5. Materials & Methods
5. MATERIAL AND METHODS

This chapter deals with the methods utilized to perform different experiments to achieve said goals.

5.1 Pharmacognostical studies:

5.1.1 Collection of Plant Material:

The plant material for the proposed study was collected from the foothills of Sinhgad, Pune.

5.1.2 Identification and authentication:

The plant was identified and authenticated from Botanical survey of India, Pune with Voucher specimen no.- SSBC1

5.1.3 Macroscopic study of plant material:

External features, dimensions and organoleptic properties of roots, stems and leaves were studied.

5.1.4 Microscopic study of plant material:

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 – 12 μm. Dewaxing of the sections was done. The sections were stained with Toluidine blue. Since Toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., wherever necessary sections also stained with safranin and fast-green and iodine (for starch).

5.1.5 Determination of physicochemical constants of powdered plant materials

5.1.5.1 Ash values:

Determination of total ash, acid insoluble ash, water soluble ash and moisture content was carried out by using procedures given in Indian Pharmacopoeia 1996.

5.1.5.2 Extractive value:

Alcohol, chloroform and water-soluble extractive values were determined according to the procedures given in Indian Pharmacopoeia 1996

5.1.6 Extraction of drug materials

1. Roots, stems & leaves were dried under shade and powdered. The powdered material was passed through no. 85 mesh, weighed & then used for extraction.
2. The separately weighed powders were placed in thimbles made up of cellulosic filter paper & successively extracted by Soxhlet extractor for 72 hours using three solvents Chloroform, Ethanol & Water respectively.

3. The resulting extracts were concentrated under reduced pressure using rotary vacuum evaporator to get the syrupy viscous masses. The viscous masses were transferred in porcelain dishes and dried.

4. The amount of extracts were weighed and stored in amber colored airtight bottle at room temperature.

5.2 Preliminary phytochemical screening

5.2.1 Tests for Carbohydrates -
Fehling’s Test and Benedict’s tests were performed.

5.2.2 Tests for Proteins –
Biuret test was performed.

5.2.3 Test for Steroids -
Salkowski test was performed.

5.2.4 Tests for Glycosides -
Borntrager’s test was performed.

5.2.5 Test for Saponin glycosides:
Foam test was performed.

5.2.6 Test for Flavonoids:
Shinoda test was performed.

5.2.7. Test for Alkaloids -
Dragendorff’s test, Hager’s test and Wagner’s test were performed.

5.2.8. Test for Tannins and phenolic compounds -
Presence of Tannins and phenolic compounds were confirmed by using 5% FeCl₃, Lead acetate and dil.KMnO₄ solutions
5.3 Thin Layer Chromatography Profile: (Wagner and Bladt, 1996: 349-364).

5.3.1 Detection of Flavanoids and their Glycosides

Table 5.1: Solvent system

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvents</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-Butanol : Acetic acid : Water</td>
<td>4 : 1 : 5 (Upper phase)</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl acetate: Formic acid: Glacial acetic acid: Water</td>
<td>100: 11: 11 : 26</td>
</tr>
</tbody>
</table>

Detection:

The developed TLC plate was observed in UV light at 269 and 336 nm. Flavanoids and their glycosides appear as yellowish orange zones /spots.

5.3.2 Detection of Saponins:

Table 5.2: Solvent system

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvents</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloroform: Methanol: Water</td>
<td>70:50:4</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform: Acetic acid: Methanol: water</td>
<td>(64:32:12:08)</td>
</tr>
</tbody>
</table>

Detection:

Detection was done by using Vanillin-Sulphuric acid reagent which gave reddish brown colored spot.

