Materials and Methods
4. MATERIALS AND METHODS

4.1 Pharmacognostical analysis

4.1.1 Identification and Collection of the Plant Material

1) *Moringa oleifera*

Seed kernels of *M. oleifera* were purchased from the local market of Ahmedabad and were identified and authenticated by Dept. of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad, India. A voucher specimen was deposited at the Dept. of Pharmacognosy, Ahmedabad.

2) *Achyranthes aspera*

The whole herb of *A. aspera* was uprooted from the L.M. College of Pharmacy campus Ahmedabad, in the month of September-October at the end of flowering season. The plant was identified by comparing it morphologically and microscopically with description given in different standard texts and floras (Kirtikar and Basu 1935). The plant was further identified and authenticated at the Dept. of Pharmacognosy, Gujarat Ayurved University, Jamnagar, India and a voucher specimen was deposited. The plant material was cleaned and dried in shade. It was powdered, passed through 40# and stored at 25 °C.

4.1.2 Macroscopic Observations

The drugs were subjected to macroscopic studies which comprised of study of organoleptic characters of the drugs viz., color, odour, appearance, taste, smell, texture, fracture, etc.

4.1.3 Microscopic Studies

4.1.3.1 *Stem/ Root/ Leaf/ Seed kernel*

For microscopical examination of drug, free hand transverse sections of the drug were taken and cleared with chloral hydrate. The sections were treated with phloroglucinol and a drop of concentrated hydrochloric acid to stain lignified material. Lignified elements were colored pink.
4.1.3.2 Powder Studies

Dried seed kernels of *M. oleifera* and dried plant of *A. aspera* were oven dried at 60°C for 4-6 hrs to make it moisture free and grounded using electric grinder and 60# powder was prepared. For microscopical examination, a slide of powdered drug was prepared in the same manner as mentioned above.

4.1.4 Evaluation of Physical Parameters

4.1.4.1 Moisture content

Five grams of accurately weighed drug powder was heated at 105 °C in an oven to a constant weight. Weight loss after drying gave the moisture content of the material.

4.1.4.2 Determination of foreign matter

100-500 g of the drug sample to be examined was weighed accurately, and spread out in a thin layer. Foreign matter was detected by inspection with the unaided eye or by the use of lens (6 X). The foreign matter was separated and weighed and percentage foreign matter was calculated.

4.1.5 Determination of Ash Values

4.1.5.1 Determination of total ash

Accurately weighed 2 g of the powdered drug was taken in a tared silica dish and it was incinerated at a temperature not exceeding 450 °C until free from carbon. The sample was cooled and weighed. If a carbon free ash cannot be obtained in this way, the charred mass was exhausted with hot water. The residue was collected on an ashless filter paper and the residue and the filter paper were incinerated the filtrate was evaporated to dryness, and ignited at a temperature not exceeding 450°C. The percentage of ash was calculated with reference to the air dried drug.
4.1.5.2 Determination of acid-insoluble ash

The ash obtained as described in the section 4.1.5.1 was boiled for 5 min. with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a Gooch crucible or on an ashless filter paper and washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air dried drug.

4.1.6 Estimation of Sodium and Potassium

The ash obtained as described in the section 4.1.5.1 was dissolved in measured quantity of water and amount of Sodium (Na) and Potassium (K) was estimated by flame photometer.

4.1.7 Determination of Extractive Values

4.1.7.1 Determination of ethanol-soluble extractive

Five grams of the coarsely powdered drug was macerated with 100 ml of ethanol (95 %) in a closed flask for twenty-four hours. The flasks were shaken intermittently during six hours and allowed to stand for eighteen hours. The extract was filtered rapidly, taking precautions against loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat-bottomed shallow dish, and dried at 100°C, to a constant weight. The percentage of ethanol-soluble extractive was calculated with reference to the air dried drug.

