4. Material and Methods

4.1 Pharmacognostic Investigations

The systematic pharmacognostic investigation of the plant material and its documentation will help the scientific community for further investigation. So efforts are made to contribute in that direction.

A. Collection and Authentication of the whole plant.

B. Microscopic characters.

C. Proximate values.

a. Extractive values
   - Alcohol soluble extractive value.
   - Water soluble extractive value.

b. Moisture content determination

c. Ash values
   - Total ash
   - Acid insoluble ash
   - Water soluble ash
   - Sulphated ash

A. Collection and authentication of plant material. (Plant I & II)

(I) The *Parkinsonia aculeata* linn leaves and bark were collected from the local areas of Ajmer road, Jaipur, Rajasthan, and identification by Dr. Gajendra Pal Singh, Asst. professor, Department of Botany, University of Rajasthan, Jaipur and herbarium submitted in herbarium, Department of Botany, University of Rajasthan, Jaipur (Specimen no. RUBL20684).
Material and Methods

(II) The Plant from *R. aquatica* identified and authenticated by Kenganora Mruthunjaya, Asst. Professor, Dept. of Pharmacognosy, JSS College of Pharmacy, Mysore, Karnataka. The voucher specimen of the plant is preserved in Dept. of Pharmacognosy, JSS college of Pharmacy, Mysore, Karnataka.

The leaves, bark and root were dried under shed, powderd and stored in a well-closed container.

B. Study of Microscopically Characters

a. Transverse section of the leaves, bark and roots

The freshly leaves and bark of *P. aculeata* and root of *R. aquatica* sections were taken by Microtome and some was stained with Saffranin and Hemotoxylin,

b. Powder microscopy

The dried leaves and bark of *Parkinsonia aculeata* and root of *Rotula aquatica* were coarsely powder and boil with chlora hydrate for 5-10 minutes and then stained with phloroglucinol and HCl in 1:1 ratio, observed under high power (40 x), for different diagnostic characters such as, calcium oxalate crystals, lignified fibers, xylem vessel, etc..

Histochemical Analysis:

Table No: 08

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Test for</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saffranin</td>
<td>Lignin</td>
<td>Pink</td>
</tr>
<tr>
<td>2</td>
<td>Toludine blue</td>
<td>Polyphosphate</td>
<td>Blue</td>
</tr>
<tr>
<td>3</td>
<td>Sudan blank-ll</td>
<td>Lipids</td>
<td>Brown</td>
</tr>
<tr>
<td>4</td>
<td>Hemotoxylin</td>
<td>Cellulose</td>
<td>Blue</td>
</tr>
<tr>
<td>5</td>
<td>10%Fecl₃</td>
<td>Tannins</td>
<td>Brown</td>
</tr>
</tbody>
</table>
C. Proximate Values:

The following proximate values were determined for the powder drug of leaves and bark of *P. aculeata* and root of *R. aquatica*.

a. Extractive values

The determination of Extractive values helps to determine the amount of soluble constituents in a given amount of medicinal plant material, when extracted with solvents. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of drug and solvent used. The use of single solvent can also be used by means of providing preliminary information of quality of a particular drug sample. Results are as on table No. 11 to 13.

- **Alcohol soluble extractive value**

5g of shade-dried leaves and bark of *P. aculeata* and root of *R. aquatica* powder was macerated with 100ml of 95% ethanol in a closed flask, shaking frequently during the first 6hrs and allowed to stand for 18hrs. Thereafter it was filtered rapidly taking precaution against loss of ethanol. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentage ethanol soluble extractive was calculated with reference to the shade-dried plant powder. Results are as on table No. 11 to 13.

