4. MATERIALS AND METHODS

4.1 PLANT MATERIAL

The plant material was bought from Botanical Source of India, Jodhpur and powdered in grinder, to get coarse powder for extraction.

4.2 EXTRACTION AND FRACTIONATION

The plant material was extracted by soxhlet with 50% alcohol and concentrated. A fresh batch of the plant material was successively extracted with petroleum ether, ethyl acetate, n-butanol and water (in the increasing order of polarity) and was concentrated in same order in a rotary evaporator at reduced pressure.

**Extraction methodology:**

50% extract – PDE (A) \(\rightarrow\) screened for the activity (using base model)

- Inflammation – Acute, sub-acute and Chronic inflammation model
- Diuretic – urine volume and electrolyte balance

Drug powder

Petroleum ether

<table>
<thead>
<tr>
<th>Petroleum ether Fraction – PDP (A)</th>
<th>Defatted powder (dry)</th>
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<tbody>
<tr>
<td>Ethyl acetate</td>
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<tr>
<th>Ethyl soluble Fraction - PDEA (B)</th>
<th>Powered drug</th>
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<tr>
<td>Powered drug</td>
<td>n- butanol</td>
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<tr>
<th>n-butanol Fraction - PDB (C)</th>
<th>Powered drug</th>
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<tbody>
<tr>
<td>Powered drug</td>
<td>Water</td>
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Water Fraction - PDW (D)

A, B, C, and D were screened for base models. Fraction with highest activity was screened for mechanism of action.
4.3 STANDARDIZATION OF PLANT MATERIAL

The quality control of herbal crude drug and bioconstituents is of paramount importance for their acceptability in modern system of medicine. One of the major problems faced by user in industry is non availability of rigid quality control profile for herbal raw material and their formulation with advanced of analytical technique and sophisticated instrument technology; it is possible to suggest a practicable quality assurance profile for a crude drug or its bioactive constituents.

4.3.1 ASH VALUE

4.3.1.1 Determination of Total Ash: 2 to 3 gm accurately weighed leaves powder was placed in silica crucible, previously ignited and weighed. Incinerated by gradually increasing the heat-not exceeding 450°C until free from carbon, cool and crucible was weighed. Calculated the percentage of ash with reference to the air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]

4.3.1.2 Determination of Acid-insoluble Ash: Boiled the ash for 5 to 10 minutes with 25 ml of dilute hydrochloric acid. Collected the insoluble matter in a dry crucible or an ashless filter paper, washed with hot water, ignited and weighed. Calculated the percentage of acid-insoluble ash with reference to the air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]

4.3.1.3 Determination of Water Soluble Ash: Boiled the ash for five minutes with 25 ml of water, collected the insoluble matter in a dry crucible, washed with hot water and ignited to constant weight at a low temperature. Subtracted the weight of insoluble matter from the weight of ash; difference in the weight represents the water-soluble ash. Calculated the percentage or water-soluble ash with reference to air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]
4.3.2  EXTRACTIVE VALUES:

4.3.2.1 Determination of Alcohol-soluble Extractive: Coarsely powdered 5 gm of the air-dried drug macerated with 100 ml of alcohol in a conical flask for 24 hours, shaked frequently for six hours and allowed to stand for 18 hours. Filtered rapidly taking precaution against loss of alcohol evaporated 25% of the filtrate to dryness in a tarred bottomed shallow dish dried at 105°C and weighed. Calculated the percentage of alcohol soluble extractive with reference to the air-dried drug.

4.3.2.2 Determination of Water-Soluble Extractive: Proceeded as directed for the determination of alcohol soluble extractive, used chloroform water, I.P., instead of alcohol.

4.3.2.3 Loss on Drying: About 1.5 g of powder / drug was accurately weighed in a petridish and kept in a hot air oven maintained at 110°C. After cooling in dessicator, the loss in weight was recorded. This procedure was repeated till constant weight was obtained.

\[
\text{Loss on drying (\%)} = \frac{\text{Loss in weight}}{W} \times 100
\]

Where \( W \) = Weight of the leaf powder in gms.

4.4  CHARACTERIZATION OF EXTRACT AND FRACTIONS BY CHEMICAL TESTS (Khandelwal, 2006)

The extracts were tested for various chemical constituents with the help of different chemical tests.

1. Alkaloids:-
   a) Dragendorff’s test: - To the extract add Dragendorff’s reagent, reddish brown precipitate indicates presence of alkaloids.
   b) Mayer’s test: - To the extract add Mayer’s reagent, cream coloured precipitate indicates presence of alkaloids.
   c) Wagner’s test: - To the extract add Wagner’s reagent, reddish brown precipitate indicates presence of alkaloids.
   d) Hager’s test: - To the extract add Hager’s reagent, yellow precipitate indicates presence of alkaloids.
e) **Tannic acid test:** - To the extract add tannic acid solution, buff coloured precipitate indicates presence of alkaloids.

2. Amino acids :-
   a) **Millon’s test:** - To the extract add about 2ml of Millions reagent white precipitate indicates presence of amino acids.
   b) **Ninhydrine test:** - To the extract add Ninhydrine solution, boil, violet colour indicates presence of amino acid.

3. Carbohydrates:-
   a) **Molisch’s test:** -To the extract add few drops of alcoholic α-naphthol, then add few drops of concentrated sulphuric acid through sides of test tube, purple to violet colour ring appears at the junction.
   b) **Barfoed’s test:** -1ml of extract is heated with 1ml of Barfoed’s reagent, if red cupric oxide is formed, monosaccharide is present. Disaccharides on prolong heating (about 10 min.) may also cause reduction, owing to partial hydrolysis to monosaccharides.
   c) **Selivanoff’s test (Test for ketones):** -To the extract add crystals of resorcinol and equal volume of concentrated hydrochloric acid and heat on a water bath, rose colour is produced. (eg. Fructose, honey)
   d) **Test for pentoses:** -To the extract add equal volume of hydrochloric acid containing a small amount of phloroglucinol and heat, red colour is produced.

4. Flavonoids: -
   a) **Shinoda test:** - To the extract add few magnesium turnings and concentrated hydrochloric acid dropwise, pink scarlet, crimson red or occasionally green to blue colour appears after few minutes.
   b) **Alkaline reagent test:** - To the extract add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicate presence of flavonoids.
   c) **Zinc hydrochloride test:** - To the extract add a mixture of zinc dust and conc. Hydrochloric acid. It gives red colour after few minutes.
5. Glycosides: -

I. General test: -

Test A: Extract 200mg of drug with 5ml of dilute sulphuric acid by warming on a waterbath. Filter it. Then neutralize the acid extract with 5% solution of sodium hydroxide. Add 0.1ml of Fehling’s solution A and B until it becomes alkaline (test with pH paper) and heat on a waterbath for 2 minutes. Note the quantity of red precipitate formed and compare with that of formed in Test B.

Test B: Extract 200mg of the drug using 5ml of water instead of sulphuric acid. After boiling add equal amount of water as used for sodium hydroxide in the above test. Add 0.1ml Fehling’s A and B until alkaline (test with pH paper) and heat on water bath for 2 minutes. Note the quantity of red precipitate formed.

Compare the quantity of precipitate formed in Test B with that of formed in Test A. If the precipitate in Test A is greater than in Test B then Glycoside may be present. Since Test B represents the amount of free reducing sugar already present in the crude drug, whereas Test A represents free reducing sugar plus those related to acid hydrolysis of any glycoside in the crude drug.