5.4 HPTLC analysis of Ethanolic extract of roots of Clerodendrum serratum Linn.:  

**GENERAL CONDITIONS**

Made/ Make of Instrument : Camag (Switzerland),  
Sample Applicator : Linomat IV  
Development Chamber : 10X10, Twin-trough chamber  
Stationary phase : Pre coated silica gel 60 F254  
Alluminium plates (Merk, KgaA, Germany)  
Plate thickness : 0.2 mm  
Plate size : 100 x 100 mm  
Syringe size : 100 µl syringe
**Materials & Methods**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application rate</td>
<td>10 s μl⁻¹</td>
</tr>
<tr>
<td>Table speed</td>
<td>10 mm s⁻¹</td>
</tr>
<tr>
<td>Distance from starting</td>
<td>15 mm</td>
</tr>
<tr>
<td>Distance from bottom</td>
<td>10 mm</td>
</tr>
<tr>
<td>Volume applied</td>
<td>5 – 10 μl</td>
</tr>
<tr>
<td>Band length</td>
<td>10 mm</td>
</tr>
<tr>
<td>Distance between tracks</td>
<td>10 mm</td>
</tr>
<tr>
<td>Development distance</td>
<td>80 mm</td>
</tr>
<tr>
<td>Reagent preparation</td>
<td>As per WHO &amp; API guidelines</td>
</tr>
<tr>
<td>Solvent used</td>
<td>Analytical Reagent grade</td>
</tr>
<tr>
<td>Mobile phase preparation</td>
<td>Solvents ratio (Vol./ Vol.)</td>
</tr>
<tr>
<td>Extract storage vials</td>
<td>5 ml glass vials</td>
</tr>
</tbody>
</table>

**Experimental:**

a) **Sample Preparation:** - Dried ethanolic extract of roots of *Clerodendrum serratum* Linn obtained, was heated in a 50 ml round bottom flask with 6% aqueous hydrochloric acid (25 ml) for 45 min on water bath in order to hydrolyze flavonoid O-glycosides. Aglycones, precipitated on cooling the solution, were removed by filtration, and dissolved in ethanol. The last traces of aglycones were removed from the filtrate by extracting with 3x20 ml quantity of diethyl ether. Latter were combined, dried over anhydrous sodium sulphate, and evaporated under reduced pressure. The diethyl ether extract, thus obtained, was pooled with methanol solution of aglycones. Finally, volume was made up to 25 ml with methanol in a volumetric flask.

b) **Sample application:** - The sample was spotted in a form of a band by means of Hamilton microsyringe on precoated silica gel F plates with the help of Linomat IV spotter (CAMAG).

c) **Chromatographic Conditions :-**

- Application mode: - CAMAG Linomat IV
- Development mode: - CAMAG Twin Trough Chamber.
- Plate material: - HPTLC Silica gel 60 F 254.(E merk)
- Solvent: - Solvents were used as per mentioned in the standard books
- Chamber Saturation: - 1 hour
Materials & Methods

- Development distance: 80mm
- Development time: 30-45min
- Scanner: CAMAG II V 3.14
- Detection: 500nm
- Integrator: CATS V 4.06 Software

The HPTLC Fingerprint Chromatogram at 269 nm and 336 nm was recorded.

5.5 Pharmacological Screening:

5.5.1 Instrument:

Instrument used in the present study

Table: 5.3 Table showing instruments used for study.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Instrument</th>
<th>Model</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Histamine chamber</td>
<td>------</td>
<td>M/S INCO, Ambala</td>
</tr>
<tr>
<td>2.</td>
<td>Plethysmometer</td>
<td>7140</td>
<td>UGO Basile 7140, Italy</td>
</tr>
<tr>
<td>3.</td>
<td>Cooling Centrifuge</td>
<td>C24</td>
<td>REMI C24</td>
</tr>
<tr>
<td>4.</td>
<td>Vacuum Distillation Unit</td>
<td>SS88</td>
<td>JSGW SS88</td>
</tr>
</tbody>
</table>

