4. EXPERIMENTAL

The present section deals with the detailed description of various methods and techniques adopted for carrying out different studies on the selected plants viz., leaves of *Rivea hypocrateriformis* Choisy and aerial parts of *Ipomoea eriocarpa* R.Br.

4.1 Pharmacogno stical studies and proximate analysis

4.1.1 Collection and identification of plant materials

*Rivea hypocrateriformis* leaves were collected in the month of October from Kapadwanj, Dist. Kheda (Gujarat), India. The leaves were authenticated in the Department of Biosciences, Sardar Patel University and a voucher specimen (RRK/RH-01/33/ARGH-11) has been deposited in Department of Pharmacognosy, A.R.College of Pharmacy & G.H.Patel Institute of Pharmacy, Vallabh Vidyanagar, Anand, India.

The fresh aerial parts of *Ipomoea eriocarpa* were collected in the month of December within the campus of New Vallabh Vidyanagar, Dist. Anand, Gujarat. These were authenticated in the Department of Biosciences, Sardar Patel University and a voucher specimen (RRK/IE-02/15/ARGH-11) has been deposited in Department of Pharmacognosy, A.R.College of Pharmacy & G.H.Patel Institute of Pharmacy, Vallabh Vidyanagar, Anand, India.

4.1.1 Preparation of powdered materials

The collected part of both selected plants were first properly cleaned and then dried under shade. The dried materials were then subjected to size reduction using a mechanical pulveriser to a coarse powder, which was used in further studies.
4.1.2 Macroscopical studies

The leaves of *Rivea hypocrateriformis* & aerial parts of *Ipomoea eriocarpa* were examined macroscopically using reported methods.

4.1.3 Microscopical Studies

Microscopical evaluation of the leaf of *Rivea hypocrateriformis* and aerial parts of *Ipomoea eriocarpa* were carried out. Free hand sections of fresh leaf of *Rivea hypocrateriformis* and aerial parts of *Ipomoea eriocarpa* were taken and studied for microscopical characters.

Further the micro powder analysis of the samples was done according to the standard procedures mentioned in text\textsuperscript{116-118} and their diagnostic features were recorded.

4.2 Proximate Analysis

Physical and physiochemical standards are to be determined for the crude drugs, wherever possible. These were determined using standard procedures.\textsuperscript{119} Fluorescence analysis was carried out according to the method described by Chase and Pratt.\textsuperscript{121}

4.2.1 Loss on drying

An excess of water in plant material will encourage microbial growth and deterioration following hydrolysis. Limits for water content should therefore be set for every given plant material. Loss on drying was determined as mentioned below:

Accurately weighed 5 gm of the dried plant material was placed in a petri dish which was previously dried and tared. The samples were dried at 100-105 °C until two consecutive weighing does not differ by more than 5 mg, the loss in weight was calculated with reference to the air dried plant materials.
4.2.2 Total ash

Controlled incineration of plant drug results in an ash residue. It usually represents the mixture of inorganic salts and silica naturally occurring in the plant drug and adhering to it, but it may also include inorganic matter added for the purpose of adulteration.

For determining the total ash about 2 gm of the powder was weighed, in a previously weighed silica crucible, and spread as a fine layer to the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450 °C until it became free from carbon. Then it was cooled and weighed. The procedure was repeated till a constant weight was obtained. The percentage of the total ash was calculated with reference to the air dried drug.

4.2.2a Acid insoluble ash

The ash obtained above (Total ash) was boiled with 25 ml of hydrochloric acid (HCl) for 5 mins. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into weight silica crucible, ignited, cooled and weighed. The procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

4.2.2b Water soluble ash

The total ash (obtained as above) was boiled for 5 min with 25 ml of hot water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible and ignited at a temperature not exceeding 450 °C. The procedure was repeated till a constant weight of the insoluble matter was detected from the weight of the total ash. The difference in weight was considered as water soluble ash. The percentage of water soluble ash was calculated with reference to air dried drug.
4.2.3 Extractive values

4.2.3 a Alcohol soluble extractive value

Extraction of plant (drug) material with solvent yields a solution of different components and the composition of this solution will depend upon the constituents present in the drug and the solvent used.

