2.1 Collection of Plant Material

The aerial root of *Ficus lacor* was collected from Panchkula (Haryana) and the roots of *Murraya koenigii* was collected from campus Chitkara University, Punjab, India, in July 2009. The taxonomically, authenticated and identified plant material by Dr. H.B. Singh, HRMHD department, with reference number NISCAIR/RHMD/Consult/2010-11/1638/236. The specimens of voucher have been submitted at the NISCAIR, Delhi for further reference in herbarium section. The roots dried, sliced into small pieces, using a mechanical grinder, coarse powder made and in tight container stored for further use.

2.2 Materials

2.2.1 Animals

The Wistar rats of any sex were used as per experimental protocols (IAEC/CCP/12/PR-005) after consent from the Institutional Animal Ethical Committee and Chitkara College of Pharmacy, Chitkara University, Rajpura. The animals (weighing 160-200g) were house in standard environmental conditions (25±2°C and rel. humidity 50±5%) and fed with standard go on a diet and water *ad libitum*. The rats were destitute of food for 24 hrs before conducting tests but allowed free access to tap water throughout the experiment. Each group comprises 6 rats.

2.2.2. Chemicals

Petroleum ether, Chloroform, Methanol, Paracetamol, Ethyl acetate, Glacial acetic acid, Acetone, Formic acid, Benzene, N-propanol, Ethanol, Tween-80, Dimethylsulphoxide (DMSO), Glycerine, conc. Sulphuric acid, Hydrochloric acid, Benzene, N-Butanol, Dichloromethane, n-hexane, pyridine, toluene, xylene, anisaldehyde, α-napthol, bismuth carbonate, calcium chloride, copper sulphate, Ferric chloride, Follin’s reagent, Iodine, Lead acetate, Magnesium chloride, Mercuric chloride, Ninhydrin, Nitric acid, Phloroglucinol, Potassium iodide, Potassium Dichromate, Potassium sodium Tartrate, Ruthenium red, Safranine, Sodium acetate, Sodium iodide, Sodium hydroxide, Sodium nitroprusside, Carrageenan (Hi-Media), DPPH, Sudan red- III. All the chemicals (S.D Fine Chemicals Pvt. Ltd. Mumbai) were purchased from local supplier.

**Solvents:** Acetic acid, Acetone, Benzene, n-Butanol, Chloroform, Dichloromethane, Ethanol, n-Hexane, Methanol, Pyridine, Petroleum ether, Tween-80, Toluene, and Xylene. All the solvents (S.D. Fine chemicals) were purchased.
2.2.3. INSTRUMENTS:
1. Double beam UV-VIS Spectrophotometer, 2201(Systronics)
2. FT-IR (SHIMADZU)
3. Buchi rota evaporator
4. UV-Chamber
5. BOD Incubator
6. Moisture balance
7. Microtome
8. Plethysmometer
9. Microscope with camera

2.3 Pharmacognostic studies

India has a rich traditional systems of medicines which is mainly consist of the highly promising systems of thearpies i.e. Ayurvedic, Siddha & Unani systems etc. since ancient times. The plant crude drugs are available easily in abundance, they are comparatively cheaper. They have negligible side effects and are commonly prescribed to all age groups patients. The pharmacological actions and uses of herbal drugs are explained in the classical literature of home-grown medicines in so many books of medicinal plant and pharmacopoeias (Chopra, 1955 and Nandkarni, 2000).

The pharmacognostical evaluation is the prelude stride in the standardization of plant drugs. It gives very important information about the morphology, microscopic and physical properties of herbal drug. The observations obtained from standardization, had been included as monographs in various pharmacopoeias. Therefore pharmacognostic studies gives the scientifically important information rabout the indentity, purity and quality of the plant drugs (Thomas et al., 2008).

There is no information in the literature regarding the pharmacognostical evaluation of aerial roots of *Ficus lacor* and roots of *Murraya koenigii*. The present study includes study of morphology, microscopy, powder study, determination of ash values, extractive value, bitterness value, haemolytic activity, microbial determination. It also includes phytochemical screening and chromatographic study of aerial roots of *Ficus lacor* and roots of *Murraya koenigii*.

2.3.1 Morphological study

The crude drug was evaluated for organoleptic properties shape, size, colour, odour, taste, fracture and texture.
2.3.2 Microscopic study
Microscopy of plant material is performed to distinguish it from the allied drugs and adulterant. The dried root was soaked overnight in water to make it smooth enough for transverse section. Paraffin wax embedded specimens were sectioned using the rotatory microtome (Weswox Optik). The thickness of section was 10-12 µm. Very fine section was selectively subjected to staining reaction with staining reagent safranin one percent solution and light green 0.2% solution. Slides were cleaned in xylol and mounted in mountant (DPX). Photomicrographs were taken using trinocular microscope (Olympus (Johnsen, 1940).

2.3.3 Histochemical Colour Reaction
Presence of different organic compounds in root of the plant is confirmed by using various histochemical tests. Care was taken to ascertain relative concentration of these chemicals by degree of colour produced in different tissues. The transverse sections of fresh root were treated with different chemical reagents for colour tests viz. phloroglucinol, millon’s reagent, iodine solution followed by sulphuric acid, Dragendorff’s reagent, Wagner’s reagent, Sulphuric acid solution, Libberman-Burchard reagent, Acetic anhydride, ferric chloride, iodine solution, Caustic alkali, Aqueous potassium hydroxide, Chloroform with sulphuric acid, aniline sulphate and sulphuric acid (Govil et al., 1993).

2.3.4 Powder studies
2.3.4.1 Microscopic study
The shade dried roots were mechanically pulverized to coarse powder and sifted through 40 mesh sieve. Take a pinch of powder was taken on slide and mounted with phloroglucinol, hydrochloric acid and glycerine. Slide was seen under microscope (Evans, 1996).

2.3.4.2 Colour reactions
To study the behaviour of root powder with different chemical reagents, a small quantity of powder was treated with different chemical reagents as 1N hydrochloric acid, sodium hydroxide, acetic acid, 5% ferric chloride, picric acid, nitric acid with ammonia solution, 5% iodine, 1N nitric acid and powder as such were performed, change in colour was observed (Hashmi et al., 2003).
2.3.4.3 Fluorescence behaviour of powder
Many herbs show fluorescence behaviour when cut surface or powder is exposed to UV light and this can help in their identification. To study the fluorescence nature of root powder, powder was treated with different chemical reagents viz. 1N sodium hydroxide, 1N hydrochloric acid, 1N sodium hydroxide in methanol, picric acid, 1N nitric acid, acetic acid, acetone, 50% sulphuric acid, nitric acid in ammonia solution and observed under day light, long UV (365 nm) and short UV light (254 nm) (Kokashi et al., 1958).