4.1.7.2 Determination of water-soluble extractive

Water-soluble extractive was obtained by following the same procedure as described for ethanolic-soluble extractive using chloroform water (0.25 % chloroform in water) instead of ethanol.
4.2 Phytochemical study

4.2.1. Preparation of plant extracts

*Moringa oleifera*

The coarse powder (500g) of the dried seed kernels was defatted using petrol ether and then it was exhaustively extracted using 95% ethanol (2000 ml) in a soxhlet extractor. The extract was concentrated under reduced pressure to yield a syrupy mass. The extracts were stored in air tight container in cool place and used throughout the project.

Cold aqueous extract of *M. oleifera* was prepared by extracting 1 part of seed kernels with 10 parts of water for 2 hrs without heating, while hot aqueous extract was prepared by heating the seed kernels with water.

*Achyranthes aspera*

The coarse powder (1.0 kg) of the dried plant was extracted with alcohol using soxhlet extractor. The extract was concentrated under reduced pressure to yield a syrupy mass.

**Preparation of Ghanvati (Tablets)**

Ghanvati is an ayurvedic form of dried aqueous extract of the drug. Dried powder of the whole plant called ‘Panchang’ in ayurveda was taken in neat and clean stainless vessel. It was heated with 2.5 times of water at 70°C -80°C until the volume of water reduces to one fourth. The whole mass was filtered. The filtrate was concentrated by heating at 80°C till it reduced to half of its volume. This was filtrate I. The marc was further heated with 1.5 times of water and similarly concentrated and filtered. This was filtrate II. The residue was discarded. Both the filtrate I and filtrate II were mixed and concentrated at 90°C till it reduced to a semisolid mass. Ghanvati was prepared from that mass in tablet form using a tablet making machine. The weight of each ghanvati was 500 mg. No other excipients were added, while preparing the ghanvatis except dry powder of *A. aspera* to help in formation of pills from the semisolid aqueous extracts. The tablets were packed in small airtight containers and properly labeled.
4.2.2. Preliminary Phytochemical Screening

Ethanolic extracts of the drugs prepared as mentioned above, were screened qualitatively for the major groups of chemical constituents using standard reagents. Small quantities of all the extracts were dissolved in ethanol and were subjected to preliminary phytochemical analysis for the detection of the individual components using specific reagents.

**Alkaloids**

A few ml (2-3 ml) of ethanolic extract was evaporated in a watch glass. One ml of dilute hydrochloric acid and a few drops of Mayer’s reagent are added to the residue. White precipitate indicated the presence of alkaloids.

A drop of ethanolic extract was spotted on a small piece of precoated TLC plate. The plate was sprayed with modified Dragendorff’s reagent. Orange coloration of the spot indicated the presence of alkaloids.

**Steroids and Terpenoids**

To one ml of ethanolic extract of drug, one ml of chloroform and 2 to 3 ml of acetic anhydride was added. To the above mixture, 1 to 2 drops of concentrated Sulphuric acid was added. Dark green coloration of the solution indicated the presence of steroids and dark pink or red coloration of the solution indicated the presence of terpenoids.

**Flavonoids**

To a 2-3 ml of ethanolic extract, a piece of magnesium ribbon and 1 ml of concentrated hydrochloric acid was added. Pink red or red coloration of the solution indicated the presence of flavonoids in the drug.

**Tannins**

To a 2-3 ml of ethanolic extract, 10 % ethanolic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicated the presence of tannins in the drug.
4.3 Clinical study

An open label, noncomparative clinical study was carried out on patients of either sex, having mild to moderate bronchial asthma and visiting Out Patient Department of Govt. Ayurvedic Hospital, Ahmedabad, India. The protocol (annexure-1) for carrying out the clinical study was approved by Director, Dept of Ayurveda and homeopathic medicine, Govt. of Gujarat, India and also by the institutional ethics committee for the clinical study. Informed consent (annexure-2) was obtained from all patients enrolled in the study.

**Inclusion Criteria:** Patients in age range of 15-75 years having mild to moderate bronchial asthma as diagnosed by their clinical history; commonly observed symptoms of bronchial asthma (Dyspnoea, wheezing, tightness in chest, cough etc.) and physical examination were enrolled in the study.