- **Water soluble extractive value**

5g of shade-dried leaves and bark of *P. aculeata* and root of *R. aquatica* powder was macerated with 100ml of water in a closed flask, shaking frequently during the first 6hrs and allowed to stand for 18hrs. Thereafter it was filtered rapidly. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C and weighed. Percentages of extractive values were calculated with reference to the shade-dried leaf powder. Results are as on table No. 11 to 13.
Material and Methods

b. Moisture content:

An accurately weighed quantity of the shade-dried coarsely powdered leaves and bark of *P. aculeata* and root of *R. aquatica* powder was taken in a tarred glass bottle and the initial weight was taken. The crude drug was heated at 105°C in an oven and weighed. This procedure was repeated till a constant weight was obtained. The moisture content of the sample was calculated as percentage with reference to the shade-dried material. Results are as on table No. 11 to 13.

c. Ash values:

- **Total ash**

2g of accurately weighed quantity of the shade-dried coarsely powdered leaves and bark of *P. aculeata* and root of *R. aquatica* was taken in a tarred silica crucible and incinerated at a temperature not exceeding 450°C until free from carbon, cooled and weighed. The percentage of total ash was calculated with reference to shade-dried leaf powder. Results are as on table No. 11 to 13.

- **Acid-insoluble ash**

Total ash obtained was boiled for five minutes with 25 ml of dilute Hydrochloric acid. The insoluble matter was collected on an ash-less filter paper, washed with hot water and ignited, cooled and weighed. The percentage of acid insoluble ash was calculated with reference to shade-dried leaves, bark and root powder. Results are as on table No. 11 to 13.

- **Water-soluble ash**

Total ash obtained was boiled for five minutes with 25ml of distilled water, cooled and collected the insoluble matter on an ash-less filter paper, washed with hot water and ignited for 15 minutes at temperature not exceeding 450°C. Subtracted the weight of the insoluble ash. The percentage of water-soluble ash was calculated with reference to shade dried leaves, bark and root powder. Results are as on table No. 11 to 13.
Material and Methods

- **Sulphated ash:**

  Silica crucible is heated to redness for 10 minutes; cooled and weighed. 1 gram of air-dried roots powder is placed in silica crucible, moistened with Sulphuric acid, ignited gently, again moistened with Sulphuric acid and ignited at about 800°C. Cooled and weighed, once again ignited for 15 minutes and weighed. The percentage of sulphated ash was calculated with reference to air-dried leaves, bark and root powder. Results are as on table No. 11 to 13.
4.2 Phytochemical Investigations

The Leaves and bark of *P. aculeata* and root of *R. aquatica* were subjected to following phytochemical investigations:

A. Extraction

- Extraction with 95% alcohol.
- Cold maceration.

B. Qualitative chemical identification tests.

C. HPTLC fingerprint profile

A. Extraction of plant material^{116-122}:

- Extraction with 95% alcohol:

The Leaves and bark of *P. aculeata* and root of *R. aquatica* were shade dried at room temperature, pulverized, and coarse powder was extracted exhaustively with 95% ethanol at temperature 40-60°C, in a Soxhlet extractor. The extract was concentrated in a rotary flash evaporator and residue was dried in a desiccator over sodium sulfite.

Method: Cold maceration.

The Leaves and bark of *P. aculeata* and root of *R. aquatica* were shade dried at room temperature, pulverized, and coarse powder was macerate exhaustively with water then being kept for 5 days in tightly sealed vessels at room temperature, protected from sunlight and shaken several times daily and adds preservative. Concentrate extract by distilling off the solvent and then evaporating to dryness on water–bath.

B. Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for leaves and bark of *P. aculeata* and root of *R. aquatica* to know the nature of constituents present in them and their distribution in total alcoholic and aqueous extracts and which extract shows maximum activity then go for further phytochemical and isolation of phytoconstituents.
The observations were recorded in Table No.15 to 17

**Tests for Carbohydrates**

- **Molisch’s test:**
  Treat the extract solution with few drops of alcoholic $\alpha$-napthol. Add 0.2 ml of concentrated H$_2$SO$_4$ slowly through the sides of the test tube, purple to violet colored ring appears at the junction.

- **Benedict’s test:**
  Treat the extract solution with few drops of Benedict’s reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath, reddish brown precipitate forms if reducing sugars are present.