Method: Powdered plant material (5 gm) was macerated with 100 ml of methanol (90 % v/v) in a closed flask for 24 hrs. The contents of the flask were shaken for first 6 hrs and then set aside for 18 hrs. The contents were filtered and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 °C and weighed. The percentage of methanol soluble extractive was calculated with reference to the air dried plant material.

4.2.3 b Water soluble extractive value

Powdered plant material (5 gm) was macerated with 100 ml of chloroform water in a closed flask for 24 hrs. The contents of the flask were shaken for first 6 hrs and then set aside for 18 hrs. The contents were filtered and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 °C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried plant material.

4.2.4 Elemental analysis

Elemental contents of different organs of *Rivea hypocrateriformis* and *Ipomoea eriocarpa* were estimated on energy dispersive X-ray analysis at Sophisticated Instrumentation Center of Applied Research and Testing, Vallabh Vidyanagar, Gujarat. It determined the relative atomic percentages of the major and minor elements on a JOEL-JSM-5200 Scanning Electron Microscope with energy dispersive X-ray analysis facility.
4.3 Phytochemical studies

4.3.1 Successive solvent extraction of plant drug

A) Petroleum ether extract

The coarsely powdered material of *Rivea hypocrateriformis* (100 gm) and *Ipomoea eriocarpa* (100 gm) were extracted separately with petroleum ether (60-80°C) via hot extraction process (Soxhlet’s apparatus). The completion of extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extract obtained with petroleum ether were collected separately, the solvent was removed by distillation and concentrated in vacuums.

B) Toluene extract

The marc left after petroleum ether extraction was dried in air and extracted in toluene by hot extraction process (Soxhlet’s apparatus). The completion of extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extract obtained with toluene were collected separately, the solvent was removed by distillation and concentrated in vacuum.

C) Chloroform extract

The marc left after toluene extraction was dried in air and extracted in chloroform by hot extraction process (Soxhlet’s apparatus). The completion of extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extract obtained with chloroform were collected separately, the solvent was removed by distillation and concentrated in vacuum.
D) Acetone extract

The marc left after chloroform extraction was dried in air and extracted in acetone by hot extraction process (Soxhlet’s apparatus). The completion of extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extract obtained with acetone were collected separately, the solvent was removed by distillation and concentrated in vacuum.

E) Methanol extract

The marc left after acetone extraction was dried in air and extracted in methanol by hot extraction process (Soxhlet’s apparatus). The completion of extraction was confirmed by evaporating a few drops of extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extract obtained with methanol collected separately, the solvents was removed by distillation and concentrated in vacuum.

F) Aqueous extract

The marc left after methanol extraction was dried in air and extracted with chloroform water by maceration process. After completion of extraction, the solvent was removed and concentrated in vacuum.

Colour, consistency and percentage yield of the all extracts were noted. The extracts were preserved under vacuum for further phytochemical studies.

4.3.2 Qualitative evaluation of successive extracts

Following qualitative chemical tests were carried on vacuum dried successive extracts of different parts of *Rivea hypocrateriformis* and *Ipomoea eriocarpa* to identify the presence of various chemical constituents.\(^{123,124}\)
**Test for Alkaloids:**

About 0.5gm of each dried successive extract was taken in 5% HCl (2ml) solution. The filtrate was tested with different alkaloidal reagents such as Dragendorff's, Hager’s and Wagner’s reagents. Presence of orange red, yellow and reddish brown precipitates with respective reagents detected the presence of alkaloids.

**Test for Flavanoids:**

*Shinoda test:* To 10 mg dried extract, 10ml ethanol (90%) was added and heated on water bath for 2 minutes. A small piece of magnesium ribbon was added to the alcoholic solution followed by 3 drops of concentrated HCl. Development of magenta red or orange color indicated presence of flavanoids.

**Test for Saponins:**

*Frothing Test:* Small portion of dried extract was vigorously shaken with 10 ml of distilled water in a test tube for 30 seconds and was left undisturbed for 20 minutes. Persistent froth indicated presence of saponins.

**Test for Phytosterols and triterpenoids:**

*Liebermann Burchard’s test:* To the small portion of each extract 5 drops of acetic anhydride was added on white clean tile. Mass was mixed properly and 2 drops of Conc. H₂SO₄ was added. Formation of red, purple or green color indicated presence of sterols and terpenoids.