2.3.5 Ash Values (Anonymous, 1996)

2.3.5.1 Total ash
Total ash is produced by incinerating the drug at the temperature possible to remove all of the carbon. A higher temperature may result in the changes carbonates to oxides. Total ash generally consists of phosphates, carbonates, silica and silicates which includes both physiological ash and non-physiological ash. e.g., sand and soil. About 2 g of air-dried powdered drug was accurately weighed and taken in a silica crucible and incinerate at a temperature not more than 450°C until free from carbon. The crucible was cooled, weighed and %age of total ash was evaluated with reference to the dried drug.

2.3.5.2 Water soluble ash
Water-soluble ash is that part of the total ash portion which was soluble in water. Then total ash obtained was boiled, about 5 minutes with 25 ml of water and insoluble material was collected in an ashless filter paper, incinerated at a temperature not exceeding 450°C, subtracted the weight of the insoluble substance from the weight of the ash and computed the rate of water soluble ash amid reference to their dried drug.

2.3.5.3 Acid-insoluble ash
The acid insoluble ash is calculated by treating, the total ash with dilute HCl and weighing the residue. This limit particularly indicating contamination with siliceous materials such as earth and sand by comparison with the total ash value for the same sample differentiation can be made between contaminating material and in the natural ash of the drug. The total ash obtained, by boiling with 25 ml of 2 N HCl for 5 min, the insoluble matter was collected in an ashless filter paper, wash with boiled water, ignited, cooled in dessicator and weigh. The proportion of acid-insoluble ash with reference to the dried drug was evaluated.
2.3.5.4 Sulphated ash
1 gm of air dried powder drug was treated with dilute sulphuric acid before ignition in a tared silica crucible to a constant weight. The ash obtained was weighed. The percentage of sulphated ash was intended with reference to the dried drug.

2.3.6 Extractive Value (Anonymous, 1996)
Extractive value is used as an evaluating crude drug which are not readily estimated by other means. It is employed for that material for which no suitable chemical or biological assay method exist.

2.3.6.1 Petroleum ether extractive
Accurately weighed 5 gm of the air dried powdered plant material was soaked in 100 ml of pet. ether (60-80°C), in a stoppered flask for 24 hrs. The mixture was vigorously shaken at regular intervals. After 24 hrs the solution was filtered without any loss of solvent. Then from the filtrate about 25 ml of solution was taken in a flat bottomed shallow disc and evaporated at 100°C till it was completely dried and also, weighed. The rate of pet. ether solvent extractive was computed with reference to air dried plant material.

2.3.6.2 Chloroform extractive
Accurately weighed 5 gm of the powdered plant drug was macerated by 100 ml of chloroform in a stoppered flask for 24 hrs. The mixture was vigorously shaken at regular intervals. After 24 hrs the solution was rapidly filtered without any loss of solvent. Then from the filtrate about 25 ml of solution was taken in a flat bottomed shallow disc and evaporated at 100°C till it was completely dried and weighed. The rate of chloroform solvent extractive was computed with reference to air dried plant material.

2.3.6.3 Ethyl acetate extractive
Accurately weighed 5 gm of the powdered plant drug was macerated through 100 ml of ethyl acetate in a stoppered flask for 24 hrs. The mixture was vigorously shaken at regular intervals. After 24 hrs solution was rapidly filtered without any loss of solvent. Then from the filtrate about 25 ml of solution was taken in a flat bottomed shallow disc and evaporated at 100°C till it was completely dried and weighed. The rate of ethyl acetate solvent extractive was computed with reference to air dried plant material.
2.3.6.4 Ethanol extractive
Accurately weighed 5 gm of the powdered plant drug was macerated by 100 ml of ethanol in a stoppered flask for 24 hrs. The mixture was vigorously shaken at regular intervals. After 24 hrs the solution was rapidly filtered without any loss of solvent. Then from the filtrate about 25 ml of solution was taken in a flat bottomed shallow disc and evaporated at 100°C till it was completely dried and weighed. The rate of ethanol solvent extractive was computed with reference to air dried plant material.

2.3.6.5 Water extractive
Accurately weighed 5 gm of dried powdered plant drug was macerated amid 100 ml of water in a stoppered flask for 24 hrs. The mixture was vigorously shaken at regular intervals. After 24 hrs the solution was rapidly filtered without any loss of solvent. Then from the filtrate about 25 ml of solution was taken in a flat bottomed shallow disc and evaporated at 100°C till it was completely dried and weigh. The percentage of aqueous soluble extract was calculated with reference to dried plant material.

2.3.7 Determination of Crude Fiber Content
2 gm of powdered drug extracted with diethyl ether and added 200 ml of boiling dilute sulphuric acid (1.25%) to the ether exhausted marc in a 500 ml flask. The mixture was refluxed for 30 min, filtered through filter paper and the residue was washed with boiling water until the effluent washing was acid free. Rinsed the residue and placed back into the flask with 200 ml of boiling sodium hydroxide solution (1.25%) and refluxed the mixture again for 30 min., filtered through ashless filter paper and washed the residue with boiling water until the last washing was neutral. It was then dried at 110°C to constant weight and then ignited to constant weight. The ash was cooled in dessicator, weighed and calculated as follows (Knevl and Digangi, 1977).

\[
\text{Percentage of Crude Fibre} = \frac{\text{Weight of the ash obtained}}{\text{Weight of the drug sample}} \times 100
\]

2.3.8 Loss on Drying
This parameter is used to determine the amount of moisture present in a particular sample. The powder (2 gm) sample was placed on a tared evaporating dish. The evaporating dish was dried at 105 ± 1°C until constant weight and weighed. The drying was continued until two successive readings match each other (Anonymous, 1996).
2.3.9 Determination of Swelling Index (WHO, 1998)
Swelling properties of medicinal plants shows specific therapeutic utility e.g. gums, pectin, or hemicellulose. One g of plant material was accurately weighed, placed into 25 ml glass stoppered measuring cylinder. 25 ml water was added and shaken the mixture thoroughly in every 10 min for one hr and allowed stand for 3 hrs at room temperature. Measured the volume in ml occupied by plant material and calculated the mean value of individual determination, related to one gm of crude plant material.