5.5.2 Drugs, solvents and chemicals:

Table: 5.4 Table showing drugs & chemicals used for study

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Drug</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Histamine dihydrochloride</td>
<td>John Baker INC. Colorado U.S.A.</td>
</tr>
<tr>
<td>2.</td>
<td>Chlorpheniramine maleate</td>
<td>Adroit Pharmaceuticals Pvt. Ltd.</td>
</tr>
<tr>
<td>3.</td>
<td>Clonidine</td>
<td>RPG Life Sciences Ltd.</td>
</tr>
<tr>
<td>4.</td>
<td>Dexamethasone</td>
<td>Cadila Healthcare Ltd.</td>
</tr>
<tr>
<td>5.</td>
<td>Egg albumin</td>
<td>Qualigens Fine Chemicals</td>
</tr>
<tr>
<td>6.</td>
<td>Sod. chromoglycate</td>
<td>Cipla ltd.</td>
</tr>
<tr>
<td>7.</td>
<td>RPMI buffer medium 1640</td>
<td>HiMedia, Mumbai. India.</td>
</tr>
</tbody>
</table>

5.5.3 Selection and maintenance of animals and tissue:

Albino mice (20-25g), Wistar albino rats (150-200g) and Guinea pigs (350-400g) were obtained from National Toxicological Centre, Pune and maintained in the institutes Animal house at room temperature of 25±2° C, relative humidity & 12h dark-
light cycle. Food and water were given ad libitum. The pharmacological work was carried out as per norms of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Institutional Animal Ethics Committee approved the experimental protocol (DYPJISR/ IAEC/08-09/P-14). Acute toxicity studies were carried out as per OECD guidelines and following Goat trachea was obtained from the slaughterhouse and kept in Krebs solution.

5.5.4 Acute Toxicity Studies:

Mice were selected for this study. They were divided into eight groups each containing six animals. C. Serrtum was administered orally in varying doses (0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 and 2.50 g/kg) to these animals. They were continuously observed for 2h to detect changes in the autonomic or behavioral responses like alertness, spontaneous activity, irritability, urination, etc. Any mortality during experimentation and the following 7 days was also recorded. A group of animals treated with vehicle (distilled water) was served as control . Based on the results of preliminary toxicity testing the doses of 50, 100 and 200 mg/kg of Clerodendrum Serrtum L. were chosen for further experiments.

5.5.5 Antiasthmatic study:

5.5.5.1 In-vitro studies

5.5.5.1.1 Isolated goat tracheal chain preparation:

Principle: - The method is used for the study of action of antispasmodic drugs on the tracheal musculature. The method is based upon the findings that the excised goat trachea will respond to many drugs with the characteristic actions for which the drugs are well known, and that with proper magnification, the response can be recorded and measured for comparative purposes. Although, the method is known for its suitability in the study of antispasmodic drugs in general, emphasis is given on its use in the testing of bronchodilators. This is because of the close anatomical and physiological association, which exists between tracheal and bronchial musculature (Castillo and Debeer, 1947: 351-355).

In isolated goat tracheal preparation, there is preponderance of H1 excitatory and a scanty population of H2 inhibitory receptors (Kulshrestha et al., 1983: 107-109; Nagchaudhari and Lahiri, 1974: 149-151) observed the dose relative contractile
responses of different agonists like acetylcholine, histamine, 5-hydroxytryptamine and bradykinin on isolated goat trachea. With these agonists, the concentration necessary to produce contraction was less with goat tracheal chain than with guinea pig tracheal chain. They also found that both goat tracheal chain and strip preparation were suitable for screening spasmogenic activity on respiratory smooth muscle and goat tracheal chain is easier to handle and prepare and is also much more sensitive than guinea-pig tracheal chain.

It is reported that isolated goat trachea contracts in response to acetylcholine (0.1-12.8 μg), histamine (0.1-102.4 μg), and barium chloride (0.1-51.2 μg) in a dose dependent manner and to 5-HT in a narrow dose range. Pheniramine maleate (H1-receptor antagonist) blocks contractions to histamine while cimetidine (H2-receptor antagonist) potentiates the contraction. These observations suggest the presence of both H1-excitatory and H2-inhibitory receptors for histamine on the isolated goat trachea (Kulshrestha et al., 1983: 107-109).