*Salkowski’s reaction:* To the small portion of each extract, 3ml of chloroform and 2ml of Concentrated H₂SO₄ were added and shaken well, Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicating presence of steroids and triterpenoids.
Test for Tannins:

Test with gelatin: To each dried extract, 1% gelatin solution containing NaCl was added. Heavy white precipitates indicated presence of tannins.

Reaction with lead acetate: To each dried extract, 2ml of 10% solution of lead acetate was added. Yellowish white precipitates indicated presence of tannins.

Test for Phenolic compounds:

Test with FeCl₃: Each dried extract was dissolved in methanol and to this 2ml of freshly prepared FeCl₃ solution was added. Development of brownish green color indicated presence of phenolic compounds.

Test for Coumarins:

With Ammonia: To the solution of each extract, NH₃ solution was added and examined in ultra-violet light. Blue fluorescence indicated presence of coumarins.

Test for Carbohydrates:

Molisch’s test: Small portion of each extract was dissolved in ethanol. To the solution, α-napthol and concentrated H₂SO₄ were added. Purple color indicated the presence of carbohydrates.

Fehling’s test: 1 ml Fehling’s solution A and 1 ml Fehling’s solution B was mixed and boiled for one minute, equal volume of test solution was added. Heated in boiling water bath for 5 minutes. Solution may appear green, yellow or red depending on amount of reducing sugar present in test solution.

Barfoed’s test: Test solution treated with reagent, boiled on a water-bath, showed brick red colour precipitate.
Tests for proteins:
The extract was dissolved in water.
Millon’s test: Test solution was treated with reagent and heated on a water bath, Protein stained red on warming.
Biuret test: Test solution treated with 40% sodium hydroxide and dilute copper sulphate solution gave blue colour.

Test for Fixed oils and fats:
Their presence was tested by taking a small quantity of petroleum ether extract residue dissolved in alcohol. Few drops of 0.5N alcoholic Potassium Hydroxide were added along with drop of phenolphthalein and the mixture heated over the water bath. Formation of soap and partial neutralization of alkali was to be considered as positive test.

A small amount of dried petroleum ether extract was pressed between two filter papers, presence of spot indicated presence of fixed oil.

4.3.3 Thin layer chromatography studies on the successive extracts of leaves of Rivea hypocrateriformis  
The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic (TLC) studies using silica gel 60 F $^{254}$ precoated plates, to confirm the presence of various constituents.\textsuperscript{125}

All the successive extracts of leaves of Rivea hypocrateriformis were subjected to TLC and detection was done at UV and after derivatization at visible light.

4.3.4 Extraction, fractionation and isolation of compound from methanolic extract of leaves of Rivea hypocrateriformis  
The methanolic extract of leaves of Rivea hypocrateriformis was subjected to liquid-liquid partition and preparative TLC, method is described in section 5.3.1 a of chapter 5.
4.3.5 Thin layer chromatography studies on the extracts, fractions and isolated constituents of *Rivea hypocrateriformis*

The extracts, fractions and compounds were then subjected to TLC studies in order to detect separation of constituents present in extract and their fractions.

Qualitative fingerprinting of extracts, fractions and isolated compounds of leaves of *Rivea hypocrateriformis* was performed by thin layer chromatography (TLC). TLC was performed on a pre-coated TLC plate silica gel 60 F$_{254}$ plates (Kieselgel 60 F$_{254}$, Merck, Germany), using the mobile phase of ethyl acetate: formaldehyde: acetic acid: water (10:1:1:2). Detection of chemical constituents was done under UV at 254nm and 366nm and alcoholic KOH as detecting agent.

4.3.6 Determination of content marker in methanolic extract of leaves of *Rivea hypocrateriformis* by HPTLC

RHI-4 is one of the marker constituent isolated and identified from the leaves. Therefore, to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPTLC method was developed for the quantification of RHI-4. The method is described in detail in section 5.3.3 of chapter 5.

4.3.7 Characterization of isolated compounds

The isolated compounds RHI-1, RHI-2, RHI-3 and RHI-4 were then subjected to determine of various physical characteristic like melting point, FT-IR, 1H NMR and Mass spectra of individual’s compounds were performed to elucidate the chemical structure.\textsuperscript{126-128}
4.4 *Ipomoea eriocarpa*

4.4.1 Thin layer chromatography studies on the successive extracts of *Ipomoea eriocarpa*

The various extracts obtained in the successive extraction process were subjected to thin layer chromatographic studies using silica gel 60 F$_{254}$ precoated plates to confirm the presence of various constituents. The successive extracts of aerial parts of *Ipomoea eriocarpa* were then subjected to TLC studies in order to detect separation of various types of constituents in different solvents using specific reagents.