2.3.10 Foaming Index Determination (Anonymous, 1996)
The medicinal plant materials contain saponins that cause the persistent foam formation when a water decoction is stunned. The foam forming capability of plant material and their extract is measured in term of foaming index. 1 gm of powdered root was accurately weighed and transferred in to a 500 ml conical flasks containing 100 ml water and boiled for 30 min, cooled and filtered into 100 ml volumetric flask and made the volume with water. The decoction was poured into ten stoppered test tubes in consecutive part of 1 ml; 2 ml; etc up to ten ml and adjusted the volume of each test tube with water to 10 ml and shaken them in lengthwise motion for 15 sec. Allowed to stand for 15 min and measured the height of the foam. The results were assessed as follows:
If height of foam in every tube was less than 1 cm the foaming index was considered less than 100. If height of the froth was higher than that of 1cm in every tube the foaming index was over than 1000. In such case repetitions was done by using a new series of dilutions of decoction in order to obtain the result. If height of foam in any test tube was 1 cm, and volume of the crude plant material decoction in that tube (a) was used to determine the index.

Formula used for calculation of foaming index = \[
\frac{1000}{a}
\]

a = Volume of decoction that was used for preparing the dilution in tube where foaming height was 1cm measured.

2.3.11 Determination of Tannins
Tannins are complex chemical compounds. They are occurring as mixture of polyphenols that are difficult to isolation and crystallize. They are capable of turning animal hide into pelt by binding proteins to form aqueous insoluble substances that are highly defiant to proteolytic enzyme. Powdered root (2 gm of each root) was
accurately weighed and placed into conical flask. Added 150 ml of distilled water and heated over boiling water for 30 min, cooled, transferred the mixture to 250 ml volumetric flask and diluted to volume with water. Allowed the solid material to settle down and filtered the liquid through filter paper, discarded the first 50 ml of filtrate. Evaporated 50 ml of extracts of root, to dryness, dried the residue in an oven at 105 °C for 4 h and weighed (T1). Took 80 ml of root extract, added 2 g of hide powder and shaken for 1 h. Filtered and evaporated 50 ml of clear filtrate to dryness. Dried the residues in an oven at 105 °C and weighed (T2). This is the amount of plant material that does not bind to hide powder. 2 g of hide powder was dispersed in 80 ml of water and shaken well for 1 hr. Filtered and evaporated 50 ml of clear filtrate to dryness. Dried the residues in an oven at 105 °C and weighed (T0).

Formula used for calculation of tannins percentage: \[ \text{Percentage of Tannins} = \left( \frac{T1 - (T2 - T0)}{W} \right) \times 500 \]

Where \( W = \) the weight of the crude plant material 2 gm (Anonymous, 1996).

**2.3.12 Determination of Bitterness Value**

Medicinal plant materials have a strong bitter taste and act as appetizing agents. The bitter properties of plant materials are calculated by comparing the highest bitter amount of a plant extract of the materials with that of a quinine hydrochloride dilute solution.

**2.3.12.1 Stock and diluted quinine sulphate solutions**

Accurately weighed 0.1 g quinine hydrochloride (R) was dissolved in safe drinking water to produce 100 ml. 5 ml of this solution was further diluted up to 500 ml by means of safe consumable water. This quinine hydrochloride (\( S_q \)) stock solution of contained 0.01 mg/ml. Nine serial dilutions were made each containing 0.042, 0.044, 0.046, 0.048, 0.050, 0.052 0.054, 0.054 and 0.0.54 and 0.058 ml solution of \( S_q \) and volume made up to 10 ml with safe drinking water and obtaining a concentration of 0.1, 0.2, 0.3 up to 1 milli gram/ml.

**2.3.12.2 Plant Materials Stock and diluted solutions** The stock solution was prepared of the concentration of 10 mg/ml in distilled water (\( S_T \)). 10 test tubes were used for serial dilution with 1 ml, 2 ml, 3 ml to 10 ml of (\( S_T \)) and final volume made up with safe drinking water to 10 ml.
2.3.12.3 Method
First of all washing the mouth with safe drinking water, 10 ml of the most weaken arrangement was tasted while twirling it in the mouth fundamentally close to the base of tongue 30 sec. After 30 sec the solution was spit out and it was ascertained for 1 min whether a delayed sensation for bitterness existed. Then mouth was rinsed water and after that highest concentration was not tasted until at least 10 min. The lowest concentration at which material continues to rouse a bitter feeling after 30 sec was referred the threshold bitter concentration. After a first series of test, rinsed the mouth systematically wash with safe consumption of water until no bitter feeling remains, wait at least 10 min before carrying out second test.

Formula used for bitterness calculation = \[\frac{2000 \times C}{A \times B}\]

Where \(A\) = Concentration of stock solution \(S_d\) mg/ml, \(B\) = Volume of \(S_T\) milli litre tube with bitter concentration, \(C\) = amount of quinine hydrochloride (in mg) tube with threshold bitter concentration (Anonymous, 1996).

2.3.13 Determination of Haemolytic Activity
Haemolytic activity of plant material is carried out for detection of saponins. It is determined by comparison of plant material extract and reference material saponin which has activity of 1000 unit per g. The erythrocyte suspension was prepared by one tenth of its volume with sodium citrate (36.5 g/L) filling in a glass stoppered flask. Sufficient volume of blood freshly collected from healthy rat was introduced to it and shaken immediately. 1 ml of citrated blood was further diluted with 50 ml phosphate buffer of pH 7.4. Reference solution was freshly prepared by dissolving 10 mg glycyrrhizinic acid, (Himedia) in phosphate buffer pH 7.4 to make 100 ml.