- **Barfoed’s test:**
  General test for monosaccharides: Heat the test tube containing 1ml reagent and 1 ml of extract solution in a beaker of boiling water; if red cuprous oxide is formed within two minutes, a monosaccharide is present. Disaccharides on prolonged heating (about 10min) may also cause reduction, owing to partial hydrolysis to monosaccharides.

- **Selwinoff’s test:**
  Hydrochloric acid reacts with ketose sugar to form derivative of furfuraldehyde, which gives red colored compound when linked with resorcinol. Add extract solution to about 5 ml of reagent and boil. Fructose gives red color within half minute. The test is sensitive to 5.5 mmol/lt. if glucose is absent. If glucose is present it is less sensitive and on addition of large amount of glucose it gives similar color.

- **Fehling’s test:**
  Equal volume of Fehling’s A (Copper sulphate in distilled water) and Fehling’s B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed along with few drops of extract solution, boiled, a brick red precipitate of cuprous oxide forms, if reducing sugars are present.

- **Caramelisation:**
Carbohydrates when treated with strong sulfuric acid, they undergo charring with the dehydration along with burning sugar smell.

- **Tollen’s test:**

  To 100mg of extract add 2ml of Tollen’s reagent, a silver mirror is obtained inside the wall of the test tube, indicates the presence of aldose sugar.

- **Bromine water test:**

  It gets decolorized by aldose but not by the ketose, because bromine water oxidizes selectively the aldehyde group to carboxylic group, giving raise to general class of compounds called aldonic acid.

**Tests for Proteins & Aminoacids**

- **Millon’s Test:**

  Extract solution + 2 ml of Millon’s reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid) white precipitate appears, which turns red upon gentle heating.

- **Ninhydrin Test:**

  Amino acids and proteins when boiled with 0.2% solution of Ninhydrin (Indane 1, 2, 3 trione hydrate), produces violet color.

**Tests for Sterols and Triterpenoids**

- **Libermann-Burchard test:**

  Extract treated with few drops of acetic anhydride, boil and cool, concentrated sulphuric acid is added from the side of the test tube, A brown ring at the junction of two layers and the upper layer turns green indicates the presence of sterols and formation of deep red color indicates the presence of triterpenoids.

- **Salkowski’s test:**

  Treat extract in chloroform with few drops of concentrated Sulfuric acid, shake well and allow to stand for sometime, red color appears in the lower layer indicates the presence of
sterols and formation of yellow colored lower layer indicating the presence of triterpenoids.

Tests for Glycosides

Test I:

Extract 200 mg of the drug by warming in a test tube with 5 ml of dilute (10%) sulphuric acid on a water bath at 100°C for two minutes, centrifuge or filter, pipette out supernatant or filtrate. Neutralize the acid extract with 5% solution of Sodium hydroxide (noting the volume of NaOH added). Add 0.1 ml of Fehling’s solution A and B until alkaline (test with pH paper) and heat on a water bath for 2 minutes. Note the quantity of red precipitate formed and compare with that formed in Test II.

Test II:

Extract 200 mg of the drug using 5 ml of and boil on water bath. After boiling add equal volume of water to the volume of NaOH used in the above test. Add 0.1 ml of Fehling’s A and B until alkaline (red litmus changes to blue) and heat on water bath for two minutes. Note the quantity of the red precipitate formed.

Compare the precipitates of Test II with Test I. If the precipitate in Test-II is greater than in Test-I, then Glycoside may be present. Since Test I represent the amount of free reducing sugar already present in the crude drug, whereas Test-II represents the Glycoside after acid hydrolysis.

Tests for Alkaloids

❖ Mayer’s test: (Potassium mercuric iodide solution).

To the extract/sample solution, add few drops of Mayer’s reagent, creamy white precipitate is produced.

❖ Dragendorff’s Test: (Potassium bismuth iodide solution).

To the extract/sample solution, add few drops of Dragendorff’s reagent, reddish brown precipitate is produced.

❖ Wagner’s test: (Solution of Iodine in Potassium Iodide).
To the extract/sample solution, add few drops of Wagner’s reagent, reddish brown precipitate is produced.