Diagnosis was based on complete clinical history, physical examination, temperamental assessment, systemic examination and Hematological examination. Pulmonary function test was also conducted before and at the end of the study.

**Exclusion Criteria:** Patients having breathlessness due to cardiovascular disorders, having very severe bronchial asthma (PEFR<20%, FEV1<20% of predicted value) or having pulmonary tuberculosis (confirmed by Chest Screening), cardiovascular disorders, pregnant women etc. were excluded from the study.

Of the patients satisfying the inclusion and exclusion criteria, baseline characteristic were measured and clinical and family history was recorded. Details of duration of bronchial asthma and other diseases if present were recorded (annexure-1).
Treatment

1) *Moringa oleifera*

Patients were given finely powdered dried seeds in dose of 3gm bid for 3 weeks and were advised to take it with water.

2) *Achyranthes aspera*

Patients were given 4 Ghanvatis bid for 3 weeks and were advised to take it with water.

Preliminary clinical study of *M. oleifera* was carried out in 25 patients by measuring the Peak expiratory flow rate (PEFR) and recorded with the help of mini Wright’s Peak flow meter. General physical examinations which include Temperature, Heart rate, Blood pressure were measured before start of the treatment and every week after start of the treatment. Hematological examination which include Hemoglobin (Hb) estimation, Total leukocyte count (TC), Differential leukocyte count (DC) and Erythrocyte sedimentation rate (ESR) were carried out before the start of treatment with *M. oleifera* and subsequently at the end of 3 weeks treatment. PEFR measurements were carried out before the start of the treatment with *M. oleifera* and subsequently at the end of 3 weeks treatment. Patients were given medication supply for 1 week and were asked to report every week. At weekly visit, patients were asked for occurrence of any untoward effect if any and improvement in the symptoms observed. Symptom score was measured for all commonly observed symptoms of bronchial asthma i.e. dyspnoea, wheezing, cough and chest tightness before starting the treatment and at the end of 3 weeks of treatment. Score was graded as 3, 2, 1 and 0 for presence of severe, moderate mild and absence of any symptom respectively.

PEFR measurement was carried out by asking the patient to keep mouthpiece of peak flow meter in mouth and hold it in the mouth firmly with the help of lips.
Materials and Methods

Patient was asked to exhale air through the mouthpiece and reading was noted down. The best of three readings were considered.

Further, detailed study of Lung functions after treatment with *M. oleifera* or *A. aspera* were carried out with the help of Spirometer. General physical examination was carried out every week after start of the treatment. The assessment was made at weekly intervals and the results were analyzed in terms of disappearance of symptoms/signs. Hematological examination was carried out before the start of the treatment with *M. oleifera* or *A. aspera* and subsequently at the end of 3 weeks treatment.

Evaluation of lung function was done with the help of computer aided spirometer at Astodia TB hospital, Ahmedabad. Spirometry was carried out before start of the treatment with *M. oleifera* or *A. aspera* and also, subsequently at the end of 3 weeks treatment. Patient was asked to keep mouthpiece of spirometer in mouth and hold it in the mouth firmly with the help of lips. Patient was asked to breathe normally a few times then to take in a deep inspiration, as much as he / she can. Then, he / she is immediately instructed to blow out as hard and as fast as possible and keep breathing out till he can do so no more. The spirogram and the values of lung volumes and lung flow rates obtained were recorded. The whole process was repeated three times and the best results were recorded. Parameters assessed were Forced vital capacity (FVC), Forced expiratory volume in 1 second (FEV₁), Peak expiratory flow rate (PEFR), and Forced expiratory flow rate (FEF 25-75%). Maximum ventilatory volume (MVV) was determined by asking the patient to breathe as hard and fast as possible for 1 minute.