2.3.13.1 Preliminary test
The alcoholic and aqueous extract (1 g) of root 0.1 ml, 0.2 ml, 0.5 ml and 1ml were taken and adjusted the volume in each tube with phosphate buffer to 1 ml. In each tube 1 ml of 2% blood suspension was added. Gently inverted to mix the tubes, to avoid the formation of foam. Tubes were shaken after 30 min interval. Then allowed to stand for 6 h at room temperature. Examined the tubes and recorded the dilution at which total haemolysis had occurred, as indicated by clear, red solution. The alcoholic extract of root has shown haemolytic activity in highest concentration i.e. 1 ml. Therefore further dilutions were done as follows.
A serial dilution of alcoholic extract of root was prepared by using 13 test tubes in a concentration of 0.40, 0.45, and 0.50 up to 1 mg/ml and adjusted the volume in each tube with phosphate buffer to 1 ml. 1 ml of 2% blood suspension was added in each tube. Tubes were observed for haemolysis after 24 h. A serial dilution of glycyrrhizinic acid was prepared in the same manner. Calculated the quantity of glycyrrhizinic acid (g) that produces total haemolysis.

The haemolytic potential of the plant material calculated using the following formula:

\[
\text{Haemolytic activity} = \frac{1000 \times a}{b}
\]

1000 = the haemolytic activity of saponin (R) which is defined.

\( a \) = amount of saponin (R) that produce total haemolysis.

\( b \) = amount of plant extract that produce total haemolysis (g) (Anonymous, 1996).

2.3.14 Determination of Microbial Count (Anonymous, 1996)

2.3.14.1 Total viable aerobic bacterial count

Culture media

**Soybean casein digest agar medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of casein</td>
<td>=15 gm</td>
</tr>
<tr>
<td>Papaic digest of soybean meal</td>
<td>=05gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>=05gm</td>
</tr>
<tr>
<td>Agar</td>
<td>=15gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

Mixed all the contents and sterilized it by autoclaving at 121°C. Adjusted the pH to 7.3 ± 0.2.

**Soybean casein digest medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of casein</td>
<td>=17gm</td>
</tr>
<tr>
<td>Papaic digest of soybean meal</td>
<td>=03gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>=05gm</td>
</tr>
<tr>
<td>Dibasic potassium phosphate</td>
<td>=2.5gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>=2.5gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

All The ingredients were dissolved in distilled water and warmed slightly. After cooling it to room temperature, the pH was adjusted to 7.1 ± 0.2. It was sterilized by autoclaving at 121°C for 30 minutes.

**Method**

Each Sample (10ml) in separate was transferred to 100 ml of SCDM and mixed well in an incubator shaker at 125 rpm for 1-4 hours, for revivification of microorganisms. 1 ml of sample was pipetted out from SCDM broth medium into pre-sterilized petri-
plates (180°C for 2 hours) and 15-20 ml of soybean casein SCDAM was added. The contents were mixed properly for uniform distribution and the SCDAM plates were incubated in a bacteriological incubator at 35°C for 48-96 h. After incubation total number of bacterial colonies was counted using colony counter and CFU / ml was calculated using the following formula:

\[
CFU / ml = \frac{\text{Total counted colony on agar plates} \times \text{dilution}}{\text{Initial sample weight taken}}
\]

2.3.14.2 Determination for *E. coli*

**Culture media**

*Macconkey agar medium*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of gelatin</td>
<td>= 17gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>= 3gm</td>
</tr>
<tr>
<td>Lactose</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>= 5gm</td>
</tr>
<tr>
<td>Bile salts</td>
<td>= 1.5gm</td>
</tr>
<tr>
<td>Agar</td>
<td>= 13.5gm</td>
</tr>
<tr>
<td>Neutral red</td>
<td>= 30gm</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>=1.0gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000ml</td>
</tr>
</tbody>
</table>

The ingredients were boiled in water for 1 minute to affect solution and after adjustment of pH to 7.1 ± 0.2, sterilization was done.

*Macconkey broth medium*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of gelatin</td>
<td>= 20gm</td>
</tr>
<tr>
<td>Lactose</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Dehydrated ox bile</td>
<td>= 5gm</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s 1000 ml</td>
</tr>
</tbody>
</table>

The adjustment of pH to 7.3 ± 0.2 was done and it was sterilized.

**Method**

Aseptically 10ml of sample was transferred to 100ml lactose broth/soyabean casein digest broth medium and the media was incubated at 37°C for 24 h. The flask was examined for growth and the contents were mixed by gentle shaking. 1 ml of the
enriched culture was pipetted into the tubes containing 10ml MacConkey's broth and incubated at 35°C for 26 h. Concomitantly, streaking on the surface Mac Conkey's agar medium was done using a loopful of enriched culture and the plates were incubated at 37°C for 24 h.

2.3.14.3 Determination for *Salmonella typhi*

**Culture media**

*Selenite F broth*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>= 05gm</td>
</tr>
<tr>
<td>Lactose</td>
<td>= 02gm</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Sodium hydrogen selenite</td>
<td>= 04gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

All ingredients were boiled in water for 1 min to affect solution and after adjustment of pH to 7.1 ± 0.2, sterilization was done.

**Method**

1.0 ml of the enriched culture was added to the tubes containing 10ml of selenite F broth. The tubes were incubated at 35°C for 48 hours and observed for the presence of turbidity.

2.3.14.4 Total fungal count

**Culture media**

(i) *Soyabean casein* digest agar medium

(ii) *Soyabean casein* digest medium

**Method**

The culture media were prepared as above and 10ml of each sample was transferred to 100 ml of SCDM and mixed well for 1-4 in incubator shaker at 125 rpm for revivification of microorganism. 1 ml of sample was pipetted out from SCDM broth medium into pre-sterilized petri plates (180°C for 2 h) and 15-20 ml of SCDAM was added. The contents were mixed properly for uniform distribution and the SCDA plates were incubated in BOD incubator at 25°C for 5-7 days. After incubation total number of fungal colonies was counted with the help of colony counter and CFU per ml was calculated using formula:
MATERIALS AND METHODS

Phytochemical, Pharmacological Evaluation and Standardization of Selected Medicinal Plants for Anti-inflammatory and Anti-arthritic activity

2.3.14.5 Determination for *Pseudomonas aeruginosa*

Culture media

*Cetrimide agar medium*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of gelatin</td>
<td>= 20gm</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>= 1.4gm</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Cetrimide</td>
<td>= 0.3gm</td>
</tr>
<tr>
<td>Agar</td>
<td>= 13.6gm</td>
</tr>
<tr>
<td>Glycerin</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

The ingredients were boiled in water for 1 minute to affect solution and after adjustment of pH to 7.1 ± 0.2, sterilization was done.

**Method:** 1.0 ml of the culture was added to the plates containing cetrimide agar media, mixed and incubated at 35°C to 37°C for 24 to 48 hours and observations for microbial growth were made.

