4. Material And Methods
4.1 Authentication of plant materials by morphological characters:

Bark of *Albizia lebbeck*, aerial parts of *Euphorbia hirta* and *Sphaeranthus indicus* were collected from local campus of Vallabh Vidhyanagar in month of March, September and December respectively. The samples were authenticated by comparing the morphological characters described in literature.\(^{105, 159, 230}\) The authenticity was further confirmed by a Taxonomist, Department of Bioscince, Sardar Patel University, Vallabh Vidyanagar. A voucher specimen Argh # 5, Argh # 6 and Argh # 7 are preserved in the Department of Pharmacognosy, A R College of Pharmacy, Vallabh Vidyanagar. The morphological characters of the parts are shown in Fig. 5, 8, and 12 and described.

4.2 Microscopical study of the plant materials.

Free hand transverse sections of fresh sample were taken and they were studied in detail. Powder study of all the three samples were also carried out\(^ {262}\). (Fig. 6 & 7, 9-11 and 13-16) Leaf constants were also determined for leaves of *E. hirta* and *S. indicus*\(^ {263, 264}\). (Table 9 & 10)

4.3 Collection and preparation of plant materials.

Bark of *Albizia lebbeck* and aerial parts of *Euphorbia hirta* and *Sphaeranthus indicus* were collected in bulk quantity after their confirmed authenticity. The plant materials were washed with water to remove soil, mud, debris and other adhering materials and dried thoroughly in air under shade at room temperature (28 ± 2\(^\circ\)C) for five
Materials and methods

days. Coarse powder of each drug was prepared, passed through sieve 
# 40 and stored in airtight container.

4.4 Quality parameters.

4.4.1 Determination of Ash values\textsuperscript{265, 266}:

a. Total ash:
Accurately weighed 2g air dried 40# powder of each sample was taken in the tarred silica crucible separately and incinerated at temperature not exceeding 450° C in muffle furnace until free from carbon. Ash obtained was cooled in desiccator and weighed. Percentage of total ash was calculated with reference to air-dried drug.

b. Acid insoluble ash:
Boiled the ash obtained in total ash content as described above separately with 25 ml of 2M hydrochloric acid for 5min. cooled, filtered and collected the insoluble matter on an ashless filter paper, washed with hot water, ignited, cooled in a desiccators and weighed. The percentage of the acid insoluble ash was calculated with reference to air-dried drug separately.

c. Water soluble ash:
Boiled the ashes obtained in total ash content as described above separately with 25 ml of water for 5min. cooled, filtered and collected the insoluble matter on an ashless filter paper, washed with hot water, ignited, cooled in a desiccators and weighed. Difference in weight of total ash and weight of water insoluble matter gave the weight of water soluble ash. The percentage of the water soluble ash was calculated with reference to air-dried drug separately.

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4.4.2 Determination of extractive values\textsuperscript{265,266}:

a. Ethanol soluble extractive value:
Accurately weighed 5g of the air-dried 40# powder of each sample were macerated with 100ml of ethanol in a closed flask for 24h, shaking frequently during first 6h. It was then allowed to stand for 18h and filtered rapidly taking precautions against loss of ethanol. 25ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish, dried at 105° C and weighed. The percentage of ethanol soluble extractive was calculated with reference to the air-dried drug.

b. Water soluble extractive value:
The above same procedure was followed using chloroform water instead of ethanol. The percentage of water-soluble extractive was calculated with reference to the air-dried drug. Moisture content/loss on drying was also determined for each sample.

4.4.3 Heavy Metal Detection:
Heavy metal analysis of all the three samples was done Inductive Coupled Plasma Spectrometer. (Perkin Elmer, Optima 3300 RL)
Procedure: For acid digestion of sample, took 0.5g sample and add 7ml of HNO\textsubscript{3} in a closed vessel device using temperature control microwave heating at 185° C for 15min then after cooling vessel device, solution was filtered and washed by deionized water and volume was made up to 100ml. Instrument was calibrated by using reference standard of 1ppm. Reference standard identification: C.P.A Ltd, Bulgaria.
The different values obtained from the quality parameters for each sample are recorded in table 11 & 12.

**4.5 Preparation of successive extracts:**

**Successive solvent extraction**

The successive solvent extraction was performed by subjecting the dry powdered drug to the extraction by various solvents in order of increasing polarity. 100g of dried coarsely powder material was packed in Soxhlet's apparatus and then it was extracted with petrol ether (60-80°), benzene, chloroform, methanol. Finally drug subjected to maceration with chloroform water I.P. for 48 hours. The completion of extraction was confirmed by evaporating a few drops of each extract from the thimble on a watch glass and ensuring that no residue remained after evaporation of the solvent. The liquid extracts obtained with different solvents were collected separately. After each extraction, the exhausted drug was taken out and kept in open to evaporate the solvent. After drying, it was packed again in Soxhlet's apparatus and extract with next polar solvent.