**Statistical analysis:**
The values were expressed as mean ± S.E.M. Statistical significance of difference in parameters before and after treatment was determined by student's paired t-test. P<0.05 was considered to be significant.
4.4 Pharmacological evaluation

Experimental study

Animal experiments
All animals were housed at ambient temperature (22±1°C), relative humidity (55±5%) and 12h/12h light dark cycle. Animals had access to standard pallet diet and water given ad libitum. The protocol of the experiment was approved by the institutional animal ethical committee as per the guidance of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

4.4.1 Treatment
Ethanolic extracts of the drugs *M. oleifera* or *A. aspera* were prepared as mentioned above. Weighted quantities of the extracts were dissolved in water and subjected to experimental study.

4.4.2 Studies on Acetylcholine and Histamine induced bronchospasm in guinea pigs
Guinea pigs of either sex weighing 350-500g were selected and randomly divided into six groups each containing six animals. The drugs were administered orally in 0.5% sodium carboxymethyl cellulose (CMC). The single dose treatments were given one and half an hour before the study. The following schedule of treatment was administered:

- **Group I:** 0.5% CMC (control)
- **Group II:** Ketotifen (1 mg/kg) (standard)
- **Group III & Group IV:** Alcoholic extract of *M. oleifera* (100mg/kg and 200mg/kg)
- **Group V & Group VI:** Alcoholic extract of *A. aspera* (150mg/kg and 300mg/kg)
Later the animals were exposed to an aerosol of 0.25% histamine and time for preconvulsion state was noted for each animal as described by Sheth et al. (1972). After about 15 days of wash out period, the same animals were given the above treatments and time for preconvulsion state was noted for 0.5% acetylcholine bromide aerosol spray.

4.4.3 Studies on isolated Guinea pig ileum
Overnight fasted guinea pigs of either sex weighing 400-600g were sacrificed using cervical dislocation method. Ileum was quickly dissected out and mounted in an organ bath maintained at 37 ±1°C and containing 20 ml Tyrode’s solution under basal tension of 500 mg. The composition of solution in mM was NaCl, 137; CaCl₂, 1.8; KCl, 2.7; glucose, 5.55; NaHCO₃, 11.9; MgCl₂, 1; NaH₂PO₄, 0.4. The solution was continuously bubbled with air. The responses to drug were recorded on a student physiograph (BioDevices) using isotonic transducer, which exerted a basal tension equivalent to 500 mg load on tissue. The issue was allowed to equilibrate for 30 min., during which, the bathing solution was changed at every 10 min. The contractile responses of ileum to various agonists (Acetylcholine, Histamine, 5-HT and BaCl₂) were recorded in presence and absence of alcoholic extract of M. oleifera or A. aspera.

4.4.4 Studies on compound 48/80 and egg albumin induced rat peritoneal mast cell degranulation
Normal saline containing 5 units /ml of heparin was injected in the peritoneal cavity of male rats lightly anaesthetized with ether. After a gentle abdominal message, the peritoneal fluid containing mast cells was collected in centrifuge tubes placed over ice. Peritoneal fluid of 4-5 rats was collected and pooled and centrifuged at 2000 rpm for 5 min. Supernatent solution was discarded and the cells were washed twice with saline and resuspended in 1 ml of saline.
Degranulation of rat peritoneal mast cell was induced in vitro by two different stimuli:

1) Non-immunological (Compound 48/80 induced)
2) Immunological (Egg albumin induced)

**Non-immunological (Compound 48/80 induced)**

0.1 ml of the peritoneal cell suspension was transferred to 6 test tubes and was treated as follows.

- Test tube no.1 & 2 - Saline
- Test tube no.3, 4 and 5 - 0.1ml of test agent in Saline (alcoholic extract of *M. oleifera* or aqueous extract of *A. aspera*)
- Test tube no.6 - 0.1ml of 10μg/ml of Ketotifen fumarate

Each test tube was incubated for 15 min at 37°C and then Compound 48/80 (0.1 ml, 10μg/ml) was added to each test tube except test tube no. 1. After further incubation for 10 min. at 37°C, the cells were stained with 0.1% toluidine blue solution made in distilled water and examined under the high power of light microscope. Percent protection of the mast cells in the control group and the treated groups were calculated by counting the number of degranulated mast cells from total of at least 100 mast cells counted.