2.3.14.6 Determination for *Staphylococcus aureus*

Culture media

*Vogel Johnson agar medium (VJA)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of casein</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>= 5 gm</td>
</tr>
<tr>
<td>Mannitol</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Dibasic potassium phosphate</td>
<td>= 5gm</td>
</tr>
<tr>
<td>Lithium chloride</td>
<td>= 5gm</td>
</tr>
<tr>
<td>Glycine</td>
<td>= 10gm</td>
</tr>
<tr>
<td>Agar</td>
<td>16</td>
</tr>
<tr>
<td>Phenol red</td>
<td>25</td>
</tr>
<tr>
<td>Water</td>
<td>q.s. 1000 ml</td>
</tr>
</tbody>
</table>

All ingredients were made in to solution by heating and cooled to approximately 45°C & 20 ml of one precent w/v solution of potassium tellurite was added to it. The pH was adjusted to 7.4 ± 0.2 and the contents were sterilized.

**Method**

Enriched culture was streaked on the surface of VJA media and incubated at 35°C for 24 hours and observed for the presence of growth.
2.3.15. Paper Partition Chromatography of Amino Acids (Das, 2005; Evans, 2006; Sharma and Ali, 1992).

Amino acids are the fundamental units of proteins. The proteins are found in every alive cell. The amino acids which can be synthesized by the living cells are called non-essential amino acids, while those which cannot be synthesized are called essential amino acids and must be supplied by diet. The essential amino acids are Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine and Valine. The Non-essential amino acids are Alanine, Aspartic acid, Cysteine, Glutamic acid, Glycine, Proline, Tryptophan, Glutamine, Asparagine, Serine and Tyrosine etc.

Carbohydrates are the most abundant organic molecule in nature. These are the carbon compounds that contain large quantities of hydroxyl groups. The simplest carbohydrates also contain either an aldehyde moiety or a ketone moiety. All carbohydrates can be classified as either monosaccharides, oligosaccharides or polysaccharides e.g. Ribose, Ribulose, Xylulose Glucose, Galactose, Mannose, Fructose, Erythrose (Styler, 1997).

2.3.15.1 Preparation of extract

Powdered (10 g) roots of *F. lacor* and *M. koenigii* were weighed and macerated with 100 ml of water and left overnight. The supernatant clear liquid was filtered. The extraction was repeated for three consecutive days so as to exhaust the root of all water soluble extractives. The combined filtrates were concentrated on a water bath and the proteins precipitated by addition of alcohol (95%) were washed with ethanol to remove unbound amino acids. The mother liquor obtained after removing the proteins was concentrated for detection of amino acids in Free State and carbohydrates

2.3.15.2 Paper chromatography

Chromatographic Whatman paper number one sheets (Qalligens) were used for paper chromatography. The starting line was marked two centimeter above from the base. To obtain the desired concentration of the extract on the paper, the spots were applied repeatedly at the same point. The spots were kept at a distance of two centimeter apart for the amino acid identification

The solvent systems used were n-Butanol: Acetic acid: Water (4:1:1) (BAW) for amino acids and *n*-butanol: glacial acetic acid: water (2:1:1) for carbohydrates.
The chamber was saturated in 16 h prior the experiment with respective solvent systems. Care was taken so as not to touch the paper with fingers. The papers were developed in descending manner. Air dried chromatograms were sprayed with 0.2% w/v solution of Ninhydrin in acetone for amino acids and aniline hydrogen phthalate for carbohydrates, heated at 110°C in oven. Pink to violet colour were visualized for amino acids and yellow to dark brown for carbohydrate.

\[ R_f = \frac{\text{Distance travelled by the solute from the start}}{\text{Distance travelled by the solvent from the start}} \]

2.3.16 Preliminary Phytochemical Screening (Rangari, 2000)

2.3.16.1 Preparation of the extract

Around 20 g of air dried powdered roots were extracted with ethanol in a soxhlet apparatus for 72 hrs and watery concentrate was arranged by maceration with refined water for 24 h to get the fluid concentrate. Concentrated ethanol and watery concentrate in revolving vaccum evaporator and unrefined ethanol concentrate was fractioned viz. petroleum ether, chloroform, ethyl acetic acid, ethanol and aqueous. The concentrates were screened for the presence of different phytoconstituents.

2.3.16.2 Test for alkaloids

A little portion of the dissolvable free petroleum ether, chloroform, ethyl acetic acid derivation, ethanol and water extricates independently with a couple of drops of dilute hydrochloric acid and filter. The filtrates were tried with different alkaloidal reagents, for example, Mayer's reagent (cream encourage), Wagner reagent (reddish brown precipitate) and Dragendorff's reagent (orange brown precipitate).

Mayer's reagent: Mayer's reagent few drops of were included every concentrate and watched development of the white or cream hued precipitates.

Dragendorff's reagent: Dragendorff's reagent few drops of were included every concentrate and development of the orange yellow or brown hued ppt.

Wagner reagent: Wagner reagent Few drops of were included every concentrate and watched development of the red colored ppt.

2.3.16.3 Test for carbohydrates: Dissolve little amounts of alcoholic and watery extracts, independently in 4 ml of refined water and filter. The filtrate may be subjected to different tests to detect the existence of carbohydrates.
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Molisch's Test: To around 2 ml of concentrate couple of drops of α-naphthol (20% in ethanol) were included. At that point around 1 ml of concentrated sulphuric acid was included at the edge of the tube. In the event that red violet ring showed up at the intersection of two layers. It showed the vicinity of carbohydrates.

*Fehling’s Test*: 1 ml of copper sulphate in alkaline conditions (Fehling’s reagent) was included to the filtrate of the root separate in refined water and warmed in a steam shower. Brick red precipitates showed up it sugars are present.

### 2.3.16.4 Test for glycosides

Hydrolysed another little part of the concentrate with weaken hydrochloric acid for couple of hrs in water bath and subjected the hydrolysate with borntrager's, and liebermann-burchard's, keller-killani tests to recognize the presence of glycosides.

Keller-Killani Test: 1 ml of glacial acetic acid containing hints of FeCl3 and 1 ml of concentrated H2SO4 was added to the concentrate deliberately. Appearance of shading showed up which affirmed the vicinity of glycosides in the root extricates.

Borntrager's test: 1 ml of benzene and 0.5 ml of dilute alkali solutions were poured in the extract. dark brown shading which shows the vicinity of glycosides in the extract.