Procedure: (Kulshrestha et al., 1983: 107-109)
1. Overnight Isolated adult Goat tracheal tissue was obtained immediately after slaughter of the animals.
2. Trachea was cut into individual rings and tied together in series to form a chain.
3. Trachea was suspended in bath of Kreb’s solution of the composition:
   NaCl 6.9, KCl 0.35, CaCl2 0.28, MgSO4 0.28, NaHCO3 2.1, KH2PO4 0.16 and Glucose 2.0 gm/litter, which was continuously aerated and maintained at 37 ± 0.5 °C.
4. One end of the tracheal chain was attached to an S-shaped aerator tube and other attached to an isotonic frontal writing lever to smoked drum (magnification 10-12 folds).
5. Tissue was allowed to equilibrate for 45 min. under a load of 400 mg (Nag Chaudhari et al., 1974: 149-151).
6. A dose response curve for histamine was taken in variant molar concentrations, by maintaining 15 min time cycle.
7. After obtaining a dose response curve of histamine on trachea, the Clerodendrum serratum Linn extracts were added to the respective reservoir and same doses of histamine were repeated.
8. Graph of percentage of maximum contractile response on ordinate and negative logarithm of molar concentration of histamine on abscissa were plotted to record dose response curve of histamine, in absence and in presence of *Clerodendrum serratum* Linn extracts.

5.5.5.1.2 Isolated guinea pig ileum preparation:

**Principle:**

Histamine is an autacoids having profound physiological effect in the body. Besides the triple response caused by it, histamine has spasmogenic response on intestinal smooth muscle. By acting on H1 receptors it causes the contraction of intestinal smooth muscle. This model was used to screen the effect of plant extract on histamine-induced contraction of intestinal smooth muscle.

**Preparation of drug solution:**

Test drug was suspended in Tyrode solution. Methanolic extract (100μg/ml), petroleum ether fraction (240μg/ml), benzene fraction (180μg/ml), chloroform fraction (140μg/ml), ethyl acetate fraction (220μg/ml). Histamine (10 μg/ml) was dissolved in distilled water.

**Procedure:**

1. The guinea pigs (overnight fasted) were sacrificed and ileum was mounted in an organ bath containing Tyrode solution and was continuously aerated at 37 ± 0.5°C.

2. One end of ileum was attached to an S-shaped aerator tube and other attached to an isotonic frontal writing lever to smoked drum (magnification 10-12 folds)

3. Tissue was allowed to equilibrate for 30 min. under a load of 500 mg. Contact time cycle was followed for recording the response of histamine.

4. After obtaining a dose response curve of histamine on ileum, the test drug extract in Tyrode solution was added to the reservoir and same dose of histamine were repeated.

5. Graph of percentage of maximum contractile response on ordinate and concentration of histamine on abscissa was plotted to record dose response curve of histamine, in absence and in presence drug extract. (Vogel,1998:362,421-426; Ghosh,1984:21)
5.5.5.2 *In-vivo* method

5.5.5.2.1 Milk-Induced Leucocytosis and Eosinophilia:

**Principle:** Several medicinal properties have been attributed to the plants in the traditional system of medicine. The presence of antistress (adaptogenic) properties in some plant materials is being one of them, as described to be tonics in the Ayurvedic system of medicine. According to Brahmans and Dardymov (1969) the most important characteristic of an adaptogen, is that it increases resistance to adverse influences of a wide range of factors of physical, chemical and biological nature; and its normalization action, which reveals itself irrespective of the direction of the previous pathologic shifts. Ayurveda provides a number of herbs for the treatment of asthma and herbal formulations used for the treatment of asthma include some antistress (nervine support) herbs to enable adoption to stress, since excessive stress or nervous debility may aggravate the symptoms of asthma. After parenteral administration of milk there is increase in TLC, and this stressful condition can be normalized by administration of an antistress or adaptogenic drug (Brekhman and Dardymov, 1969: 419-430). Furthermore leukocytes recruited during asthmatic inflammation release the inflammatory mediators like cytokines, histamine, and major basic protein and promote the ongoing inflammation. This model was used to evaluate the protective effect of *Clerodendrum serratum* Linn. against milk-induced leukocytosis.