❖ **Hager’s Test**: (Saturated solution of Picric acid)

To the extract/sample solution, add few drops of Hager’s reagent, yellow precipitate is produced.

### Tests for Phenolic Compounds

❖ **Ferric chloride test**:

Extract solution gives **blue-green** color with few drops of FeCl₃.

❖ **Shinoda Test**: (Magnesium Hydrochloride reduction test)

To the extract solution, add few fragments of magnesium ribbon and concentrated Hydrochloric acid drop wise, yellowish, yellow- orange occasionally orange color appears after few minutes.

❖ **Zinc- Hydrochloride reduction test**:

To the extract solution, add a mixture of Zinc dust and concentrated Hydrochloric acid. It gives yellowish, yellow- orange occasionally orange color appears after few minutes.

### Tests for Flavonoids

❖ **Shinoda test**: (Magnesium Hydrochloride reduction test)

To the extract solution add few fragments of magnesium ribbon and concentrated Hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.

❖ **Zinc- Hydrochloride reduction test**:

To the extract solution, add a mixture of Zinc dust and con. Hydrochloric acid. It gives red color after few minutes.
Alkaline reagent test:

To the extract solution, add few drops of Sodium hydroxide solution, formation of an intense yellow color that turns to colorless on addition of few drops of dilute acetic acid indicates the presence of flavonoids.

Tests for Tannins

Gelatin test:

Extract solution with 1% gelatin solution containing 10% sodium chloride gives white precipitate.

Ferric chloride test:

Extract solution gives blue-green color precipitate with FeCl₃.

Vanillin Hydrochloride test:

Extract solution when treated with few drops of Vanillin Hydrochloride reagent gives purple red color.

Alkaline reagent test:

Extract solution with sodium hydroxide solution gives yellow to red precipitate within short time.

Test for Steroidal Glycosides

Kedde’s test:

Extract the root powder with chloroform, evaporate to dryness, and add one drop of 90% of alcohol and 2 drops of 2% 3,5-dinitro benzoic acid (3,5, dinitrobenzene carboxylic acid - Kedde’s reagent) in 90% alcohol. Make alkaline with 20% sodium hydroxide solution. A purple color is produced. The color reaction with dinitrobenzoic acid depends upon the presence of an α,β unsaturated – γ lactone in the aglycone.

C. HPTLC fingerprint profile:

The high performance thin layer chromatography (HPTLC) finger print profile of the aqueous alcoholic extracts of all the selected plants were carried out using pre-coated Silica Gel plates.
as the stationary phase. 100 mg of each dried extract was reconstituted separately in 10 ml of methanol, filtered and clear filtrate was used for the HPTLC fingerprint analysis. It was spotted as a band in different concentrations using a Camag Linomat IV applicator.

The plates were eluted with two different solvent systems separately. Plates were then densitometrically scanned with CAMAG TLC scanner IV using the Wincats software at multi wavelengths either under UV or visible light using Deuterium lamp or Tungsten lamp. Photo documentation was carried out using a Linomat Reprostar unit under UV light at 254 and 366 nm.

4.3 Pharmacological Screening

Pharmacological Screening
A) Animal selection:
The experiment was carried out using Swiss albino mice and rats of either sex weighing between 20-30gm for acute toxicity study and Swiss albino mice and rats of either sex weighing around 20-30 gms and 150-180 g were used for the biological activity. Animals were maintained at normal laboratory conditions and were given standard animal feed.

B) Acute Toxicity Studies:
Albino mice of either sex weighing between 20-30gm were used during investigation. The animals were fast overnight. The OECD guideline no-420 fixed dose method was adopted and accordingly doses of total alcoholic and aqueous extract of leaves and bark of *P. aculeata* and root of *R. aquatica* was calculated.

As per following the OECD guideline no. 420 fixed dose method procedure, the safest dose of total alcoholic extract and aqueous extract were 2000mg/kg body weight. The safe dose was found to be 2000mg/kg body weight; hence 1/10th of the dose was taken as effective dose.