### 2.3.16.5 Test for phenolic compound and tannins

Taken little amounts of different concentrates independently in water and test for the vicinity of phenolic mixes and tannins with weaken ferric chloride arrangement (5%) and lead acetic acid test.

Ferric chloride test: Added ferric chloride solution (5%), if green/ blue colour was seen in all the segments it was because of the vicinity of phenolic components. Colour showed up which demonstrate the vicinity of phenolic compound.

Lead acetic acid vation test: Added few drops of lead acetic acid solution (5%) were added to the different extract of the root. The appearance of white ppt affirm the vicinity of phenolic components.

### 2.3.16.6 Test for flavonoids

Ammonia test: Filter paper strips were dunked in the different extracts and ammoniated. The filter paper transformed its colour to yellow which demonstrates the vicinity of flavonoids.

Pew test for flavonoids: To 1 ml of the every concentrates, a bit of metallic magnesium/zinc was included trailed by expansion of 2 drops of concentrated
hydrochloric acids. An brown shading the vicinity of flavonoids in the different extracts.

2.3.16.7 Test for proteins and free amino acids
Added a couple of drops of various extracts in a couple ml of refined water and subjected to ninhydrin and million’s and biuret tests.

Millon’s test: To 2 ml of extract filtrate, few drops of million’s reagent was added. A red colour affirms the vicinity of proteins and free amino acids.

Biuret test: In the ammoniated filtrate few drops of 0.02% copper sulphate solution was included. A red colour confirms the vicinity of free amino acids and proteins.

Ninhydrin test: To each of the extract filtrate, lead acetate solution was included to accelerate tannins and filtered. The Filtrate was spotted on a paper chromatogram, splashed with ninhydrin reagent and dried at 110o C for 5 minutes. Violet spots if seen affirmed the vicinity of proteins and free amino acids.

2.3.16.8 Test for saponin

Foam test: Dilute 1 ml of alcoholic and fluid concentrates independently with refined water to 20 ml and shake in a graduated barrel for 15 minutes. An one centimeter layer of froth shows the vicinity of saponin.

Sodium bicarbonate test: To the couple of milligrams of concentrate couple of drops of sodium bicarbonate were included and shaken well. Arrangement of honeycomb like foaming shows positive test for saponins.

2.3.16.9 Test for phytosterol and triterpenes

Liebermann-Burchard's test: The hydro-alcoholic concentrate was shaken with chloroform and couple of drops of acidic anhydride were included chloroform extricate alongside a couple of drops of concentrated sulphuric corrosive from the side of the tube. The presence of blue to block red shading shows the vicinity of sterol and triterpenes.

Hesse's response: The deposit was broken down in chloroform (4 ml) and equivalent amount of concentrated sulphuric corrosive was then at the edge of the tube. The arrangement of the pink hued ring, which is on shaking diffused in both the layers, demonstrated the vicinity of sterols in the concentrate.

2.4 Column chromatography: Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or
the support coated with a liquid stationary phase may fill the whole side volume of the tube (packed column) corning glass column was used for the present study.

**Extraction:** The dried powdered aerial root of *Ficus lacor* (2.5 kg) was subjected to hot continuous extraction with ethanol using soxhlet apparatus for 72 h till solvent became colourless. The liquid was concentrated in rotary vacuum evaporator. A thick dark brownish viscous mass (100 g) was obtained.

The dried powdered root of *Murraya koenigii* (2 kg) was subjected to hot continuous extraction with ethanol using soxhlet apparatus for 72 h till solvent became colourless. The liquid was concentrated in rotary vacuum evaporator. A thick dark brownish viscous mass (85 g) was obtained.

**Packing of the column:** Silica gel for column (100-120 mesh size)

**Mobile phase:** Solvent system employed for column in increment of polarity were pure petroleum ether, petroleum ether: chloroform (19:1), (9:1), (3:1), (1:1), pure chloroform, chloroform : methanol 19:1), (9:1), (3:1), (1:1), methanol.

**2.5 Pharmacological screening** (Ghose, 1984; Kulkarni, 1999)

The various extracts and fractions were subjected to pharmacological screening to identify fractions responsible for anti-inflammatory and anti-oxidant activities. Screening was done by using various models to identify the mechanism of action.

**2.5.1 Determination of LD$_{50}$ value and acute toxicity:** (Kulkarni, 1999)

Rats in groups of 6 were administered intraperitonealy with different doses of the fractions from the three drugs by the staircase method, starting from 10 mg/kg and increasing dose by a factor 1.5 if there was no mortality and decreasing subsequent dose by a factor .7 in case there was mortality (Ghosh 1984). Least tolerated (100 % mortality) and most tolerated (0 % mortality) were determined by hit and trial method for various extracts and fractions. Corrections for 0% and 100% mortality were done by the formulas:

$100 (0.25/n)$; For 0% mortality: and, $100 (n - 0.25 / \sqrt{n})$; For 100% mortality.

S.E. of LD$_{50}$ = Log Dose with highest mortality- Log Dose with lowest mortality/ n

Doses were selected between these two and any mortality observed for 24 hrs and the number of deaths noted. A curve of log dose versus probit value was plotted to get dose for probit value 5 which was taken to be LD$_{50}$. Dose range well below LD$_{50}$ was selected for study.

**Dose planning and Grouping of animals:**

Doses equivalent of or less than 1/10$^{th}$ of the doses corresponding to LD$_{50}$ values and producing no gross behvioural changes or cvs changes were used. Fractions showing
no CNS or CVS effects even at higher doses were used in wider dose ranges as compared to fractions which showed some changes at higher doses. For the later fractions a narrower dose range (10, 20, 40, 60, 80 and 100mg/kg) was used for study of the dose response relation for different parameters. For other fractions 10, 20, 50, 100, 120 and 150 mg/kg was the dose gradient used to study the dose response relationships.

Grouping:
In most models, the animals were divided into groups on the following basis:
1. Vehicle treated Normal control Group
2. Untreated diseased control Group
3. Reference group treated with standard drugs
4. Test Groups depending on number of doses and fractions for study.

