{\text{1000}} \]

\[ = \frac{\text{T.P.-B}}{\text{S-B}} \times \text{X 6} \]

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<table>
<thead>
<tr>
<th>Albumin (gm/100ml)</th>
<th>[ \text{O.D. of A} - \text{O.D of B} ] [=] [\frac{\text{O.D of S} - \text{O.D. of B}}{100} \times \text{X} \times 6]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[\frac{\text{O.D of A} - \text{O.D of B}}{0.1} \times 1000]</td>
</tr>
<tr>
<td></td>
<td>[\frac{\text{A} - \text{B}}{\text{S} - \text{B}} \times 6]</td>
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Globulin (gm/100 ml) = Total protein – Albumin.

c) **Estimation of serum alkaline phosphatase activity (Bessey, et al., 1964)**

**Reagents**

**Barbitone buffer**: 424 mg of barbitone was dissolved in 500 ml of distilled water. The pH of the solution was adjusted to 7.4 by adding 1 N sodium hydroxide.

**Phosphate standard (40 μg of Phosphate/ml)**: 175.5 mg of potassium dihydrogen phosphate was dissolved in distilled water and the solution was made up to 100 ml in a volumetric flask. 10 ml of this solution was pipette out and made upto 100 ml in another volumetric flask of 100 ml capacity.

**Substrate**: 500 mg of sodium β-glycero-phosphate was dissolved in 50 ml of distilled water.

**20 % Trichloro acetic acid**: 20 g of trichloro acetic acid was dissolved in 100 ml of the distilled water.
10 % Trichloro acetic acid: 10 g of trichloro acetic acid was dissolved in 100 ml of the distilled water.

2.5 % of Ammonium molybdate: 2.5 g of ammonium molybdate was dissolved in 100 ml of 10 N sulphuric acid.

Color reagent (ANSA): 250 mg of amino napthol sulfphonic acid was added to 19.5 ml of 15% sodium bisulfite solution and 0.5 ml of 20% sodium sulfite solution in to a conical flask. Shake until it is dissolved and filtered into a brown bottle through a Whatmann no. 1 filter paper. ANSA is unstable to light so it should be prepared fresh at the time of each experiment.

Procedure

Eight reagent tubes were taken and arranged into two groups of four each. The reaction mixture containing 0.8 ml of barbitone buffer and 1 ml of substrate (sodium β-glycero-phosphate) was added to all the tubes. To the four test tubes marked as T₁, T₂, T₃ and T₄, 0.2 ml of serum of corresponding group was added and all the eight tubes were incubated at 37°C for 30 min. The reaction was terminated by adding 1 ml of 20% of trichloro acetic acid. Then to the test tubes marked as B₁, B₂, B₃ and B₄, 0.2 ml of serum of corresponding group was added after adding 1ml of 20% trichloroacetic acid. The solutions of all the eight tubes were centrifuged and the supernatant was collected separately. 1 ml of 2.5% of ammonium molybdate, followed by 0.5 ml of ANSA was added to the supernatant solution. The solution were mixed by gentle shaking and allowed to stand for 10 min. at room temperature.

Optical density was read at 680 nm. The difference in O.D. between the corresponding control and experimental ones gave the actual O.D. The alkaline phosphate activity was calculated as micro molar product released/mg of protein at 37°C.
Reagents

**Phosphate buffer (0.1 M):** 17.41 g of dipotassium phosphate (dibasic) and 13.61 g of potassium dihydrogen phosphate (monobasic) were dissolved separately in distilled water and made up to one liter. The pH of the solution was adjusted to 7.4.

**Substrate for AST:** Dissolve 2.66 g of L-aspartic acid and 30 mg of \( \alpha \)-ketoglutaric acid in 20 ml of phosphate buffer; adjust the pH to 7.4 by adding 1 N sodium hydroxide, drop wise and stirring make up to 100 ml with phosphate buffer of pH 7.4.

**Substrate for ALT:** Dissolve 2.66 g of L-alanine and 30 mg of \( \alpha \)-ketoglutaric acid in 20 ml of phosphate buffer and adjust the pH to 7.4 by adding 1 N sodium hydroxide, drop wise and with stirring make up to 100 ml with phosphate buffer of pH 7.4.

**Sodium pyruvate standard (100 \( \mu \)g/ml):** 100 mg of sodium pyruvate was dissolved in phosphate buffer, taken in a volumetric flask of 100 ml capacity. The volume was maintained up to 100 ml. 10 ml of this standard solution was pipetted out and transferred to another volumetric flask of 100 ml capacity. The volume of this solution was also diluted up to 100 ml. This gives the working standard solution containing 100 \( \mu \)g/ml of sodium pyruvate.