4.4.2 Extraction, fractionation and isolation of compound from extracts of aerial parts of *Ipomoea eriocarpa*

Unsaponified fraction

Coarsely powdered aerial parts of *Ipomoea eriocarpa* was extracted with petroleum ether (60-80 °C) in Soxhlet’s apparatus until exhaustion; the extract was concentrated in vacuum by evaporator and dried in desiccator. The dried petroleum ether extract was saponified to obtain the unsaponifiable matter.

The unsaponifiable matter (USM) was subjected to liquid-liquid partition and column chromatography, method is described in section 6.3 of chapter 6.

4.4.3 Thin layer chromatography studies on the extracts, fractions and isolated constituents of *Ipomoea eriocarpa*

The various extracts, fractions and compounds were then subjected to TLC studies in order to detect separation of constituents present in extract and their fractions.

Qualitative fingerprinting of extracts, fractions and isolated compounds of *Ipomoea eriocarpa* aerial parts was performed by thin layer chromatography
(TLC). TLC was performed on a pre-coated TLC plate silica gel 60 F\textsubscript{254} plates (Kieselgel 60 F\textsubscript{254}, Merck, Germany), using the different solvent systems. Detection of chemical constituents was done under UV and detected by spraying with reagent as visualization agent.

4.4.4 High Performance Thin Layer Chromatography studies on defatted methanolic extract of \textit{Ipomoea ericarpa}

10g of powdered material was defatted with petroleum ether and then extracted with methanol (50ml x 3) on a water bath for 30 min, concentrated on a rotary evaporator and dried. A stock solution (10mg/ml) was prepared in methanol. Suitably diluted stock solutions were spotted on precoated silica gel G60 F\textsubscript{254} TLC plates (Merck) with the help of CAMAG Linomat V applicator. Plates were developed in solvent systems of different polarities to resolve polar and non polar components of the extract. The developed plates were scanned using TLC Scanner 3 (CAMAG). The photographs were made with the help of Reprostar 3 (CAMAG) digital camera.

The non polar components (steroids and terpenoids) in the extract were resolved using a solvent system of (Solvent 1) toluene: chloroform: ethyl acetate (10:2:1) and the characteristic peaks of separated compounds were recorded under UV light at 254nm and 366nm. Subsequently the plate was derivatized using anisaldehyde sulphuric acid reagent and the characteristic peaks of the detected compounds were recorded at 540nm. Similarly the polar components (Phenolic compounds) in the extract were separated using toluene: ethyl acetate: formic acid: methanol (6:6:1.6:0.4) (Solvent 2), and the developed plate was derivatized using ferric chloride reagent, characteristic peaks of the detected compounds were recorded at 540nm.

4.4.5 Characterization of isolated compounds

The isolated compounds IEI-1 & IEI-2 were then subjected to determinations of various physical characteristic like melting point absorption maxima in UV
light etc. The FT-IR, NMR and Mass spectra of individual’s compounds were performed.129-131

4.5 Biological studies

4.5.1 Experimental work

4.5.1a Animals

Albino mice of Swiss strain weighing 20-30 g and Albino Wistar rats of body weight 150-200 g acclimitized in the Animal House of Pinnacle Biomedical Research Institute, Bhopal. The animals were fed *ad libitum* with normal rat pellet diet (Golden feed Industries, New Delhi) and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute, Bhopal. (1283/c/09/CPCSEA). The animal study was carried out at Pinnacle biomedical research institute, Bhopal. (Protocol approval no: PBRI/11/IAEC/PNS-29)

In all undertaken experiments, 6 groups of animals were taken viz.control, standard and two test groups at two different dose levels and 6 animals were used in each group.

4.5.1b Preparation of test extracts

Five hundred grams each of dried powdered leaves of *Rivea hypocrateriformis* and dried aerial parts of *Ipomoea eriocarpa* were extracted with required quantity of methanol for eight hours, filtered and named as MRH & MIE respectively. All extracts were stored at 0-4 °C until used. When needed, the residual extract was suspended in distilled water and used in the study.