2.5.2 Screening for anti-inflammatory and anti-arthritic Activity
2.5.2.1 Inhibition of Carrageenan induced, paw edema in rats (Winter et al 1962):
Rats were divided into groups of 06 each (120-150gm).
1. Normal saline treated control
2. Untreated diseased animals
3. Reference group treated with indomethacin before carrageenan
4. Experimental Groups
Control group I was given normal saline one hour before the carrageenan infusion. Experimental groups were given doses of different portions in 0.5ml of ordinary saline, infused intraperitonealy one hour prior to infusion of 0.1 ml of 1% carrageenan arrangement in the right rear paw under the plantar aponeurosis (s.c) for affectation of edema. The volume of paw edema was controlled by Plethysmometer and a measurement reaction relationship was built for both oral and i.p. dosage and a connection built between i.p. furthermore, oral dosages delivering most extreme mitigating impact. Reference gathering was given Indomethacine 2.5 mg/kg 1hr preceding the carrageenan infusion. Rate restraint of edema in respect to a control gathering was ascertained as depicted by Winter et al.

2.5.2.2 Inhibition of histamine and serotonin induced paw edema in rats (Singh et al 1996).
In another set of experiments serotonin and histamine (0.1ml 0f 1mg/ml of both) were used as phlogistic agents. Groups of 6 animals were made as earlier;
1. Vehicle treated normal control
2. Untreated diseased animals
3. Reference group treated with indomethacin before carrageenan
4. Experimental Groups depending on the number of extracts and fractions.

The extract, various fractions, standard pyrilamine and control vehicle (arrangement of 2.5% DMSO and 2.5% Tween 20) were regulated intraperitonealy one hour prior to the infusion of incendiary arbiters in their particular gatherings. Various doses of concentrate or fractions were infused intraperitonealy in vehicle to discover dosage reaction relationship. 0.1ml Serotonin (1mg/ml) or histamine (1mg/ml) was infused and reaction noted at 30 mins for Serotonin and 60 mins for Histamine bunches. Pyrilamine maleate (1 mg/kg) was utilized as the enemy (reference) of histamine and as a standard medication in the reference bunch. The volume of paw edema was dictated by plethysmometer.

2.5.2.3 Inhibition of formalin induced paw edema in rats (Hosseinzadeh and Younesi 2002)
Animals were separated into groups of six each group. Acute inflammation was actuated by subaponeurotic infusion of 0.1 ml of 2% formalin one hour after i.p. organization of different doses of extracts or fractions, Diclofenac (5 mg/kg), or just vehicle (solution of 2.5% DMSO and 2.5% tween 20) in individual gatherings. The volume of paw was resolved one, two, and four hours taking after the infusion of formalin by plethysmometer. For ceaseless aggravation mull over, the above creatures were further treated with the parts, Diclofenac or vehicle, once day by day, for 9 sequential days and a second infusion of formalin was given on the third day. The every day changes in the volume of paw were measured plethysmographically.

2.5.2.4 Inhibition of Adjuvant induced arthritis in rats (Newbould 1963)
Animals were divided into groups of 6 each group. Creatures were separated into gatherings of 6 each as prior. Joint inflammation was affected by intradermal infusion of 0.5 ml of a 5 mg/ ml suspension of warmth executed Mycobacterium tuberculosis in fluid paraffin into the plantar surface of the rear paws. The clinical highlights of adjuvant actuated joint pain (AIA) showed as erythema, induration and edema, and displayed in various joints as takes after: (a) onset: clinical signs around days 8–10; (b) early stage: dynamic seriousness of the clinical signs through the following 7–10 days; and (c) late stage: unconstrained relapse amid the following 10–14 days.
Every one of the four paws were inspected and evaluated for seriousness and loci of
the joint injuries (erythema, swelling and induration) which grew on the paws by the
15th day and surveyed on a subjective 5-point size of ligament score (0-4). Rats were
evaluated day by day for indications of joint inflammation upto 28th day post-CFA.
The maximal ligament score every rodent was situated at 16 (greatest of 4 points× 4
paws), where;
0 = no indications of illness;
1 = signs including the lower leg/wrist;
2 = signs including the lower leg in addition to tarsals (proximal piece of the rear
paw) and/or wrist in addition to carpals of the forepaw;
3 = signs reaching out to the metatarsals or metacarpals;
Furthermore, 4 = extreme signs including the whole rear or fore paw.
Animals with affirmed joint pain were differentiated into group of 6, with comparable
mean ligament scores and infused with various fractions, at different doses of
fractions or reference drug (indomethacin 2.5 mg/kg) intraperitonealy in two dosages
(one measurement relating to ED50 dosage for portions as decided for hindrance of
carrageenan induced inflammation, (or 20 mg/kg) and a higher dosage well beneath
the poisonous measurement, which was 100-150 mg/kg for different parts) day by day
for next 7 days from 15th day onwards. Mean joint scores got for every after a long
time for treated gatherings were contrasted and fitting scores of the control group.
The progressions in body weight were recorded on 7th, 14th and 21st day and toward
the end of 28th day. Rats were yielded on the 29th day by beheading. The blood was
gathered and biochemical parameter like hemoglobin substance, RBC, WBC and ESR
measured. Plasma was differentiated from the blood gathered with EDTA. IL-1 and
TNF-α measured. Promptly in the wake of yielding, liver, kidney and spleen were
divided, organ weight changes on 28th day & homogenized enzyme estimation.

2.5.2.5 Lysosomal enzyme inhibitory activity (Geeta and Varalaxmi 1999)
It was performed along with determination of adjuvant arthritis inhibition studies. As
described, rats were divided into groups of 6. Reference control group was given
Indomethacin (2.5 mg/kg)

Enzyme assays: The activities of lysosomal enzymes were investigated in liver,
plasma, kidney and spleen.
Acid phosphatase: It was measured by the method of King (1965), on the basis of the action of the enzyme on disodium phenyl phosphate substrate mixture containing 1.5ml 0.01 M substrate, 1.5ml 0.1M citrate buffer pH 4.8 and 0.5ml tissue homogenates) to liberate phenol after incubation at 37°C for 30 mins. The reaction was then arrested by addition of 1ml 10% Trichloro acetic acid and the released phenol treated with 1 ml 15% Sod. Bicarbonate and Folin’s Phenol reagent, incubated at 37°C for 10mins and colour developed measured at 640 nm and compared to standard phenol solutions and blank treated in similar fashion. The activity of acid phosphatase was expressed as µ mol. of Phenol liberated /min/mg protein at 37°C.