C) Extract used:
Total ethanol extract, aqueous extract of leaves and bark of *P. aculeata* and root of *R. aquatica* were screened for their biological property. The dried alcoholic extract and aqueous extract were suspended in distilled water using tween 80.

4.1.1 Analgesic Activity:
A very large number of methods have been introduced for the laboratory assessment of analgesic activities. This is partly a reflection of the difficulties inherent in using animal preparations in the search for capable of alleviating pain in man. These difficulties can be summarized as follows:

(a) It is impossible to know whether the sensation experienced by animal is response to noxious stimuli is qualitatively similar to the pain felt by human being in similar circumstances.
(b) The relief of pain in human beings may involve more than an interference with pain producing mechanisms or an inhibition of activity in the nervous pathway for pain i.e. physiological factors play prominent part.

(c) The most serious deficiency concerns the nature of the stimulus used to provoke pain.

**1 Peripheral analgesic activity**

Most of the so called peripheral analgesic possess anti-inflammatory properties and in some cases also antipyretic activity besides analgesia. For many of them the mode of action has been elucidated as an inhibition of cyclooxygenase in the prostaglandin pathway. Nevertheless, new peripheral analgesics have to be tested not only for their in vitro activity on cyclooxygenase but also for their in vivo activity.

**2 Central analgesic activities.**

Pain is a symptom of many diseases requiring treatment with analgesics, that means opioids drugs. The addiction liability of opioids led to intensive research for compounds without this side effect. Many approaches have been used to differentiate the various action of strong analgesic by developing animal models not only for analgesic activity but also for addiction liability. Several types of opioids receptors have been identified in the brain allowing in vitro binding tests. However the in vitro tests can only partially substitute for animal experiments involving pain. Pain is a common phenomenon in animals at least in vertebral animal similar to that felt by man.

Analgesic effects in animal are comparable with the therapeutic effect in the man. Needless to say, that in every instance painful stimuli to animal must be restricted as much as possible. Painful stimuli can be consist of direct stimulation of the efferent sensory nerves or stimulation of pain receptor by various means such as heat or pressure. The role of endogenous peptides such as enkephalins and endorphins gives more insight into brain processes and the action of central analgesics.

Pain can also be elicited by inflammations. Progress has been made in elucidating the role of various endogenous substances such prostaglandins and peptides in the inflammatory process. Most of the so called non-steroids anti-inflammatory agents have
also analgesic activity. Lim and Guzman differentiated between antipyretic analgesic causing analgesic by blocking impulse generation at pain receptor in the periphery while the narcotic analgesic block synaptic transmission of impulses signaling pain in the central nerves system. An old but excellent survey on methods being used to test compounds for analgesic activity has been provided. Today the classification into central and peripheral analgesic is definitively too simplified but provides a guide for differentiation by pharmacological methods.

The various methods to screen analgesics using different pain stimuli are summarized below-

**Table No: 09**

<table>
<thead>
<tr>
<th>Pain stimulus</th>
<th>Species</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Thermal method</td>
<td>Mice/Rat</td>
<td>1. Hot plate method.</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>2. Tail flick test.</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td>3. Tail immersion test.</td>
</tr>
<tr>
<td>B. Mechanical methods</td>
<td>Mice/Rat</td>
<td>1. Tail pressure.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Skin pressure.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Distension of hollow viscera.</td>
</tr>
<tr>
<td>C. Chemical methods</td>
<td>Mice/Rat</td>
<td>Writhing test (collier1964) or peritoneal method.</td>
</tr>
<tr>
<td>D. Electrical stimulation</td>
<td>Dog, Rabbit, Guinea pig, Mice</td>
<td>Tooth pulp stimulation. Pododolorimeter.</td>
</tr>
</tbody>
</table>

(a) **Tail Immersion Method:**

**Principle:**
The method has been developed to be selective for morphine-like compounds. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 55°C.