II. Chemical tests for specific glycosides: -

A. Anthraquinone glycosides: -

a) Borntrager’s test: - Boil the test material with 1ml of sulphuric acid in a test tube for 5 minutes. Filter while hot. Cool the filtrate and shake with equal volume of dichloromethane or chloroform. Separate the lower layer of dichloromethane or chloroform and shake it with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammoniacal layer.

b) Modified Borntrager’s test: - Boil 200mg of the test material with 2ml of dilute sulphuric acid. Treat it with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, shake it with equal volume of chloroform and continue the test as above. As some plants contain anthracene aglycone in a reduced form, if ferric chloride is
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used during the extraction, oxidation to anthraquinones takes place, which shows response to Borntrager’s test.

c) Hydrolyze ether extract with methanolic magnesium acetate gives pink colour in daylight and greenish orange colour under ultraviolet (UV) light.

d) **Test for hydroxyanthraquinones:** - Treat the sample with potassium hydroxide solution red colour is produced.

B. Cardiac glycosides:

a) **Kedde’s test:** - Extract the drug with chloroform, evaporate to dryness. Add one drop of 90% alcohol and 2 drops of 2% 3,5-dinitro benzoic acid in 90% alcohol. Make alkaline with 20% sodium hydroxide solution, purple colour is produced. The colour reaction with 3,5-dinitro benzoic acid depends on the presence of α,β-unsaturated lactones in the aglycone.

b) **Keller-Killiani test** (test for deoxy sugars): - Extract the drug with chloroform and evaporate it to dryness. Add 0.4ml of glacial acetic acid containing trace amount of ferric chloride. Transfer to a small test tube, add carefully 0.5ml of concentrated sulphuric acid by the side of the test tube. Acetic acid layer shows blue colour.

c) **Raymond’s test:** - Treat the extract with hot methanolic alkali, violet colour is produced.

d) **Legal’s test:** - Treat the extract with pyridine and add alkaline sodium nitroprusside solution, blood red colour appears.

e) **Baljet’s test:** - Treat the extract with picric acid or sodium picrate, orange colour is formed.

C. **Coumarin glycosides:** - Place a small amount of sample in test tube and cover the test tube with a filter paper moistened with dilute sodium hydroxide solution. Place the covered test tube on water bath for several minutes. Remove the paper and expose it to ultraviolet (UV) light, the paper shows green fluorescence.
D. Cyanogenetic glycosides: -

a) Place 200mg of drug in a conical flask and moisten with few drops of water. (There should be no free liquid at the bottom of the flask as the test will not work because the hydrogen cyanide produced will dissolve in the water rather than come off as a gas to react with the paper). Moisten a piece of picric acid paper with 5% aqueous sodium carbonate solution and suspended by means of cork in the neck of the flask. Warm gently at about 37°C. Observe the change in colour. Hydrogen cyanide is liberated from cyanogenetic glycosides by the enzyme activity and reacts with sodium picrate to form the reddish purple sodium isopurpurate.

b) Prepare solution of Guaiacum resin in absolute alcohol and allow it to dry on paper. Treat it with copper sulphate solution. The paper turns blue due to prussic acid with HCN.

E. Saponin glycosides: -

a) Froth formation test: - Place 2ml solution of drug in water in a test tube, shake well, stable froth (foam) is formed.

b) Haemolysis test: - Add 0.2ml of extract to 0.2ml of blood in normal saline and mix well. Centrifuge and note the red supernatant Compare with control tube containing 0.2ml of 10% blood in normal saline diluted with 0.2ml of normal saline.

6. Phenolic compounds (Tannins):-

a) Ferric chloride test: - Treat the extract with ferric chloride solution, blue colour appears if hydrolysable tannins are present and green colour appears if condensed tannins are present.

b) Phenazone test: - Add about 0.5 gm of sodium acid phosphate to 5ml of extract warm it and filter. To the filtrate add 2% phenazone solution, bulky precipitate is formed, which is often coloured.

c) Gelatin test: - To the extract add 1% gelatin solution containing 10% sodium chloride. Precipitate is formed.
7. Proteins:-
   a) **Biuret test:** - To the extract (2ml) add Biuret reagent (2ml), violet colour indicates presence of proteins.
   b) **Hydrolysis test:** - Hydrolyze the extract with hydrochloric acid or sulphuric acid. Then carry out the Ninhydrine test for amino acids.
   c) **Test with trichloroacetic acid:** - To the extract add trichloroacetic acid, precipitate is formed.
   d) **Xanthoproteic test:** - To the (5ml) of extract, add 1ml of concentrated nitric acid and boil, yellow precipitate is formed. After cooling it, add 40% sodium hydroxide solution, orange colour is formed.

8. Steroids and Triterpenoids: -
   a) **Libermann-Burchard test:** - Treat the extract with few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid from the side of the test tube, brown ring is formed at the junction two layers and upper layer turns green which shows presence of steroids and formation of deep red colour indicates presence of triterpenoids.
   b) **Salkowski test:** - Treat the extract with few drops of concentrated sulphuric acid red colour at lower layer indicates presence of steroids and formation of yellow coloured lower layer indicates presence of triterpenoids.
   c) **Sulfur powder test:** - Add small amount of sulfur powder to the extract, it sinks at the bottom.
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4.5 SEPARATION AND ISOLATION OF PHYTO-CONSTITUENTS BY CHROMATOGRAPHIC METHODS: (Evans, 1997; Stahl, 1969)

HPTLC analysis

Thin-layer chromatography was performed on 10 cm×20 cm high-performance thin-layer chromatographic (HPTLC) silica gel 60 F254 plates coated with 0.25mm layer (Merck). Silica gel plates were pre-washed with developing solvent methanol. Six microliters of each standard solution and/or 7 microliters of the n-butanol test solution were applied as 6mm bands, 5mm from the bottom, 23mm from the left edge, 8mm apart by means of Linomat IV (Camag). The plate was developed using 4ml of developing solvent n-hexane-ethyl acetate (5:1, v/v) and ethyl acetate-acetonitrile (3:2) in a horizontal developing chamber (Camag) with a tank containing 15 ml of developing solvent. Developing distance was 8 cm Anisaldehyde detection reagent was prepared by mixing 20 ml acetic acid with 170 ml methanol. During cooling with ice and water, 16ml of sulfuric acid and 1ml of p-methoxybenzaldehyde were added. After developing and drying, the plate was dipped for 2 s in the anisaldehyde detection reagent using a Camag immersion device II, dried with the hair dryer and heated with Camag TLC plate heater for 2 min at 110 °C.

Preparative Thin Layer Chromatography: -

A thick layer of silica gel G was coated on the square shaped plate and activated at 110°C for one hour. The broad band (2 mm width) of ethanolic extract was applied on the plate. The details of plate were as follows-

Plate dimension: 10 x 20 cm
Adsorbent: Silica gel G
Activation: 110°C for 1 hr
Band parameter: Width-2 mm, Length-8 cm

The plate was developed in a saturated chamber having solvent ethyl acetate: acetonitrile (3:2). After developing the plate was dried and small portion of the band was identified under UV light and based on Rf value observed in HPTLC. The
scraped silica gel was suspended in alcohol and filtered on Whatmann filter paper no.1 and washed several times with alcohol. Each spot indicated presence of single spot on TLC and Rf values of each scrap was recorded. They were as follows:

Scrap No.1:  Rf = 0.20;  Scrap No.2:  Rf = 0.22
Scrap No.3:  Rf = 0.28;  Scrap No.4:  Rf = 0.31
Scrap No.5:  Rf = 0.40;  Scrap No.6:  Rf = 0.59
Scrap No.7:  Rf = 0.80

The isolated compound was then subjected I.R. spectroscopic studies for identification.

4.6 THE SPECTRAL ANALYSIS

4.6.1 UV – Visible Spectrophotometric analysis

4.6.1.1 UV – Visible Spectrophotometric analysis of n-butanol fraction

The n-butanol fraction of *P. daemia* were dissolved in methanol and scanned in entire UV-Visible range (200-800 nm) (Shimadzu UV 1800).