4.5.2 Acute toxicity studies132-135

The procedure was followed by OECD (Organization of Economic Cooperation and Department) Guidelines 423 [Acute Toxic Class Method].

Ph. D. Thesis Page 65
The Acute Toxic Class Method is stepwise procedure with 3 animals of a single sex per step. Depending upon the mortality and/or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substances. This procedure resulted in the use of a minimal number of animals while allowing for acceptable data based scientific conclusion.

The method uses defined doses orally (5, 50, 300, 2000 mg/kg body weight of the animals used) and the results allowed a substance to be ranked and classified according to the Globally Harmonised System (GHS) for the classification of chemicals, which cause acute toxicity (Figure 4.1)
Figure 4.1: Flow chart for Acute Toxic Class Method (OECD guidelines-423)

FLOW CHART FOR ACUTE TOXIC CLASS METHOD (OECD GUIDELINES 423)

- 0, 1, 2, 3 - Number of Moribund or dead animals at each step
- GHS - Globally Harmonized classification system (mg/kg body weight)
- X - Unclassified
The six albino rats weighing 150-200 gms were used for study. The methanolic extract from leaves of *Rivea hypocrateriformis* (MRH) and aerial parts of *Ipomoea eriocarpa* (MIE) were used for the following study.

The starting dose level for MRH and MIE was 2000mg/kg body weight; p.o. as most of the crude extracts possess LD value more than 2000mg/kg body weight of animal used. Dose volume administered was 0.1ml/100gm body weight of the animal orally which were fasted over night with water *ad libitum*. Food was withheld for a further 3-4 hours after administration of drug.

Body weights of the rats were noted before and after termination of drug. The onset of toxicity and sign of toxicity were also noted. As no toxicity or death was observed for these dose levels, the same dose level was repeated and the data were recorded. Any change in skin and fur, eyes and mucous membrane and also respiratory, circulatory, autonomic and central nervous system and somatomotor activity behaviour pattern were observed. And also signs of tremors, convulsions, salvation, lethargy, sleep, salivation and coma were noted if any and the observations were recorded.

**4.5.3 Hot plate method**¹³⁶,¹³⁷

4.5.3a Purpose and rationale

The paws of mice and rats are very sensitive to heat at temperature which is not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws. The time until these responses occurs is prolonged after administration of centrally acting analgesics, whereas peripheral analgesic of the acetylsalicylic acid or phenyl-acetic acid type does not generally affect these responses.

4.5.3b Procedure

The hot plate, which is commercially available, consists of an electrically heated surface. The temperature is controlled for 55°C to 56°C. This can be a
copper plate or a heated glass surface. The albino rats were placed on the hot plate and the time until either licking or jumping occurs is recorded by a stop watch. The latency was recorded before and after 20, 60 and 90 min following administration of pentazocine (10 mg/kg, S.C.) as standard, MRH 250 mg/kg; p.o. and 500 mg/kg; p.o. and MIE 250 mg/kg; p.o. and 500 mg/kg; p.o. as test doses respectively. The analgesic activity was determined by comparing with the control group.

4.5.4 Paw edema

4.5.4a Purpose and rationale

Among the many methods used for screening of anti-inflammatory drugs, one of the most commonly employed techniques is based upon the ability of such agents to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as Brewer’s yeast, formaldehyde, dextran, egg albumin, kaolin, aerosol, sulfated polysaccharides like carrageenan or naphthoylheparaminne. The effect can be measured in several ways. The hind limb can be dissected at the talocrural joint and weighed. Usually, the volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls. Many methods have been described how to measure the paw volume by simple and less accurate and by more sophisticated electronically devised methods. The value of the assessment is less dependent on the apparatus but much more on the irritant being chosen. Some irritants induce only a short lasting inflammation whereas other irritants cause the paw edema to continue over more than 24 hr.

4.5.4b Procedure

Albino rats were divided into six groups and were administered orally diclofenac sodium (15 mg/kg) as standard, MRH 250 mg/kg; p.o. and 500 mg/kg; p.o. and MIE 250 mg/kg; p.o. and 500 mg/kg; p.o. respectively.
The rats were challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the planter side of the left hind paw. The paw was marked with ink at the level of the lateral malleous and immersed in mercury up to this mark. The paw volume was measured plethysmographically immediately after injection, again after 1, 3 and 5 hr. The difference between two readings was taken as the volume of edema and the percentage anti-inflammatory activity was calculated and reported.