**Procedure:**

Young female Wister rat (150-180g body weight) was placed into individual restraining cages leaving the tail hanging out freely. The animals were allowed to adapt to the cages for 30 min before testing the lower 5cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55°C within a few seconds the rat reacts by withdrawing the tail. The reaction time is recorded in interval of 0.5s by a stopwatch. After each determination the tail was carefully dried. The reaction time was determined before and periodically after either oral or subcutaneous administration of test substance e.g, after 0.5, 1, 2, 3, 4 and 6h. The withdrawal time of untreated animals was between 1 and 5.5s. A withdrawal time of more than 6sec therefore was regarded as a positive response.

**(b) Hot Plate Method:**

**Principle:**

The paw of the mice and rats was very sensitive to heat at temperatures which was not damaging the skin. The response was jumping, withdrawal of the paws licking of the paws. The time until these responses occur was prolonged after administration of centrally acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenyl-acetic acid type do not generally affect these responses.

**Procedure:**

Groups of 6 mice of either sex with an initial weight of 20 to 30 gm were used for each dose. The hot plate, which is commercially available, consists of an electrically heated surface. The temperature is controlled for 55°C to 56°C. This can be copper plate and a heated glass surface. The animal was placed on the hot plate and time until either licking or jumping occurs is recorded oral or subcutaneous administration of the standard or the test compound.
Evaluation:

The prolongation of the latency times comparing the values before and after administration of the test compounds or the values of the control with the experimental groups can be used for statistical comparison using mean ±SEM. ANOVA by Dunnett Compare all Vs Control.

Statistical analysis

All the results were expressed as mean ±SEM. ANOVA by Dunnett Compare all Vs Control.

4.1.2. Anti pyretic activity:

Materials and Methods:

Preparation of extract

Total ethanol extract, aqueous extract, *P. aculeata* and *R. aquatica* was screened for its biological property. The dried alcoholic extracts and aqueous extracts were suspended in distilled water using teen was employed to assess biological activity.

Animals used

Albino rats of either sex weighing 150-180 gm each were used for this experiment. The animals were housed in standard metal cages and provided with food and water ad libitum.

Preparation of standard:

Paracetamol was used as standard drug (positive control). Paracetamol (150 mg/kg body wt) was dissolved in normal saline.

Induction of yeast-induced pyrexia

Rats were divided into eight groups of six each for this experiment. The normal body temperature of each rat was measured rectally at predetermined intervals and recorded.
Fever was induced per the method described by Smith and Hambourger (1935). The rats were trained to remain quiet in a restraint cage. A thermister probe was inserted 3-4 cm deep into the rectum and fastened to the tail by adhesive tape. The temperature was measured on a thermometer. After measuring the basal rectal temperature, animals were given a subcutaneous injection of 10 ml/kg body wt. of 15% w/v yeast suspended in 0.5% w/v methyl cellulose solution. Rats were then returned to their housing cages. After 19 h of yeast injection, the animals were again restrained in individual cages for another recording of their rectal temperature as described above.

**Drug administration**

After 19 h of yeast injection, the extracts were administered orally at doses of 200 mg/kg body wt. to six groups of animals, respectively. A similar volume (5ml/kg body wt.) of normal saline solution was administered orally to the control group. The eighth group of animals received the standard drug Paracetamol (150mg/kg body wt.) orally. Rats were restrained for recording of their rectal temperature at the nineteenth hour, immediately before Extracts, saline or Paracetamol administration, and again at one-hour intervals up to the twenty-third hour after yeast injection.

**Statistical analysis**

All the results were expressed as mean ±SEM. ANOVA by Dunnett Compare all Vs Control.

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**4.1.3. Anti-Inflammatory activity**\(^{101,105,128}\):

**Carrageenan Induced Rat Paw Oedema- (Acute Model):**

**Preparation of extract**
Total ethanol extract, aqueous extract, *P. aculeata* and *R. aquatica* were screened for its biological property. The dried alcoholic extract and aqueous extract will suspended in distilled water using Tween 80 as an emulsifying agent and was employed to assess biological activity.

**Animals use.**

Albino rats of either sex weighing 180-200 g each will use for this experiment. The animals were house in standard metal cages and provided with food and water *ad libitum.*

**Preparation of standard:**

Acute inflammation was induced by injecting carrageenan (0.1 ml of 1% suspension in 0.9% saline) in sub-plantar region and paw volume will measured at 0, 1, 2 and 4 hours.