4.6.1.2 UV – Visible Spectrophotometric analysis of n-butanol fraction

The water fraction of *P. daemia* were dissolved in methanol and scanned in entire UV-Visible range (200-800 nm) (Shimadzu UV 1800).

4.6.1.2.1 Determination of total phenol

Total phenolic contents in the extracts were determined by the using the Folin-Ciocalteu method (Miliauskas et al., 2004). A dilute solution of PDB and PDW (0.5 ml of 1:10 g ml-1) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1M). The mixtures were allowed to stand for 15 min and the total phenols were determined by UV-VIS spectrometer at 765 nm and the content expressed in terms of gallic acid equivalent (GAE) using the following formula:

\[ C = c.V/m \]

Where:  
\[ C = \text{total content of phenolic compounds (mg/g) plant extract in GAE; } \]
\[ c = \text{the concentration of gallic acid established from the calibration curve (mg/ml); } \]
\[ V = \text{the volume of extract in ml; } \]
\[ m = \text{the weight of pure fraction in g} \]
4.6.1.2.2 Determination of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination (Chang et al., 2002). PDB and PDW (0.5 ml of 1:10 g ml-1) in methanol was separately mixed with 1.5 ml of 1M potassium acetate and 2.8 ml of distilled water. It was maintained at room temperature for 30 min and then the absorbance of the mixture was measured at 415 nm with UV-VIS spectrometer. The total flavonoids content was expressed in terms of catechin equivalent using the following formula:

\[ C = c \cdot \frac{V}{m} \]

Where: 
- \( C \) = total content of flavonoid (mg/g) test extract in catechin equivalent;
- \( c \) = the concentration of catechin established from the calibration curve (mg/ml);
- \( V \) = the volume of extract in ml;
- \( m \) = the weight of pure fraction in g

4.6.2 Infra Red (IR) spectra

I.R. spectrum of the n-butanol fraction and water fraction of *P. daemia* were recorded on FTIR spectrophotometer, by applying KBr disc.

4.7 ANIMALS

Wistar albino rats (180 - 250 g) and Swiss albino mice (20 - 25 g) of either sex were acclimatized for 7 days under standard husbandry conditions, i.e. room temperature 25±1°C, relative humidity 45-55% and light/dark cycle 12/12 h. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (Regd. No. 717/02/a/CPCSEA)
4.7.1 Husbandry Practices

**Caging:** Polypropylene rat cages covered with stainless steel grid top were autoclaved. Clean rice husk was used as the bedding mate.

**Water Bottle:** Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.

**Housing:** Single rat per cage

**Room Sanitation:** Each day, the floor of the experiment room was swept and all work tops and the floor were mopped with a disinfectant solution.

4.7.2 Animal Identification:

Each rat was uniquely numbered on the tail using a tattoo machine. Appropriate labels were attached to the cages indicating the study number, sex, and dose, type of study, cage number and animal number.

4.8 PHARMACOLOGICAL EVALUATION

For pharmacological evaluation all test drugs i.e. PDE, PDP, PDEA, PDB and PDW were suspended in 1% carboxymethyl cellulose and administered orally with not more than 20 ml/kg for rat and 10 ml/kg for mouse, unless and otherwise specified. For all studies freshly prepared solutions were used.

4.8.1 ACUTE TOXICITY TEST

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method) (Ecobichon, 1997). Albino mice (n = 6) of either sex selected by random sampling technique was used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the extract (50% alcoholic extract) was administered orally at the dose level of 5 mg / kg body weight by gastric intubation and observed for 14 days. If mortality is observed in 2 out of 3 animals, then the dose administered will be assigned as toxic dose. If mortality is observed in 1 animal, then the same dose will be repeated again to confirm the toxic dose. If mortality is not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg / kg body weight. According to the results of acute toxicity test, the doses were chosen for experiments.
4.8.2 Carrageenan-induced rat pedal inflammation:

Rats were divided in twelve groups of 6 animals each. Group A: saline control; Group B: 10 mg/kg indomethacin; Group C: ethanolic extract- 50 mg/kg; Group D: ethanolic extract – 100 mg/kg; Group E: Petroleum ether – 100 mg/kg; Group F: Petroleum ether – 200 mg/kg; Group G: ethyl acetate – 100 mg/kg; Group H: ethyl acetate – 200 mg/kg; Group I: n-butanol – 100 mg/kg; Group J: n-butanol – 200 mg/kg; Group K: Water – 100 mg/kg; Group L: Water – 200 mg/kg. One hour after the oral administration of drugs, acute paw oedema was induced by injecting 0.1 ml of 1% carrageenan in 0.9% saline. Paw volume was measured with the help of plethysmometer by mercury displacement method from 0 to 3 hours (Winter et al., 1962).

The percentage inhibition of paw oedema in treated groups was then calculated by using the formula:

\[
\text{Percentage inhibition} = \left(1 - \frac{V_t}{V_c}\right) \times 100
\]

Where \(V_t\) = is the oedema volume in the drug treated

\(V_c\) = is the oedema volume in the control group

In the secondary experiment groups with maximum activity (n-butanol and water fraction), the whole right hind paw whole right hind paw and liver tissues were taken at the third hour. The right hind paw tissue was rinsed in ice-cold normal saline and was taken at the third hour. The right hind paw tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline four times their volume and finally homogenized at 4\(^0\) C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the TNF-\(\alpha\), NO and malondialdehyde (MDA) assays.

On the other hand, the whole liver tissue was rinsed in ice-cold normal saline and immediately placed in cold normal saline of the same volume and finally homogenized at 4\(^0\) C. Then, the homogenate was centrifuged at 11,270 g for 5 min. The supernatant was obtained for the anti-oxidant enzyme Like Superoxide dismutase - SOD, Glutathione peroxidase - GPx activity assays.
4.8.2.1 Tissue TNF-α by ELISA

Tissue levels of TNF-α were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instruction. The measurements were performed according to the manufacturer’s protocols. The absorbance at 450 and 540nm was measured on a microplate reader. TNF-α was determined from a standard curve for the combination of these cytokines (Chun et. al., 2007).

4.8.2.2 NO Assay

NO was measured according to the method of Moshage et al. (1995). For nitrite determination, NO$_3^-$ was converted into nitrite after enzymatic conversion by nitrate reductase; NO$_2^-$ was measured by using the Griess reaction (Green et al., 1982). Values obtained by this procedure represented the sum of nitrite and nitrate.

4.8.2.3 MDA Assay

MDA was evaluated by the thiobarbituric acid-reacting substance (TRARS) method (Nakhai et. al., 2007) First, the paw tissues were homogenized in buffered saline (1:4); then, 400 ml of 1,1,3,3-tetraethoxypropan trichloroacetic acid (28% w/v) was added to 200 ml of this mixture and centrifuged in 3000g for 30 min. After that, 300 ml of the supernatant was added to 150 ml of 2-thiobarbituric acid (1% w/v). The mixture was incubated for 45 min in a boiling water bath, and then 450 ml n-butanol was added; the solution was centrifuged and cooled, and absorption of the supernatant was recorded at 532nm. Tetramethoxypropane was used as standard. MDA levels were expressed as nanomoles per milligram of protein. Protein concentration was measured by Lowry method (Lowry et. al., 1951). Bovine serum albumin was used as standard.
4.8.2.4 Anti-Oxidant Enzymes’ Activities

SOD enzyme activity was determined at room temperature according to the method of Misra and Fridovich. One hundred microliters of tissue extract was added to 880 ml (pH 10.2, 0.1mM EDTA) of carbonate buffer. Twenty microliters of 30mM epinephrine (in 0.05% acetic acid) was added to the mixture at 480nm for 4 min on a Shimadzu model 1800 Spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit.