**Immunological (Egg albumin induced)**

Rats were sensitized by administering three doses of 350 μg of egg albumin adsorbed on 60 mg of aluminum hydroxide gel, the doses being given on the first, third and fifth day subcutaneously. The mast cells were collected on the tenth day of sensitization. The study was conducted in the same manner as above and the sensitized cells were degranulated using egg albumin (1mg/ml). Percent protection of the mast cells in the control group and the treated groups were calculated by counting the number of degranulated mast cells from total of at least 100 mast cells counted. Control group consisted of positive control group in which egg albumin was added without addition of test agent and a negative control group in which neither egg albumin nor the test agent was added to correct for spontaneous degranulation of mast cells without any degranulating agent.
4.4.5 Anti inflammatory study

Carrageenan induced rat paw edema
Albino rats of either sex weighing 200-250 g were divided in 4 groups of 6 animals each.
Group I - 0.5% CMC
Group II - Diclofenac sodium (20 mg/kg p.o.)
Group III & IV- Alcoholic extract of *M. oleifera* (200mg/kg & 400mg/kg p.o.) or alcoholic extract of *A. aspera* (250mg/kg & 500mg/kg p.o.) in 0.5% CMC

Animals were treated with drugs and subsequently 1 hr after treatment; 0.1 ml of 1 % carrageenan was injected subcutaneously into the planter region of right hind paw to induce edema. The paw volume was measured initially and at 1, 3 and 5 hr after carrageenan injection using plethysmographic method of Harris and Spencer (1962). Percentage increase in paw volume from baseline was calculated and compared with control.

4.4.6 Anti microbial Studies
The in-vitro antimicrobial activity of the *M. oleifera* was studied by broth dilution method and minimum inhibitory concentration (MIC) was found out. Cold aqueous extract, hot aqueous extract and the alcoholic extract were prepared from the seeds of *M. oleifera* and aqueous and alcoholic extract were prepared from *A. aspera*. These extracts at different concentrations (5-100mg/ml) were tested against the organisms *Escherichia coli*, *Staphylococcus aureus* and *pseudomonas aeruginosa*.

Statistical analysis: The values were expressed as mean ± S.E.M. Statistical significance was determined by student’s t-test. P<0.05 was considered to be significant.
4.5 Isolation of active principle.

Extraction and isolation of active compound was done by following method. Defatted seed kernels were incubated with 10 parts of water for two hours and the filtrate was extracted with EtoAc. The concentrated EtoAc solution was chromatographed on silica gel (3 x 60 cm) with EtoAc/MeOH/H₂O (95:4:1). The fractions were chromatographed on TLC plate using the same solvent. The fractions giving single spot were trapped and subjected to the structure characterization.

Characterization and purity check by HPTLC, UV, IR, LCMS, and NMR.

Thin Layer Chromatography

For TLC experiments, precoated plates of silica gel 60F₂₅₄ (E. Merck) were used and spotting was done on CAMAG LINOMAT IV Automatic TLC spotter. For purity assessment of the isolated compound, and for recording UV spectrum of the compound, the plates were scanned on CAMAG TLC Scanner 3. The purity of the compound isolated was checked by carrying out TLC in different solvent systems and co-chromatography and recording chromatogram and UV absorption spectrum of compound separated on TLC at start, middle and end position of the band.

Characterization by UV, IR, LCMS and NMR

The sample was spotted and developed in a solvent system containing ethyl acetate:methanol: water (95: 4: 1). The UV absorption spectra were recorded on a CAMAG TLC Scanner. UV absorption spectrum of the isolated sample in methanol was recorded on UV/VIS spectrophotometer (ELICO). IR spectra was recorded on BUCK SCIENTIFIC IR Spectrophotometer (model 500). Atmospheric pressure ionisation with ion spray mass spectra of molecular ions were obtained on a PE SCIEX API 165 MS with Waters LCMS. NMR spectra was recorded in CDCL₃ and DMSO using BRUKER DRX FTNMR spectrometer.