2.5.2.6 Screening for Antioxidant activity:
Plants are a usual source of pharmacologically active chemical compounds are known as phytoconstituents (Farnsworth, 1994). Phytoconstituents found to go about as cell by searching free radicals, and numerous have helpful potential with the expectation of complimentary radical related ailments. Responsive oxygen species (ROS) including singlet oxygen, hydroxyl radicals, superoxide radicals and hydrogen peroxide are often produced as by results of natural response (Kikuzaki and Nakatani 1993). In any case, these ROS created by daylight, UV light, ionizing radiation and metabolic process of action have a wide range of obsessive impacts, for example, carcinogenesis, Arthritis, DNA harm and different degenerative ailments, for example, neuro-degenerative disease, maturing and cardiovascular diseases (Osawa, 1994; Noda et al. 1997).

2.5.2.6.1 Total phenolic content
The total phenolic substance in the concentrate was calculated using the Folin-Ciocalteu’s reagent (FCR) as indicated by Molan et al. 2009. Every sample (0.5 ml) was blended with 2.5 ml of FCR (diluted 1:10, v/v), and 2 ml of Na₂CO₃ (7.5%, w/v) was included. The absorbance was then measured at 765 nm after brooding at 30°C for 90 minutes. Results were communicated as gallic corrosive equivalents (mg of gallic acid/g of dried extract).

2.5.2.6.2 Total flavonoid content
Total flavonoid substance of ethanol concentrate was calculated utilizing a colorimetric process (Zhishen et al., 1999). Quickly, every specimen (0.5 ml) was blended with 2 ml of refined water and after this 0.15 ml of a NaNO₂ solution 15%, w/v. Following 6 minutes, 0.15 ml of an AlCl₃ solution 10% w/v was added and
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permitted to remain for 6 minutes, then 2 ml of NaOH solution 4%, w/v was added to the mixture. Momentarily, water was added to convey the last volume to 5 ml, and after that the mixture was precisely blended and permitted to remain for an additional 15 minutes. Absorbance of the solution was calculated on 510 nm versus arranged blank water. Results were shown as catechin equivalent (mg of catechin/gm of dried concentrate).

2.5.2.6.3 Free radical scavenging activity

Scavenging potency of diphenyl- 2-picrylhydrazyl (DPPH) radicals of ethanol extract or catechin was preformed according to the method reported by Molan et al., 2009, with minor modifications. Assays process was performed in 3 ml of reaction mixtures composed of 2.0 ml of 0.1 mM DPPH ethanol solution, 0.9 ml of 50 mM Tris-HCl buffer (pH 7.4) and 0.1 ml deionized water (as control) or test plant concentrate. Following 30 minutes of standing at room temperature, absorbances of the reaction mixtures at 517 nm were noted. The inhibitory impact of DPPH was computed by following formula:

\[ \text{Percentage inhibition} = \left( \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right) \times 100\]

2.5.2.6.4 NO scavenging activity

The scavenging impact of ethanol concentrate on nitric oxide was measured according to Marcocci et al., 1994. Sodium nitroprusside (5 mM) in phosphate-buffered saline pH 7.4 was blended with diverse concentrations of the test sample (100, 200 -1000 mg/ml) and incubated at 25 ºC for 150 minutes. After incubation, nitrite delivered from sodium nitroprusside was measured by Griess reagent, one precent sulfanilamide solution in 5 precent phosphoric acid and 0.1 precent 1-naphthylethyelenediamine dihydrochloride in H₂O. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and consequent coupling with 1-naphthylethyelenediamine dihydrochloride was noted at 570 nm. Catechin used as a positive control. The percentage of NO scavenging was calculated using following formula:

\[ \text{Percentage inhibition} = \left( \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \right) \times 100\]

2.5.2.7 Histological analysis

All preparations were stored in six percent formaldehyde for 24 hours. Decalcification in ethylenediamine tetra acetic acid was subsequently done and the planning were set in paraffin.then after the exclusion of paraffin, 5-μm thick section were cutted.
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For the measurement of the histological arthritis scores, the joints sections were stain with haematoxylin and eosin. All cases of diseased and other, three sections per knee joint were evaluated using Almicro 50Hz 200AC Microscope with camera at 10 X 0.25 X magnification and scored using a semi quantitative scale.

The extent of acute joint inflammation: was defined by the quantity of infiltration of the synovial membrane by polymorph nuclear leucocytes, the exudation of granulocytes cells in the joint space, the presence or absence of fibrin exudation in the joint space and periarticular inflammation. Each parameter was scored using a 0-3 scale, where:

0 = there was no changes, 1= mild affect; 2 = moderate changes, and 3 = severe change.

A maximum total score of 12 was therefore possible for different groups for acute Inflammation.

Chronic joint inflammation was evaluated based on the hyperplasia of synovial lining cells, infiltration by mononuclear cells and fibrosis of synovial membrane or periarticular tissue. These were evaluated with a score of 0-3, resulting in a maximum total score of 9 in a particular group.

The degree of the injures to articular cartilage and adjoining bone structures was evaluated on the basis of cell necrosis, structural bone defects and cartilage defects and evaluated on a scale of 0-4, where,

Score 0=no damage, 1= <5% of the cartilage surface affected, 2 = 5-10% of the cartilage surface influenced, 3 = 10-50% of the ligament surface influenced, 4= > 50% of the ligament surface influenced (maximal aggregate score of 4).

Safranin O recoloring was performed to gauge the proteoglycan content in the ligament. Keeping in mind the end goal to acquire similar histological result all slides were recolored utilizing precisely the same system.

First and foremost, the recoloring power (red) at the epiphyseal development plate of the femoral condyle of non-joint and ligament creatures was measured. The number juggling mean got from these qualities was utilized as a kind of perspective esteem (100%). The estimations of articular ligament occurred at the most distal purpose of the arch of the femoral condyle. For every situation, qualities were gotten for the shallow layer, center layer, and profound layer of the hyaline ligament, and in addition for the calcified ligament layer. Information was communicated as a rate of the reference esteem. Accordingly, the estimations of the contra parallel, non-
ligament knee joint (left) were subtracted from the ligament knee (right), bringing about negative values on account of proteoglycan loss.

**2.5.2.8 Statistical analysis**

The data acquired from animal trials were communicated as mean standard error (±S.E.M.). Statistical differences between the treatment and the control were assessed by ANOVA and Students–Newman–Keuls post hoc tests. Significance of data was expressed as * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. 