Eosinophilia is an abnormal increase in peripheral eosinophil count to more than 4 % of total leukocytes. In the late phase, especially in the development of allergic asthma, eosinophils play role as an inflammatory cell. Eosinophil secretes mediators such as eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDNT), granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), and Prostaglandin (PG), which results in epithelial shedding, bronchoconstriction and promotion of inflammation in respiratory tract (Osama and Joshi, 1984: 321-329). Eosinophilia is associated with respiratory disorder, often allergic in nature together with pulmonary infiltrates that are detectable on chest films. Most of the literatures do not include a diagnostic evaluation and precise practical clinical approach to eosinophilia (Karnick, 1995: 185-187) performed clinical studies and assessed the role of eosinophil in asthmatic response. It was also demonstrated that parental administration of milk produces a marked and significant increase in the leukocytes/eosinophils count after 24hr of its administration.
Materials & Methods

In this model milk in a dose of 4 ml/kg (s.c.) was administered and absolute eosinophil count was taken before and after administration of milk was calculated.

Procedure: - (Brekhman, 1969: 419-430; Vadnere et al., 2007: 487-494)

1. Mice were divided into five groups, five animals in each group.
2. Animals belonging to group-I received distilled water (DW) 10 ml/kg, (p.o.).
3. Animals belonging to group II, III, IV, V received boiled and cooled milk injection in dose of 4 ml/kg, (s.c.).
4. Animals belonging to groups III, IV and V received test extracts of *Clerodendrum serratum* Linn. in dose 50, 100 and 200 mg/kg, p.o. respectively, 1 hr before milk injection.
5. Blood samples were collected from each mouse from the retro orbital plexus, under light ether anesthesia.
6. Total leukocyte / Eosinophilia count was done in each group before drug administration and 24 hr after milk injection.
7. Difference in Total leukocyte / Eosinophilia count before and 24hr after drug administration was calculated.

5.5.5.2.2 Passive Paw Anaphylaxis in Rat:

Principle: - Allergic asthma is a chronic inflammatory process occurring due to exposure of allergen resulting in the activation of T-lymphocytes with subsequent release of inflammatory mediators. Immune-modulating agents are useful in asthma by inhibiting the antigen-antibody (AG: AB) reaction their by inhibiting release of inflammatory mediators (Pungle et al., 2003: 1460-1462). In this method antibodies against egg albumin were raised in rats. Injecting this antibody sensitized the animals. 24 hr after sensitization, the plant extract was administered. After 1 hr of plant extract administration animals were challenged with egg albumin. This model was used to evaluate the protective effect of plant extract against allergen-induced passive paw anaphylaxis and thus to study the effect of plant extract on AG: AB reaction mediated inflammatory response.

Procedure: - (Gokhale and Saraf, 2000: 228-232)

1. Anti serum to egg albumin was raised in rats using aluminum hydroxide gel as an adjuvant.
2. Animals were given three doses of 100 mcg of egg albumin (s.c.) absorbed on 12 mg of aluminum hydroxide gel prepared in 0.5 ml of saline on 1st, 3rd, 5th day.

3. On 10th day of sensitization, the blood was collected from the retro orbital plexus. The collected blood was allowed to clot and the serum was separated by centrifugation at 1500 rpm.

4. Animals were divided into five groups each containing 5 animals. Animals belonging to group I served as control and were administered only the vehicle (10 ml/kg, p.o.).

5. Animals belonging to group II were administered Dexamethasone (0.5 mg/kg, i.p.).

6. Where as animals belonging to groups III, IV and V received Chloroform, ethanolic and aqeous extracts of *Clerodendrum serratum* Linn. in doses 50, 100 and 200 mg/kg, p.o. respectively.

7. The animals were passively sensitized with 0.1 ml of the undiluted serum into the left hind paw. The contra lateral paw received an equal volume of saline.

8. The Chloroform, ethanolic and aqueous extract of *Clerodendrum serratum* Linn. were administered 24 hr after sensitization.

9. 1 hr. after test drug administration, the animals was challenged in the left hind paw with 10 µg of egg albumin in 0.1 ml of saline and the paw inflammation was measured by using a Plethysmometer (UGO Basile, 7140).

10. The difference in the reading prior to and after antigen challenge represented the edema volume and the percent inhibition of edema was calculated by using the formula,

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

    T - Mean relative change in paw volume in test group.