GPx enzyme activity was determined according to the method of Flohe and Gunzler at 37° C. The reaction mixture composed of 500 ml phosphate buffer, 100 ml 0.01M GSH (reduced form), 100 ml 1.5mM NADPH and 100 ml GRx (0.24 units). One hundred microliters of the tissue extract was added to the reaction mixture and incubated at 37° C for 10 min. Then, 50 ml of 12mM t-butyl hydroperoxide was added to 450 ml of tissue reaction mixture and measured at 340nm for 180 s. The molar extinction coefficient of 6.22 X10⁻³ was used to determine the enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein.

4.8.3 Formaldehyde-induced sub-acute inflammation:

Formaldehyde induced sub chronic inflammatory state was carried out as previously described (Umukoro and Ashorobi, 2006). Rats were divided in twelve groups of 6 animals each. Group A: saline control; Group B: 10 mg/kg indomethacin; Group C: ethanolic extract- 50 mg/kg; Group D: ethanolic extract – 100 mg/kg; Group E: Petroleum ether – 100 mg/kg; Group F: Petroleum ether – 200 mg/kg; Group G: ethyl acetate – 100 mg/kg; Group H: ethyl acetate – 200 mg/kg; Group I: n-butanol – 100 mg/kg; Group J: n-butanol – 200 mg/kg; Group K: Water – 100 mg/kg; Group L: Water – 200 mg/kg. One hour after the oral administration of drugs, 0.1 ml of 2% formaldehyde was injected into the right hind paw of each rat. Paw volume was measured with the help of plethysmometer by mercury displacement method at 48
hours after formaldehyde injection and the percentage inhibition of oedema was calculated as described above.

4.8.4 Carrageenan air pouch granuloma

The inflammation was produced in rats by the method described by Hambleton and Miller (1989). The dorsal subcutaneous space was injected with 20 ml of air to create a pouch, which was reinflated on the fourth day with 10 ml of air. On the seventh day, rats were divided into twelve groups of 6 animals each. Group A: saline control; Group B: 20 mg/kg prednisolone; Group C: ethanolic extract– 50 mg/kg; Group D: ethanolic extract – 100 mg/kg; Group E: Petroleum ether – 100 mg/kg; Group F: Petroleum ether – 200 mg/kg; Group G: ethyl acetate – 100 mg/kg; Group H: ethyl acetate – 200 mg/kg; Group I: n-butanol – 100 mg/kg; Group J: n-butanol – 200 mg/kg; Group K: Water – 100 mg/kg; Group L: Water – 200 mg/kg. Drugs were administered orally and, 1 hour later, 1 ml of 1% carrageenan suspended in normal saline was injected into the pouch. The drug treatment was given daily for four days and 24 hours after the last dose the rats were sacrificed. Five millilitres of ice cold normal saline containing 0.1% EDTA was injected into the pouch and the exudate was collected in a graduated tube and its volume was measured. The granulomas were also resected and their wet and dry weights were recorded. Mean difference from control in the fluid volume, wet and dry weights were calculated.

In secondary Experiment, in groups with maximum activity (n-butanol fraction and water fraction) following parameters are evaluated. Cell migration, exudation, MPO, NOx, and TNF-α level and were evaluated 24 h after phlogogen administration.

4.8.4.1 Quantification of cell migration and exudation

After killing animals with an overdose of ether, samples from the air pouch exudate were collected for determinations of total and differential leukocytes. Total leukocyte counts were performed in a Neubauer chamber diluting the exudate in Türk solution (1:20), and cell preparations of exudate were stained with Field stain for the differential leukocyte counts, which were performed under an oil immersion objective (Benincá et al., 2007). To evaluate the degree of exudation, animals were previously
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challenged with a solution of Evans blue dye (25.0 mg/kg) administered by intravenous route (i.v.), 5 min after treatment with carrageenan (Benincá et al., 2007). The amount of dye was estimated by spectrophotometrically at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50.0 μg/ml.

4.8.4.2 Quantification of nitrate/nitrite concentrations

Nitric oxide was measured as its breakdown product of nitrite (NO\(^2-\)) and nitrate (NO\(^3-\)) using the Griess method (Green et al., 1982). Samples of the air pouch cavity lavage obtained from control and treated animals that did not receive Evans blue dye injection were separated and stocked at −70 °C. On the day of the experiments, the samples were thawed and deproteinized by the addition of 6 mM sodium hydroxide and 0.6% of zinc sulfate. Afterwards, 250 μl of air pouch cavity lavage was diluted in 30 μl of ammonium formate, 30 μl of hydrated disodium hydrogen phosphate-12 and 30 μl of Escherichia coli (EC ATCC 25922: diluted (1:10) in PBS), and then the mixture was incubated for 2 h at 37 °C. After centrifugation at 50 ×g for 5 min, 250 μl of the supernatant was transferred to cuvettes and the same volume of fresh Griess reagent (5% v/v) of H\(_3\)PO\(_4\), 1% of sulfanilic acid and 0.1% of N-(1-naphthyl)-ethylenediamine was added and incubated for 10 min at room temperature. The reaction of NO\(^2-\) with this reagent produces a pink colour, which was quantified at 543 nm against standards (0–150 μM) on a Shimadzu model 1800 Spectrophotometer (Saleh et al., 1999). Results were expressed as μM.

4.8.4.3 Quantification of myeloperoxidase

In-house assays of both myeloperoxidase an activity was employed according to the methods described in the literature (Rao et al., 1993). Using conventional reagents, the concentration of each enzyme was estimated by means of colourimetric measurements (absorbances of 450 nm) on Shimadzu model 1800 Spectrophotometer. Results were expressed as mU/ml (MPO).
4.8.4.4 Quantification of TNF-α

Samples of exudate were collected and immediately prepared for the analysis of TNF-α level. In this protocol, commercially available kits were used with specific antibodies for TNF-α. The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. When necessary the fluid exudate samples were diluted 1:3 or 1:10 with specific diluents (buffered with 0.09% sodium azide) to determine the TNF-α levels. The range of values detected by these assays was: TNF-α (5.0–2000.0 pg/ml). TNF-α levels were estimated by means of colourimetric measurement at 450 nm with an ELISA plate reader by interpolation from a standard curve.

4.8.5 Cotton pellet granuloma

The granulomas were developed by the method described by Naik et al. (1980). Rats were divided into twelve groups of 6 animals each. Group A: saline control; Group B: 10 mg/kg indomethacin; Group C: ethanolic extract- 50 mg/kg; Group D: ethanolic extract – 100 mg/kg; Group E: Petroleum ether – 100 mg/kg; Group F: Petroleum ether extract – 200 mg/kg; Group G: ethyl acetate – 100 mg/kg; Group H: ethyl acetate – 200 mg/kg; Group I: n-butanol – 100 mg/kg; Group J: n-butanol – 200 mg/kg; Group K: Water – 100 mg/kg; Group L: Water – 200 mg/kg. Following one hour of oral administration of drugs, sterile cotton pellets weighing 10 mg were implanted subcutaneously in both the axillae of rats under ether anesthesia. Drugs were given daily for 10 days. On the 11th day, rats were sacrificed and the cotton pellets with the surrounding granulomas were resected out and their wet and dry weights were recorded.

Further, in groups with maximum activity following parameter are evaluated i.e Plasma MDA and histopathology.
Materials and Methods

4.8.5.1 Plasma MDA (malondialdehyde) Estimation

After 11 days of drug treatment in the cotton pellet-granuloma method, 3-5 ml of blood was collected from inner canthus of eye from each animal using capillary tube, in a vial containing EDTA as an anticoagulant. Plasma was separated by centrifugation at 3000 rpm for 10 minutes. It was stored at -20°C and used to estimate MDA levels. The reduced levels of MDA were taken as indicator of anti-lipoperoxidative activity which can be taken as the index of reduced oxidative stress.