**Assessment of Anti-inflammatory activity**

After grouping separately, each group was orally administered with one of the following treatments.

All the treatments was administer 30 min prior to carrageenan, except sodium iodide which was give 20 minutes prior to carrageenan. Acute inflammation was induced in each group by injecting 0.1 ml of 1% carrageenan into the subplantar region of right hind paw. A mark was put on the leg at the mallaleous to facilitate the dipping of the leg to the same level at the second and subsequent times.

The initial reading was taken as zero hour, that is, immediately after injecting carrageenan and the procedure was report at zero an hour, one, two, three and four hours after carrageenan injection. The difference between zero hour reading and one of the subsequent readings provides the actual Oedema volume at that time. The mean paw volume at different times was calculate for all groups and the percentage inhibition will then calculated by using the formula;

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

Where \( V_t \) = the amount of oedema formed in drug treated group.

\[ V_c = \text{the amount of oedema formed in the control group.} \]

The results were analyzed by ANOVA followed by Dunnet's test (p-value ≤ 0.05 was taken as significant).

4.1.4. In vitro free radical scavenging antioxidant activity:

(a) **Free radical scavenging activity by DPPH Method\textsuperscript{106-110}:**

Free radical scavenging potentials of the extracts were tested against a methanolic solution of \( \alpha,\alpha \)-diphenyl-\( \beta \)-picryl hydrazyl (DPPH). Antioxidants reacts with DPPH and
convert it to $\alpha,\alpha$-diphenyl-$\beta$-picryl hydrazine. The degree of discoloration indicates the scavenging potentials of the antioxidant extract. The change in the absorbance produced at 517 nm has been used as a measure of antioxidant activity.

**Fig. 06. Reduction of DPPH free radical**

\[ \text{In-Vitro Antioxidant was carried out on total alcohol and aqueous extracts of } P. \text{ aculeata and } R. \text{ aquatica using BHT as standard antioxidant and results are shown in figure no. 124.} \]
The total alcohol and aqueous extracts of *P. aculeata* and *R. aquatica* had shown dose dependant antioxidant activity in DPPH model. The IC$_{50}$ values for total alcohol and aqueous extracts, BHT.

**Materials:** DPPH stock solution (100µM): 39.4 mg of DPPH was dissolved in one liter of analytical grade methanol.

**Preparation of sample solutions of extracts:**
Total ethanol extract, aqueous extract, *P. aculeata* and *R. aquatica* were dissolved in 25 ml of ethanol and filtered, filtrate was used for experiment. The alcoholic extracts and aqueous extracts of all the plants were first dissolved in 5 ml of distilled water, added with 20 ml of ethanol, filtered and the filtrate was used for the experiment.

Preparation of Standard solutions: BHT (Butylated Hydroxy Toluene) was taken as standard. 25 mg dissolved in 20 ml methanol and volume made up to the mark in 25 ml volumetric flask.

**Procedure:** Total ethanol extract, Aqueous extract, *P. aculeata* and *R. aquatica* aliquots of 10, 20, 40, 60, 80 and 100 µl/ml of extracts and 10, 20, 40, 60, 80 and 100 µl/ml of standard solutions were taken in different test tubes, volume was adjusted to 500 µl. To this 5 ml of methanolic solution of DPPH was added, shaken well and the mixture was allowed to stand at room temperature for 20 minutes. The control was prepared as above without extract. The readings were read at 517 nm using methanol as blank. The absorbance of control was first noted at 517nm. The change in absorbance of the samples was measured.

10 to 100 µl (10 to 100 µg) of BHT were taken in different test tubes.

Scavenging activity was expressed as the inhibition percentage calculated using the following formula,
Each experiment was carried out in triplicate and percentage scavenging activities of various extracts are listed.

\[
\% \text{ Anti radical activity} = \frac{\text{CONTROL Abs.} - \text{SAMPLE Abs.}}{\text{CONTROL Abs.}} \times 100
\]