    C - Mean relative change in paw volume in control group.

5.5.5.2.3 Clonidine-induced Catalepsy in mice.

**Principle:** Catalepsy is a condition in which the animal maintains imposed posture for long time before regaining the normal posture. Catalepsy is a sign of extra pyramidal effect of drugs that inhibits dopaminergic transmission or increase/release histamine (inhibitory neurotransmitter) in brain. Clonidine, a α₂-adrenoreceptors agonist induces dose dependent catalepsy in mice, which is inhibited by histamine H₁ receptor antagonist but not by H₂ receptor antagonist (Jadhav et al., 1983: 671-673; Muley et. al., 1979: 671-673). Histamine acts as a modulator of pre-synaptic catecholamine processes in the
CNS by causing depletion of the transmitter stores in the nerve terminals. (Muley et al., 1983: 671-673) showed that intracerebroventricular (i.c.v.) injection of histamine in conscious mice induced catalepsy, which was inhibited by H₁ receptor antagonist, Chlorcyclizine and not by metiamide, an H₂ receptor blocker.

There are histamine containing mast cells in brain. Brain histamine does play a definite role in the production of the extra pyramidal motor symptoms of catalepsy. Therefore it has been suggested that the cataleptic effect of Clonidine in the mouse be mediated by histamine (via H₁ receptors), which is released from the brain mast cells in response to stimulation of α₂ adrenoreceptors by Clonidine (Balsara J.J., 1983: 671-673; Schwartz, 1997: 325-339).

Procedure: - (Ferre et al., 1990: 753-757; Taur et al., 2007: 470-477)

1. Bar test was used to study the effect of test drug extracts on Clonidine induced catalepsy.
2. Mice were divided into five groups, five animals in each group.
3. Animals belonging to group I served as control and were administered the vehicle (10 ml/kg, p.o.).
4. Animals belonging to group II received standard drug Chlorpheniramine maleate (10 mg/kg, i.p.).
5. Animals belonging to groups III, IV and V received three doses 50, 100 and 200 mg/kg p.o. respectively of Chloroform, ethanolic and aqueous extracts of Clerodendrum serratum Linn.
6. The forepaws of mice were placed on a horizontal bar (1 cm in diameter, 3 cm above the table) and the time required to remove the paws from bar was noted for each animal.
7. All the groups received Clonidine (1 mg/kg, s.c.), 1 hr after the test drug administration and the duration of catalepsy were measured at 15, 30, 60, 90, 120, 150 and 180 min.

5.5.5.2.4 Mast Cell Degranulation.

Principle:- The histamine concentration has been calculated to be around 0.3 m. (Uvnas, 1969: 23-32). The clonidine and compound 48/80 act through the dynamic expulsion of granules without causing any damage to the cell wall. Clonidine releases histamine from mast cells in a similar manner to a selective liberator like compound.

**Procedure:** (Lakadawala et al., 1980: 790-791)

1. Rats were divided into five groups, five animals in each group.
2. Animals belonging to group-I received vehicles 5 ml/kg, (p.o)
3. Animals belonging to group-II received Sodium cromoglycate 50 mg/kg, (i.p.).
4. Animals belonging to group-III, IV and V received Chloroform, ethanolic and aqueous extract of *Clerodendrum serratum* Linn. in dose 50, 100 and 200 mg/kg, p.o. respectively.
5. The treatment was continued for 7 days. On day 7th, 2 hour after the assigned treatment mast cells were collected from the peritoneal cavity.
6. 10 ml of normal saline solution was injected into peritoneal cavity and abdomen was gently massaged for 90 second.
7. The peritoneal cavity was carefully opened and the fluid containing mast cells was aspirated and collected in siliconised test tube containing 7 to 10 ml of RPMI-1640 Medium (pH 7.2- 7.4).
8. The mast cells were then washed thrice by centrifugation at low speed (400-500 rpm) and the pallet of mast cells were taken in the medium.
9. The mast cells suspension approximately (1 x 10^6 cells/ml) was challenged with 0.5 μg/ml of clonidine solution and stained with 1 % toluidine blue and observed under high power microscope field (400 X).
10. Total 100 cells were counted from different visual areas and the number of intact and degranulated cells was counted. The percent protection was calculated.