The extracts were concentrated by distillation until thick viscous residue remained in distillation flask. The final volumes of the extract were evaporated to dryness below 60° C, dried in vacuum desiccator and weighed. The percentage (w/w) of dry extract was calculated on the basis of dried material. (Table 13-15)

**4.6 Qualitative Analysis of Extracts:**

The extract obtained after successive solvent extraction were subjected to several chemical tests to indicate the presence or absence of particular plant constituents. The tests for particular plant constituent
were only to those solvent extracts by which they are normally extracted.

**Test for alkaloids:**

About 0.5g of each dried successive extract was taken in 5% HCl (2ml) solution. The filtrate was tested with different alkaloidal reagents such as Dragendorff’s, Hager’s and Wagner’s reagents. Presence of orange red, yellow and reddish brown precipitates with respective reagents detected the presence of alkaloids.

**Test for flavanoids:**

*Shinoda test:* To 10mg dried extract 10 ml ethanol (95%) was added and heated on water bath for 2min. A small piece of Magnesium ribbon was added to the alcoholic solution followed by 3 drops of concentrated HCl. Development of magenta red or orange color indicates presence of flavanoids.

*Fluorescence test:* To 10mg of extract 10ml methanol was added, boiled and filtered while hot and filtrate was evaporated to dryness. To the residue, 0.3ml of boric acid (3% w/v) and 1ml oxalic acid (10% w/v) were added. The mixture was evaporated on boiling water bath and the residue was extracted with 10ml ether. Greenish yellow fluorescence of the ethereal solution confirms presence of flavanoids.

**Test for saponins:**

*Froth test:* Small portion of dried extract was vigorously shaken with 10ml of distilled water in a test tube for 30sec and was left undisturbed for 20min. Persistent froth indicated presence of saponins.
**Materials and methods**

Haemolytic zone test: 0.5ml of blood was mixed with gelatin solution (3g gelatin powder dissolved in 100ml of 0.85% NaCl solution at 60°C) and taken on a glass slide. A thick section of the each sample was placed on it. A clear haemolytic zone was formed around the section indicating the presence of saponins in the drug.

**Test for carbohydrates:**

Molisch’s test: Small portion of each extract was dissolved in ethanol. To the solution add α-napthol and concentrated H$_2$SO$_4$. Purple color indicates the presence of carbohydrates.

**Test for phytosterols and triterpenoids:**

Liebermann Burchardt test: To the small portion of each extract 5 drops of acetic anhydride was added on white clean tile. Mass was mixed properly and 2 drops of concentrated H$_2$SO$_4$ was added. Formation of red, purple or green color indicates presence of sterols and triterpenoids.

Salkowski reaction: To the small portion each extract add 3ml of chloroform and 2ml concentrated H$_2$SO$_4$. Shake well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence indicates presence of steroids and triterpenoids.

**Test for tannins:**

Test with gelatin: To each dried extract add 1% gelatin solution containing NaCl. Heavy white precipitates indicate presence of tannins.

Reaction with lead acetate: To each dried extract add 2ml of 10% solution of lead acetate. Yellowish white precipitates indicate presence of tannins.
**Materials and methods**

**Test for phenolic compounds:**

Test with FeCl₃: Each dried extract was dissolved in methanol and to this 2ml of freshly prepared FeCl₃ solution was added. Development of brownish green color indicates presence of phenolic.

Test with Folin ciocalteu reagent: Each dried extract was dissolved in methanol and to this a drop of Folin ciocalteu reagent was added. Development of bluish green color indicates presence of phenols with catechol or hydroquinone nuclei.

**Test for coumarins:**

With Ammonia: To the solution of each extract NH₃ solution was added and examined in ultra-violet light. Blue fluorescence indicates presence of coumarins.

With Hydroxylamine hydrochloride: Each extract was dissolved in ether and filtered. To the ethereal extract one drop of saturated solution of alcoholic Hydroxylamine hydrochloride and a drop of alcoholic KOH were added. It was heated, cooled and acidified with 0.5 N HCl and a drop of 1% FeCl₃. Development of violet color indicates presence of coumarins.

**Test for Anthraquinone glycoside:**

Borntrager's test: Each dried extract was dissolved in water. It was then shaken with benzene. To the benzene layer ammonia was added. Pink coloration of aqueous phase indicates presence of anthraquinone glycosides.

Modified Borntrager's test: To the aqueous solution 5% FeCl₃ and concentrated HCl was added and it was heated for 5min. The solution
was extracted with benzene. To the benzene layer ammonia was added. Pink coloration of aqueous phase indicates presence of anthraquinone glycosides (C- glycosides).

**Test for cardiac glycoside:**

Keller- Kiliani’s test for deoxysugars: Each dried extract was dissolved in water. To this acetic acid containing trace amount of FeCl₃ was added and then transferred to the surface of concentrated sulphuric acid. A reddish brown ring at the junction and upper layer slowly become blue indicate presence of deoxysugars.