4.8.6 Freund’s complete adjuvant induced arthritic model

Rats were divided into twelve groups (n = 6). Group A: saline control; Group B: 5mg/kg Prednisolone; Group C: ethanolic extract - 50 mg/kg; Group D: ethanolic extract – 100 mg/kg; Group E: Pet ether extract – 100 mg/kg; Group F: Pet ether extract – 200 mg/kg; Group G: ethyl acetate – 100 mg/kg; Group H: ethyl acetate – 200 mg/kg; Group I: n-butanol – 100 mg/kg; Group J: n-butanol – 200 mg/kg; Group K: Water – 100 mg/kg; Group L: Water – 200 mg/kg. Eleven groups, except normal group, were made arthritic by injecting 0.1 ml Freund's complete adjuvant (Sigma, Germany) into the subplantar region of left hind paw on day '0'. This adjuvant consists of dead mycobacterium tuberculosis bacteria suspended in heavy paraffin oil to give final concentration of 0.5 mg/ml. Saline or extracts or dexamethazone were administered orally once daily, from the initial day i.e. from the day of adjuvant injection (0 day), 30 minutes before adjuvant injection, and continued till 21st day (Vogel, 2002). The anti-arthritic effect of the extracts as well as dexamethazone was evaluated by measuring paw volume of injected paw on 3rd, 5th, 9th, 13th, and 21st day of study by using Plethysmometer.

The percentage inhibition of paw oedema in treated groups was then calculated by using the formula:

Percentage inhibition = \( (1 - \frac{V_t}{V_c}) \times 100 \)

Where \( V_t = \) is the oedema volume in the drug treated

\( V_c = \) is the oedema volume in the control group
Further, in groups with maximum activity following parameter were evaluated. The changes in body weight were recorded daily. The primary and the secondary lesions were measured.

Primary lesions refer to the edema formation in the injected hind paw that peaks 3–5 days after injection of the phlogistic agent and is measured on day 5 by calculating the percent inhibition of the edema volume of the injected paw using the formula described above. Secondary lesions are immunologically mediated changes characterized by inflammation of the non-injected sites (hindleg, forepaw, ears nose and tail) decrease in weight and occur after a delay of 11–12 days. Accordingly, secondary lesions were evaluated by calculating the percent inhibition of paw volume of the noninjected right paw over control on day 21 and using an arthritic index as the sum of scores according to the method of Schorlemmer et al., 1999; Vogel., 2002.

<table>
<thead>
<tr>
<th>Lesion Site</th>
<th>Nature of lesion</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear</td>
<td>a. Absence of nodules and redness</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>b. Presence of nodules and redness</td>
<td>1</td>
</tr>
<tr>
<td>Nose</td>
<td>a. No swelling of connective tissue</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>b. Intense swelling of connective tissue</td>
<td>1</td>
</tr>
<tr>
<td>Tail</td>
<td>a. Absence of nodules</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>b. Presence of nodules</td>
<td>1</td>
</tr>
<tr>
<td>Forepaw</td>
<td>a. Absence of inflammation</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>b. Inflammation of at least one joint</td>
<td>1</td>
</tr>
<tr>
<td>Hind paw</td>
<td>a. Absence of inflammation</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>b. Slight inflammation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c. Moderate inflammation</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>d. Marked inflammation</td>
<td>4</td>
</tr>
</tbody>
</table>

On the day 22nd blood was withdrawn from the each animal through retro-orbital plexus under light ether anaesthesia. The blood was collected into vials containing EDTA and the biochemical parameters like haemoglobin content, total WBC count, differential WBC count, ESR and RBC Serum albumin, C-rective protein (CRP), RF and Histopathology of synovial joints were analyzed.
4.8.6.1 Body Weight:

Body weight of each animal was measured on the day of CFA administration, and later on 21st days. The mean percentage reduction in body weights with respect to that on day of CFA administration were calculated for each drug treated group and compared with that of disease control group.

4.8.6.2 Pain score:

Scores were assigned for evaluation of the pain associated with the arthritis as shown in following table (Kumar et. al., 2006 and Laird et. al., 2001)

<table>
<thead>
<tr>
<th>Score</th>
<th>Flexion pain test score</th>
<th>Mobility Score</th>
<th>Stance Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No squeaking and no leg withdrawal</td>
<td>Normal</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>Either squeaking or leg withdrawal</td>
<td>Limping</td>
<td>Paw lifted continuously</td>
</tr>
<tr>
<td>2</td>
<td>Both squeaking and leg withdrawal</td>
<td>Walking with difficulty</td>
<td>Paw touching but with no weight bearing</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>Walking without touching the injected paw</td>
<td>Some weight bearing on the paw</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td></td>
<td>Normal</td>
</tr>
</tbody>
</table>

4.8.6.3 Erythrocyte Sedimentation Rate (ESR):

Higher erythrocyte sedimentation rate is indicator of inflammatory disorder.

Method: WESTERGREN’S METHOD

Requirements:
1) Westergren’s pipette and Westergren’s stand, (2) Oxalate bulb, (3) Sodium Citrate solution

Westergren’s pipette: It has a bore of 3mm and a length of 300 mm. It is marked with graduations from 0 to 300 mm. It is open from both the ends.

Westergren’s Stand: It can accommodate six pipettes. There is base containing six grooves each containing a rubber cushion to prevent leakage of blood from Westergren’s pipette. The stand allows the pipettes to remain in exactly vertical position.

Procedure:

1) A sample of blood (about 3 ml) was obtained by puncturing retro-orbital plexus and was mixed with 3.8% sodium citrate solution in proportion of four
Materials and Methods

parts of blood to one part of citrate soon. The mixing of blood was done by rotating the sample gently between the palms of hands.

2) The blood was sucked slowly up to the mark zero in the Westergren’s tube.
3) The tube was set upright in the Westergren’s stand, taking care that no blood escapes. The tube was fixed with the help of screw cap.

4) At the end of one hour and two hours, the upper level of red blood cell column was read. It was indicator of mm of clear plasma or ESR.

4.8.6.4 Serum Rheumatoid Factor (RF):

Serum RA factor estimation was done by turbidimetry method.

Principle of the method:
The RF- Turbilatex is a quantitative turbidimetric test for the measurement of RF in human serum or plasma. Latex particles coated with human gammaglobulin are agglutinated when mixed with samples containing RF. The agglutination causes an absorbance change, dependent upon the RF content of sample that can be quantified by comparison from a calibrator of known RF concentration.

Clinical Significance:
Rheumatoid factors are a group of antibodies directed to determinants in the Fc portion of the immunoglobulin G molecules. Although rheumatoid factor are found in a number of rheumatoid disorders, such as systemic lupus erythematosus (SLE) and sjogren syndrome, as well as in non rheumatic condition, its central role in cliniclies its utility as an aid in the diagnosis of rheumatoid arthritis.

Reagents:
The following reagents were contained in the kit:

<table>
<thead>
<tr>
<th>Diluent (R1)</th>
<th>Tris buffer 20mmol/L, pH 8.2. Sodium azide 0.95g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latex (R2)</td>
<td>Latex particles coated with human gamma globulin, pH 7.4 Sodium azide 0.95g/L</td>
</tr>
<tr>
<td>RF-CAL</td>
<td>Calibrator. Human serum. The RF concentration is stated on the vial label.</td>
</tr>
<tr>
<td>Optional</td>
<td>Ref.: 1102114 Control serum ASO/CRP/RF level L</td>
</tr>
<tr>
<td></td>
<td>Ref.: 1102115 Control serum ASO/CRP/RF Level H.</td>
</tr>
</tbody>
</table>
Materials and Methods

Preparations:

For RF Calibrator:

RF Calibrator was reconstituted with 2.0 ml of distilled water. It was mixed gently and brought to room temperature for 10 minutes before use.

For Calibration curve (range from 20 to 160 IU/ml): The following RF calibrator dilutions were prepared in the normal saline. The concentration of the RF calibrator was multiplied by the corresponding factor stated in table below to obtain the RF concentration of each dilution.