**5.5.5.2.5 Bronchial alveolar lavage in rats:**

**Principle:** - Allergic inflammation associated with airway hyper reactivity is the main feature of allergic asthma. The inflammatory response is characterized by an increase in the numbers of eosinophils and mast cells (Wardlaw, 1988: 62–69) mucus hyper secretion and activation of T cells (Lukacs, 2001: 108–116). Several studies have shown that T-helper type (Th2) cells play a major role in the initiation and maintenance of allergic airway inflammation and asthma through their increased production of Th2-type

**Procedure:** (Richard W. Chapman, et al., 2007: 215–221)

1. Animals was divided into five groups each group containing five animals. (n=5)

2. All the animals was sensitized by an intraperitoneal injection of 1ml alum precipitate antigen containing 20μg of ovaalbumin and 8mg of alum suspended in 0.9% sodium chloride solution.

3. A booster injection of this alum-ovalbumin mixture was given 7 days later.

4. Non sensitized animals was injected with alum only.

5. Seven days after (15 day) second injection animals was exposed to aerosolized ovaalbumin(1%) for 30 min.

6. Standard & test group was received Dexamethasone (1mg/kg, i.p.) as standard and Chloroform, ethanolic and aqueous extracts of Clerodendrum serratum Linn. In doses 50, 100 and 200 mg/kg as test drug 5 hr before antigen challenge.

7. The rats were sacrificed at the end of study (24 hr after sensitization) and tracheal catheter was inserted in trachea.

8. Bronchoalveolar lavage fluid was collected by lavaging the lung with 2 aquilots of 5 ml of 0.9% sodium chloride solution total recovery volume per rat was approximately 8ml.
Total leukocytes and eosinophil, neutrophil was counted under microscope and histopathological evaluation of lung tissue was carried out.

Fig. No: 5.1: Plethysmometer, 7140 UGO Basile, Italy

Fig. No. 5.2: Rat paw edema measurement
5.5.5.3 Statistical Analysis:

All observations were presented as Mean ± SEM. The data was analyzed by student’s t-test or one-way ANOVA followed by Dunnett’s test. P < 0.05 was considered as significant.

5.6 Isolation and characterization:

5.6.1 Isolation of Compound 1:

Procedure.

Dried powdered roots (500g) was extracted with ethanol using soxhlet apparatus. The extract obtained was dried in rotary vacuum evaporator at 40°C, yielding a dark brown colored viscous mass 50g (10.0%). 10g of the extract was treated with lead acetate, resulting a yellow precipitate. The precipitate was suspended in methanol, treated with hydrogen sulphide to remove the lead and then filtered. After evaporating the filtrate, the residue was treated with boiling water and extracted with ether. Ether extract was concentrated and filtered and the filtrate was then extracted with sodium hydrogen carbonate solution and acidified with hydrochloric acid. Recrystallization from alcohol-water resulted an yellowish brown amorphous residue (50mg). The resultant compound was then characterized by using UV, FTIR, $^1$H-NMR and FAB-MS spectroscopy (Kaneta M., et. al., 1971: 40-44).
5.6.2 Isolation of Compound 2:

Procedure.

Ethanolic extract of roots of *Clerodendrum serratum* (15 gm) was taken into the minimum amount of methanol. Solution was stirred until the extract gets well dissolved into the methanol. Then cold ether was slowly added into the methanolic solution. A yellow colored ppt. was separated out on addition of cold ether (Noorwala Mushtaq, et. al., 1995 : 170). The solution was filtered and dried under vacuum to get brownish yellow powder (8.20 g). Further purification was done on silica gel column chromatography (60-120) (column dimension (3 × 120 cm) using solvent system CHCl₃:MeOH with increasing polarity. The fraction with solvent ratio (7:3) gave an brown colored amorphous compound (2.87gm), which was further subjected to characterization by using UV, FTIR, IH-NMR and DART-MS spectroscopy.