4.5.5 Freund's adjuvant (F.A.) induce arthritis in rat\textsuperscript{140-142}

4.5.5a Principle

Freund's complete adjuvant induced arthritis in rat model which is the best and most widely used experimental model for arthritis with clinical and laboratory features which closely mimic the clinical features of human rheumatoid disease. This model is sensitive to anti inflammatory and immune inhibiting medicines and considers being relevant for the study of phathophysiological and pharmacological control of inflammation process as well as for the evaluation of anti-nociceptive potential of drugs.

4.5.5b Procedure

On day zero, animals were injected into the sub plantar region of the left hind paw with 0.1 ml of complete Freund’s adjuvant (FA). This consists of 6mg \textit{Mycobacterium butyricum} suspended in heavy paraffin oil by through grinding with motor and pestle to give a concentration of 6mg/ml.

MRH 250 mg/kg; p.o. and 500 mg/kg; p.o. and MIE 250 mg/kg; p.o. and 500 mg/kg; p.o. and diclofenac sodium at 20 mg/kg/alternate day; p.o doses were administered for 12 days from the day of Freunds’s adjuvant injection. Paw volumes of both side and body weight were recorded on the day of injection.

On the day 4, the volume of the injected paw was measured again, indicated the primary lesion and the influence of therapeutic agents on this phase. The
severity of induced adjuvant disease was followed by measurement of the non-injected paw.

Purposefully, from day 13 to 21, the animals were not dosed with the test compound or standard. On 21st day, blood was withdrawn from retro-orbital plexus of all the groups and various haematological and biochemical parameters were estimated.\textsuperscript{143,144} Rats were sacrificed by cervical dislocation and hind limb collected for further histological examination. The severity of the secondary lesion was evaluated by the following parameters:

1. Body weight
   Body weight of each animal was taken on the day of administration, and later on day 21 as prescribed.

2. Biochemical estimation
   Biochemical changes in groups of rats were estimated on 8\textsuperscript{th} and 21\textsuperscript{st} day. Serum was separated from the blood samples and SGOT, SGPT, CRP and ALP were determined by the colorimetric methods using kits. Results for biochemical estimations were recorded.

3. Paw edema
   Paw volumes of both hind limbs were recorded on the day of F.A injection and on 1, 4, 8, 14 and 21\textsuperscript{st} day using mercury plethysmograph. The fifth day measurement is indicative of primary lesions and 14\textsuperscript{th} day measurement is indicative of secondary lesions. On day 21\textsuperscript{st}, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund’s adjuvant. Effects of plant extracts on Freund’s adjuvant induced paw edema were recorded.
4. Arthritic index

All the animals were closely observed and scored as described in the following manner:

<table>
<thead>
<tr>
<th>Organ</th>
<th>Indication</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ears</td>
<td>• Absence of nodules &amp; redness</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Presence of nodules &amp; redness</td>
<td>1</td>
</tr>
<tr>
<td>Nose</td>
<td>• No swelling of connective tissue</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Intensive swelling of connective tissue</td>
<td>1</td>
</tr>
<tr>
<td>Tail</td>
<td>• Absence of nodules</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Presence of nodules</td>
<td>1</td>
</tr>
<tr>
<td>Forepaws</td>
<td>• Absence of inflammation</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Inflammation of at least 1 joint</td>
<td>1</td>
</tr>
<tr>
<td>Hind paws</td>
<td>• Absence of inflammation</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Slight inflammation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>• Moderate inflammation</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>• Marked inflammation</td>
<td>3</td>
</tr>
</tbody>
</table>

An arthritic index for each animal was calculated as the sum of these scores. The average scores for each group of drug treated animals were compared with that obtained for disease control animals.
1. Histopathology of synovial joints

21 days after the Freund’s adjuvant administration, few animals from each group were selected and their synovial joint was isolated for the biopsy examination of synovium proliferation, synovial lining angiogenesis and inflammation.

4.6 Statistical analysis

Results are represented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one way analysis of variance (ANOVA) followed by Dunnet’s ‘t’ test. Data were considered statistically significant at P value< 0.05.