For one point calibration (linear range up to 100 IU/ml):

Preparation for RF calibrator dilution: 30μl RF calibrator + 70 μl normal saline (9 gm/L). The RF calibrator concentration was multiplied by 0.33 to obtain the RF concentration of the diluted calibrator.

Procedure:

1. The reagents and the photometer (cuvette holder) were brought to 37°C.
2. The following assay conditions were described:
   - Wavelength: 650 nm (600-650 nm)
   - Temperature: 37°C
   - Cuvette light path: 1 cm
3. The instrument was adjusted to zero with distilled water.
4. The following reagents were pipette into a cuvette and mixed
5. Absorbance was read after 2 minutes (A2) of the sample addition.

<table>
<thead>
<tr>
<th>Calibrator dilution</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF (μl)</td>
<td>100</td>
<td>90</td>
<td>75</td>
<td>50</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Factor</td>
<td>0</td>
<td>0.1</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Calculations:
Materials and Methods

For calibration curve:
Calculated the absorbance difference \((A_2 - A_{blank \text{ reagent}})\) of each point of the calibration curve and plotted the values obtained against the RF concentration of each calibrator dilution. Rheumatoid factor concentration in the sample was calculated by interpolation of its \((A_2 - A_{blank \text{ reagent}})\) in the calibration curve.

For one point calibration:

\[
\frac{[(A_2 - A_{blank \text{ reagent}}) \text{ sample}]}{[(A_2 - A_{blank \text{ reagent}}) \text{ calibrator}] \times \text{Diluted calibrator concentration}} = \text{IU/ml RF}
\]

The reading of RF factor of all the groups obtained was compared with the control animals and was expressed as IU/ml RF.

4.8.6.5 Serum C - reactive protein (CRP): Principle:
C-reactive protein is measured by turbidimetry method of analysis. Turbidimetry is the measurement of the reduction in light transmission caused by particle formation. Light transmitted in forward direction is detected. The amount of light absorbed by suspension of particles depends on specimen concentration and on the particle size. Solution requiring quantization by turbidimetry is measured using visible spectrophotometers.

Reagents:

| Diluent (R1) | Tris Buffer 20 mmol/L, pH 8.2
|             | Sodium azide 0.95g/L |
| Latex (R2)  | Latex particle coated with goat IgG anti-human CRP, pH 7.3
|             | Sodium azide 0.95 g/L |
| CRP-CAL     | Calibrator. C-reactive protein concentration is stated on the vial label |
| Optional    | Ref: 1102114 Control serum ASO/CRP/RF level L |
|             | Ref: 1102115 Control serum ASO/CRP/RF level H |

Calibration:
The assay was calibrated to the reference material CRM 470/RPPHS.

Reagent Preparation & Stability:
Working Reagent: Swirl the latex vial gently before use. Prepare the necessary amount as follows:
1 ml Latex Reagent + 9 ml Diluent
This working reagent was stable for 30 days at 2-8° C.

**CRP Calibrator:** Contents of vial were reconstituted with 1.0 ml of distilled water and kept for 10 minutes before use. This CRP calibrator was stable for 30 days at 2-8° C or 3 months at -30° C.

**Samples:**
Fresh serum is preferred, though samples stored for 8 days at 2-8° C or 3 months at -20° C may also be used.

**Procedure:**
Assay protocol

1. Bring the working reagent and the photometer (cuvette holder) to 37°C.
2. Assay condition
   - Wavelength: 540 nm (530-550 nm)
   - Blank: Distilled water
   - Temperature: 37°C
   - Cuvette Path Length: 1 cm
3. Adjust the instrument to zero with distilled water.
4. Pipette into a cuvette:

**Pipetting System:**

<table>
<thead>
<tr>
<th>Dispense</th>
<th>Calibrator</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Calibrator or</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>5.0 µl</td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>

5. Mix and read the absorbance immediately (A1) and after 2 minutes (A2) of the sample addition.

**Calculation:**

\[
\frac{(A2-A1) \text{ sample}}{(A2-A1) \text{ Calibrator}} \times \text{ Calibrator Concentration}
\]

**Reference Values**
Normal values up to 6 mg/L.
Each laboratory should establish its own reference range.
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4.8.6.6 Serum A/G ratio

Estimation of Protein Contents:

Principle:
Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex; the intensity of the color formed is directly proportional to the amount of protein present in the sample.

Reagents:
Reagent A: Biuret reagent.
Reagent B: Protein standard.

Pipette into clean dry test tubes labeled as blank, standard and test.

<table>
<thead>
<tr>
<th>Addition Sequence</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biuret Reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein standard</td>
<td>---</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>---</td>
<td>---</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C for 10 min. or at room temperature for 30 min. Measure the absorbance of the standard, test sample against the blank within 60 min at 550nm.

Calculation:
Total proteins in gm/ dl = (Abs T/ Abs S) X 8
Normal Range: 6-8gm/dl

Determination of Serum Albumin: Name of the method: Bromocresol green method.

Principle: Albumin present in serum binds specifically with bromocresol green at pH 4.1 to form green colored complex, intensity of which can be measured colorimetrically by using 640 nm.

Normal Range: 3.3-4.8 g/dL.

Preparation of reagents:
1) Albumin reagent (ready to use): It is prepared by mixing following chemicals in 900 ml distilled water.
Materials and Methods

a) Succinic acid: 8.85 gm
b) Bromocresol green: 108 mg
c) Sodium azide: 100 mg
d) Tween-20: 4.0 ml

pH of this solution is adjusted by using 1 N sodium hydroxide to 4.1. Final volume is made to one litre by using distilled water. It is stable at room temperature for one year.

2) Albumin standard (4gm/dl): Bovine albumin 4.0 gm in 100 ml of normal saline containing 0.1 gm/dl sodium azide. It is stable at 2-8°C for one year.

Procedure:

Mono step method

➢ Take three test tubes and labeled them as test, standard and blank.
➢ Add 5 ml of albumin reagent in each of the test tube.
➢ Then add 0.05 ml of serum, albumin standard and distilled water respectively.
➢ Mix thoroughly and keep at room temperature (25°C± 5° C) for exactly 10 minutes.
➢ Measure the intensities of the test and standard by setting at 100% T, by using 640 nm (red filter).

Calculation:

Serum albumin g/dl = O.D. test/ O.D. Std. x 4

Determination of Globulins:
Normal range = 1.8-3.6 g/dl

A/G ratio = serum albumin (g/dl) / serum globulins (g/dl)
Normal range = 1.2:1 to 2:1

4.8.6.7 Histopathology of synovial joints:

Rats were killed at day 21 by ether narcosis; hind limbs were removed and fixed in 10% buffered formalin. The limbs were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5 μm thickness, and subsequently stained with haematoxylin–eosin for examination under a light microscope. Sections were examined for the presence of hyperplasia of the synovium, pannus formation and destruction of the joint space.
**4.8.7 DIURETIC ACTIVITY**

The method described by Wiebelhaus et al. 1965 was employed, with modification, for the assessment of diuretic activity. Healthy albino rats of either sex (160-200 g) were divided into twelve groups of five animals each. They were fasted 18 hours prior to the test, with free access to water. On the day of the experiment, animals were given 25ml/kg of body weight normal saline orally. Group A received vehicle (0.2 ml of 5% tween 80) and served as control group. Group B: Furosemide 100 mg/kg; Group C: ethanolic extract- 200 mg/kg; Group D: ethanolic extract – 400 mg/kg; Group E: Pet ether extract – 200 mg/kg; Group F: Pet ether extract – 400 mg/kg; Group G: ethyl acetate – 200 mg/kg; Group H: ethyl acetate – 400 mg/kg; Group I: n-butanol – 200 mg/kg; Group J: n-butanol – 400 mg/kg; Group K: Water – 200 mg/kg; Group L: Water – 400 mg/kg. All drugs/vehicle were administered orally (p.o.). Immediately after dosing, the rats were placed in the metabolic cages with special provision to collect faeces and urine. Animals were kept at room temperature of 35±1°C throughout the experiment. Urine excreted for the next 5 h was collected and the total 5 h urine volume for each rat was compared with the volume of urine produced after the administration of normal saline.