**Procedure for AST**

Pipette out 0.5 ml of AST substrate to eight labeled test tubes, arranged in two groups of four each and were marked as test (T\(^1\), T\(^2\), T\(^3\) and T\(^4\)) and blank (B\(^1\), B\(^2\), B\(^3\) and B\(^4\)) 0.4 ml of distilled water was added to all the test
tubes and were incubated at 37°C for 5 min. 0.1 ml of serum of corresponding animal group was added to the test tubes marked as T (group-I T¹, group-II to T², group-III to T³ and group IV to T⁴). The contents of all the test tubes were mixed well and all the eight test tubes were incubated for one hour at 37°C. 0.5 ml of color reagent was added to all the eight tubes to stop the reaction. To the test tubes marked as B¹, B², B³ and B⁴, 0.1 ml of serum of corresponding animal group was added. The test tubes were allowed to stand for 20 min. and 5 ml of 0.4 N sodium hydroxide was added to all the test tubes with gentle shaking. Then the tubes were allowed to stand for about 10 min. (not longer than 30 min.) and O.D. was measured at 505 nm.

The difference in O.D. between the test (T) and the blank (B) was calculated. The AST activity was expressed as micromoles product released/mgs of protein at 37°C. Statistical significance was determined by Critical Difference (C.D.) method.

**Procedure for ALT**

The assay method was similar with that of the AST but the difference was the change in the substrate (ALT) and the period of incubation was only up to 30 min.

**Histological studies of the wound** (Kanai L.Mukhergee, 2000)

After fixing in 10% neutral formalin solution, liver tissues are dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 μm section, stained with haematoxylin-eosin dye (H&E stain) and then observed under a photomicroscope for morphological changes which includes, cell gross necrosis, sinusoidal congestion, fatty changes, ballooning degeneration, infiltration of lymphocytes and Kuffer cells.
iii). Anti-inflammatory activity.

a) Instrument

Instrument used: Plethysmograph filled with mercury. (Fig. 31)

b) Extracts and compounds used for screening

- Aqueous extract of *Elephantopus scaber*
- Ethanol extract of *Elephantopus scaber*
- Deoxyelephantopin (sesquiterpene lactone) of *Elephantopus scaber*

c) Drug formulations

For oral administration, of *Elephantopus scaber* suspensions of 60 mg/ml of the aqueous extract, 60 mg/ml of ethanol extract and 10 mg/ml of the isolated compound deoxyelephantopin were incorporated with 1% w/v of gum tragacanth.

d) Animal

For the assessment of the anti-inflammatory activities of *Elephantopus scaber* five groups containing six animals in each were used for carrageenan induced paw edema models. The group first was considered as control, which received 1ml of 1% gum tragacanth. The group second served as reference standard that received oral administration of 10 mg/kg.b w. of the standard drug diclofenac sodium. (Sankar, *et al.*, 2001) The animals of the group III and group IV was treated with oral suspensions of 300 mg/kg.b w. of the aqueous extract and ethanol extract respectively. While, the animals of the group V, received deoxyelephantopin orally in the dose of 10 mg/kg.b. w. (higher dose).

**Carrageenan induced paw edema model**

**Procedure**

All the animals were fasted for 18 hrs. Providing water *ad libitum*. Before starting of the experiment either hind paw of each animal was marked at
the tibiotarsal junction, such that, each time the paw is dipped in the mercury column till the mark. This ensures a constant paw volume. The initial paw volumes for both paws were determined by dipping each paw in the column of mercury and measuring the volume of displaced mercury.

Thirty minutes after the oral administration of the formulated drugs, 0.1 ml of 1% carrageenan was injected into the sub plantar region of the hind paws. A mark was put on the leg at the malleous to facilitate uniform dipping at subsequent readings. This paw edema volume was measured with the help of plethysmograph (Fig. 31) by mercury displacement method at zero hour (immediately after injecting carrageenan). The same procedure was repeated at the time intervals of 30, 60, 90, 120 and 180 min. The difference between zero hour and subsequent reading was taken as actual edema volume.

The percentage inhibition of edema in the drug-administered groups was calculated by using the formula.

\[
\text{% Inhibition} = 1 - \frac{V_t}{V_c} \times 100
\]

Where, \( V_t \) = Edema volume in the drug treated animals

\( V_c \) = Edema volume in the control group.