The volume of urine excreted during 5 h for each animal in the group is expressed as the percent of the liquid (normal saline) administered. This percentage gives a measure of urinary excretion independent of the animal weight. The ratio of urinary excretion in the test group to urinary excretion in the control group is used as a measure of the diuretic action for the given dose of the drug. As the diuretic action is prone to variability, a parameter known as diuretic activity was calculated instead. To obtain the diuretic activity, the diuretic action of the extract is compared to that of the standard drug in the test group (Mukherjee, 2002).

$$\text{Percentage of saline load excreted} = \frac{\text{volume of urine}}{\text{volume of saline load}} \times 100$$
Materials and Methods

Phytopharmacological action of *Pergularia daemia* with special reference to its actions and mechanism of action as diuretic and anti-inflammatory agent

The parameters taken to study were pH, Na\(^+\), K\(^+\) and Cl\(^-\) concentration in urine. Further, in groups with maximum activity Plasma electrolyte level, Plasma urea level, Hematocrit, Urine conductivity and GFR (as a creatinine clearance) were evaluated.

**Parameter studied**

4.8.1 **Diuretic action:**

Diuretic action of plant was estimated by ration of urinary excretion of treated group by urinary excretion of standard drug.

4.8.2 **Urine and plasma electrolytes**

Blood was collected in capillary tubes containing ethylenediamine tetra-acetic acid by retro-orbital puncture under light diethyl ether anesthesia. Plasma was obtained by centrifugation (600 times 10\(^3\) g at 4 °C), and stored at −20 °C until analyzed. Plasma and urinary levels of sodium and potassium were quantitated by flame spectrophotometry, while Cl\(^-\) concentration will be determined titrimetrically.

4.8.3 **Plasma urea level**

Blood was collected in capillary tubes containing ethylenediamine tetraacetic acid by retro-orbital puncture under light diethyl ether anesthesia. The plasma urea level was estimated by DAM method.

4.8.4 **Hematocrit**

Blood sample at the end of experiment were collected and was analyze by autoanalyzer for the estimation of hematocrit.

4.8.5 **Urine conductivity**

Conductivity of urine was measured by conductometer (SYSTRONIC LTD).

\[
\text{Urinary excretion} = \frac{\text{total urinary output}}{\text{total liquid administered}} \times 100
\]

\[
\text{Diuretic action} = \frac{\text{urinary excretion of treated group}}{\text{urinary excretion of control group}}
\]

\[
\text{Diuretic activity} = \frac{\text{diuretic action of test drug}}{\text{diuretic action of standard drug}}
\]
4.8.6 GFR

Glomerular filtration rate was evaluated by the clearance of creatinine. Concentration of creatinine in plasma and urine was determined by the Jaffe alkaline picrate method. For urinary creatinine excretion 24 h urine was collected and its volume measured. Glomerular filtration rate (GFR) was estimated from creatinine clearance ($C_{Cr} = U_{Cr} \times V / P_{Cr}$, where $U_{Cr}$: urinary excretion of creatinine and $P_{Cr}$: plasma level of creatinine, $V$: volume of urine).

4.8.8 Soybean lipoxygenase inhibition study

*In vitro* study was carried out as reported previously (Block et al., 1988). The tested compound was dissolved in ethanol and incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution (1/9 $\times$ 10$^4$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded in presence of n-butanol fraction (100-1000 μg/ml), water fraction (100-1000 μg/ml) and in absence of extracts (Block et al., 1988).

**Experimental design for Lipoxygenase estimation:**

A. Preparation of Borate Buffer, pH 9.0 (Reagent A): 6.18g of boric acid was dissolved in 300ml water. The pH was adjusted to 8.00 with 50% NaOH solution. The final pH was adjusted to 9.0 with 10% NaOH solution. The final volume was made upto 500ml with distilled water.

B. Substrate Solution: 0.05ml Linolic acid was added to 50.0ml volumetric flask. 0.05mlk of 95 % ethanol was added and mixed gently to form the emulsion and brought to the final volume up to 50 ml. For assay 5.0ml of this solution was diluted to 30ml with Reagent A.

C. Enzyme solution (Reagent C): Prepared a solution of approx 10,000 units per ml of enzyme in ice cold reagent A. For assay: approx 500 units/3ml reaction volume was used.
Materials and Methods

**Procedure:**

In two cuvets of quartz of 1cm light path, which have been labeled blank and test the following quantities of the reagent have been added.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer Solution (Reagent A)</td>
<td>1.0 ml</td>
<td>0.95ml</td>
</tr>
<tr>
<td>Substrate Solution (Reagent B)</td>
<td>2.0ml</td>
<td>2.00ml</td>
</tr>
<tr>
<td>Enzyme solution (Reagent C)</td>
<td>-</td>
<td>0.05ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>3.0ml</td>
<td>3.0ml</td>
</tr>
</tbody>
</table>

Reagent C was quickly added to the cuvet labeled ‘Test’ and mixed well. The cuvet was quickly placed in a spectrophotometer and the increase in absorbance at 234nm per min against blank was recorded. The maximal ΔA234nm per min between 1-3min interval was recorded.

4.8.9 Ulcerogenic activity

The method of Vogel, 2002 was employed. Food was withheld from the animals for 48 h prior to the experiment. The fasted animals received the n-butanol fraction (100 and 200 mg/kg), water fraction (100 and 200 mg/kg) administered orally. Control animals received equivalent volume of vehicle (3% v/v Tween 85) or indomethacin (30 mg/kg). Three hours later, animals were killed and the stomachs removed and cut open along the lesser curvature. The opened stomach was washed with normal saline and observed with a magnifying lens (x10). Lesions on the mucosal surface were scored according to an arbitrary scale: 0 = no lesion; 0.5 = hyperemia; 1 = one or two lesions; 2 = severe lesions; 3 = very severe lesions; 4 = mucosa full of lesions (Bani et al., 2000).

Gastric ulcer lesion area was measured as described by Khan 2004. In brief the stomach samples were flattened and carefully sandwiched between the two layers of a transparent plastic folder of A4 size. The specimens within the plastic folder were scanned and using a Scion Image software lesion area was measured.
4.8.10 Mouse Carrageenan Peritonitis

Inflammation was induced by the modified method of Griswold et al., 1987. Male Swiss albino mice weighing 20-25 g were divided into six groups (n=6). Group I served as control, Group II served as standard and was dosed with indomethacin (10 mg/kg, p.o.), Group III received n-butanol fraction (100 mg/kg, p.o.), Group IV received n-butanol fraction (200 mg/kg, p.o.) and Group V and VI were dosed with water fraction at the doses of 100 and 200 mg/kg p.o., respectively. The control (2% gum acacia), standard drug and extract doses were administered orally one hour prior to the induction of peritonitis. After one hour, carrageenan (0.25 ml, 0.75% w/v in saline) was injected intraperitonially. Four hours later, the animals were sacrificed by cervical dislocation and 2 ml of Ca\(^{2+}\) and Mg\(^{2+}\) free phosphate buffered saline (PBS) was injected into the peritoneal cavity. Following a gentle massage, peritoneal exudates were removed. The total leukocyte count was determined in a Neubauer chamber and the differential cell count was determined (Wintrobe et al., 1961 and Amour et al., 1965).

The percentage of leukocyte inhibition was calculated using the following formula:

\[
\% \text{ of Leukocyte Inhibition} = (1 - \frac{T}{C}) \times 100
\]

Where ‘T’ represents the treated groups’ leukocyte count and ‘C’ represents the treated control group leukocyte count.

Inhibition of Neutrophil migration was calculated by the following equation:

\[
\text{Inhibition of Neutrophil Migration} = 100 - \{(N T/NC) \times 100\}
\]

Where NT = Neutrophil counts of treated groups
NC = Neutrophil counts of control groups.

4.8.11 Acetic acid induced capillary permeability in mice

The method used by Whittle, 1964 was used in this study with some modifications to evaluate the effect of the fractions on vascular permeability in adult albino mice of both genders. One hour after oral administration of the n-butanol fraction (100 or 200 mg/kg) and water fraction (100 or 200 mg/kg) ; 0.1 ml of Evans
Blue dye (4% in Normal saline) was intravenously administered through the tail vein. Animals in the positive control group received indomethacin (10 mg/kg) and equivalent amounts of vehicle were given to the animals in the vehicle control group. Thirty minutes later, animals received an intraperitoneal injection of 0.4 ml of acetic acid (0.5%, v/v). Treated animals were sacrificed 30 min after the injection of acetic acid and the peritoneal cavity was washed with normal saline (5 ml) into heparinized tubes and centrifuged. The dye content in the supernatant was measured at 590 nm using a spectrophotometer and the conc. of dye leaked into the peritoneal fluid was calculated using a standard curve of Evans blue dye. The percentage of inhibition was also calculated.

4.8.12 Mast cell Stabilization

The method described Vyas and Vyas, 2009 was employed, with modification, for mast cell stabilization. Mice were divided in six groups, five animals each. A three-day drug treatment schedule was followed. Group-I received vehicle (10 ml/kg, i.p.). Group-II was treated with standard drug sodium cromoglycate (200µg/ml, i.p.). Group-III and IV were treated with n-butanol fraction of P. daemia with 100 mg/kg and 200 mg/kg orally, respectively. Group-V and VI received water fraction of P. daemia with 10 mg/kg and 200 mg/kg orally, respectively. On fourth day, each animal was injected with 4 ml/kg, 0.9 % saline solution, into peritoneal cavity. By gentle massage, peritoneal fluid was collected after 5 mins and transferred into siliconised test tubes containing 7-10 ml RPMI-1640 buffer medium (pH 7.2-7.4). This solution was then centrifuged at 400-500 RPM. Pellet of mast cell was washed with same buffer medium twice by centrifugation, discarding supernatant. These cells were challenged with clonidine (80 µg), incubated at 37°C in a water bath for 10 mins. Followed by staining with 1% toluidine blue and observed under microscope (45X). Total 100 cells were counted from different visual area. Percent protection against degranulation was calculated.
4.8.13 Membrane stabilizing activity

Preparation of erythrocyte suspension:

Whole blood was obtained with heparinized syringes from rats through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 minutes at 3000 g.

Hypotonic solution-induced rat erythrocyte haemolysis:

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis (Shinde et al., 1999). The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the n-butanol fraction (0.5–5.0 mg/ml), water fraction (0.5 – 5 mg) or indomethacin (0.1 mg/ml). The control sample consisted of 0.5 ml of RBC mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to modified method described by Shinde et al., 1999.

\[
\% \text{ Inhibition of hemolysis} = 100 \times \frac{\text{OD1} - \text{OD2}}{\text{OD1}}
\]

Where:

\[
\text{OD1} = \text{Optical density of hypotonic-buffered saline solution alone}
\]
\[
\text{OD2} = \text{Optical density of test sample in hypotonic solution}
\]

4.8.14 Neutrophil adhesion

The method describe by Wilkonson, 1978 was used for evaluating the effect of extracts on neutrophil adhesion. The animals were randomly divided in five groups of 6 animals each. Group I served as control, Group II received n-butanol fraction (100 mg/kg, p.o.), Group III received n- butanol fraction (200 mg/kg, p.o.) and Group IV and V were dosed with water fraction at the doses of 100 and 200 mg/kg p.o.,
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respectively. After 14 days of treatment of all groups, blood samples were collected by retro-orbital puncture in heparinized vial and subjected to total as well as differential count. After the initial counts, blood samples were incubated with 80 mg/ml of Nylon fibers for 10 min. at 37°C. The incubated blood samples were again analyzed for total and differential count. The product of total leukocyte count and percent neutrophils known as neutrophil index was determined for each test group. Percent neutrophil adhesion was calculated from the following formula:

\[
\text{Neutrophil adhesion (\%) = \frac{N_{\text{Nu}} - N_{\text{It}}}{N_{\text{It}}}}
\]

Where:

\( N_{\text{Nu}} \) = neutrophil index of untreated blood sample; and

\( N_{\text{It}} \) = neutrophil index of treated blood sample.

4.8.15 In-Vitro antioxidant activity

4.8.15.1 Measurement of the H-donor activity

The free radical scavenging activity of n-butanol fraction (PDB), water fraction (PDW) and butylated hydroxyl toluene (BHT) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (Blois, 1958). DPPH solution (0.1 mM) in ethanol was prepared and 1 ml of this solution was added to 3 ml of extract solution in water at different concentrations (10-300 μg/ml). After 35 min, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

\[
\% \text{ DPPH scavenged} = \frac{A(\text{cont}) - A(\text{test})}{A(\text{cont})} \times 100
\]

where \( A(\text{cont}) \) is the absorbance of the control reaction and \( A(\text{test}) \) is the absorbance in the presence of the sample of the extracts.

4.8.15.2 NO radical scavenging activity
Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent (Ganesh et al., 2004). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate-buffered saline (PBS) was mixed with 3 ml of different concentrations (10 – 300 μg/ml) of the PDB, PDW and BHT dissolved in the suitable solvent systems and incubated at 25 °C for 150 min. The samples from the above were reacted with Greiss reagent (1% sulphanilamide, 2% H3PO4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The nitric oxide radicals scavenging activity was calculated as in the case of DPPH.

*4.8.15.3 Superoxide anion radical scavenging activity*

Measurement of super oxide anion scavenging activity of the PDB and PDW based on the method described by Liu et al., 1997 with slight modification. Super oxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the super oxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8) containing1.0ml of NBT (50 μM) solution, 1 ml NADH (78 μM) solution and sample solution of the extracts (10-300 μg/ml) in water. The reaction started by adding 1.0ml of phenazin methosulphate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 250 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. L-Ascorbic acid was used as a reference compound. Decreased absorbance of the reaction mixture was indicated an increased superoxide anion scavenging activity. The % inhibition of super oxide anion generation was calculated as in the case of DPPH.

*4.8.15.4 H2O2 radical scavenging activity*

The ability of extracts to scavenge H2O2 was determined according to the method of Ruch et al., 1989. A solution of H2O2 was prepared in phosphate buffer (pH 7.4). Extracts (10-300 µg/ml) in distilled water were added to a H2O2 solution (0.6 ml, 40 mM). Absorbance of H2O2 at 230 nm was determined10 min later against a blank solution containing the phosphate buffer without H2O2. The % of H2O2...
scavenging of both the extracts and standard compounds was calculated as in the case of DPPH.

4.8.15.5 Reducing power

The reducing power of the extracts was determined according to the method of Oyaizu, 1986. Various concentrations of the PDB, PDW and L-Ascorbic acid (10-300 μg/ml) in 1.0 ml of de ionized water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 1036 x g for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared FeCl₃ solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of their action mixture indicated increased reducing power.

4.9 STATISTICAL ANALYSIS

The data were expressed as mean ± SEM. The significance of the difference was evaluated by one-way ANOVA followed by Dunnett’s multiple comparisons test for parametric data, whereas the statistical significance was analyzed by Wilcoxon simple paired test for arthritic index and percentage body weight change of control and by the Kruskall–Wallis test followed by Dunn’s multiple comparison tests for the pain scored data. Data were considered statistically significant if P value <0.05.