Legal’s test: to the each extract few drops of pyridine and sodium nitroprusside were added and was made alkaline. A pink or red color indicates presence of cardiac glycoside.

**Test for Fixed oils and fats:**

Their presence was tested by taking a small quantity of petroleum ether extract residue dissolved in alcohol. Few drops of 0.5 N alcoholic Potassium Hydroxide were added along with drop of phenolphthalein and the mixture heated over the a water bath. Formation of soap and partial neutralization of alkali was to be considered as positive test.

A small amount of dry petroleum ether extract was passed between two filter papers to indicate presence of oil.

**Test for volatile oils:**

Presence of volatile oils was confirmed by hydrodistillation method using Clavanger’s apparatus.

Results of qualitative tests are reported in table 16-18. The TLC studies were also performed using different solvent system for
different successive extract of each drug as shown below and results are reported in table 19, 20 and 21.

<table>
<thead>
<tr>
<th>Successive extract</th>
<th>Solvent system</th>
<th>Visualizing agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether (60-80(^\circ))</td>
<td>Benzene : Ethyl acetate :: 9:1</td>
<td>Anisaldehyde sulphuric acid</td>
</tr>
<tr>
<td>Benzene</td>
<td>Benzene : Ethyl acetate :: 8:2</td>
<td>Anisaldehyde sulphuric acid</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Chloroform : Methanol : ammonia :: 9.5 : 0.5 : 0.1</td>
<td>Dragendorff's reagent</td>
</tr>
<tr>
<td>Methanol</td>
<td>Toluene : Ethyl acetate : Acetone : Formic acid :: 10:5:15:1</td>
<td>i) UV light    ii) FeCl(_3)   iii) I(_2) chamber</td>
</tr>
<tr>
<td>Water</td>
<td>Acetone : Water :: 9:1</td>
<td>Anisaldehyde sulphuric acid</td>
</tr>
</tbody>
</table>
4.7 Separation and analysis of phytomarkers:

4.7.1 Isolation and identification of phytomarker for *Albizia lebbeck*:

Preliminary phytochemical examinations revealed that phytosterols/triterpenoidal constituents of *A. lebbeck* can be extracted with petroleum ether. It was therefore decided to extract the marker from petrol ether extract. TLC study of the petroleum extract was done according to procedure given in standard book and it showed presence of betulinic acid$^{276}$. Betulinic acid is reported as anti anaphylactic compound$^{277}$, therefore it was thought worthwhile to separate the same by preparative TLC.

**Procedure:** For preparative TLC, the glass plates (20x10 cm) were used. 50g of silica gel G (S. D. fine) were suspended in 80ml water to make slurry. This slurry was spread uniformly over the plates, the plates were dried at R.T. and activated at 110$^\circ$ C for half an hour in the oven. The petroleum ether (500mg) extract containing betulinic acid was applied as thin band (4mm broad) along with standard betulinic acid (Aldrich) spotted on both sides and were developed in the mobile phase Toluene: Acetone: Acetic acid :: 100: 1: 0.1. The bands developed were visualized in the iodine chamber; the bands corresponding with standard betulinic acid were marked. The bands were scrapped from the plates after complete vaporization of iodine. Silica gel was taken in a round bottom flask; 100ml methanol was added in it and refluxed for 30min on boiling water bath. Methanolic extract was filtered through Whatmann filter paper no, 40. Two washings each of 5ml were given to silica gel. The filtrates were combined in clean dry beaker and the solvent was evaporated to dryness. White amorphous powder of betulinic acid was obtained.
Isolated compound was identified by comparing m.p. and mixed m.p. with that of standard betulinic acid (Aldrich). Its identity was further confirmed by TLC by comparing R_f value and color reaction with standard betulinic acid using silica gel G (precoated TLC plate) as stationary phase, Toluene: Acetone: Acetic acid::100: 1: 0.1 as a mobile phase and anisaldehyde H_2SO_4 as spraying reagent. Finally the compound was confirmed as betulinic acid by comparing IR spectrum (FTIR, Perkin Elmer) of isolated and standard betulinic acid (Aldrich) (Table 22 & 23 Fig. 17a & 17b)

4.7.2 Estimation of betulinic acid in bark of *Albizzia lebbeck* by HPTLC method:

**Instrument:**
A Camag TLC system comprising of a Linomat V sample applicator (Camag, Switzerland), TLC scanner III controlled by CATS software (version 4.06). Camag 100 µl HPTLC Syringe, Camag twin trough chambers (20*10 cm²) and automatic TLC sampler III were used for sample application and quantitative estimation.

**Reagent and other materials:**
Standard betulinic acid (isolated), toluene, acetone, acetic acid, distilled water, methanol (HPTLC grade), silica gel 60F_254 precoated HPTLC plate (E-Merck), Anisaldehyde sulphuric acid reagent (0.5% anisaldehyde in sulphuric acid in methanol). All the reagents used were of AR Grade.

**Spotting parameters:**
- Start position:10 mm from bottom edge
- Bandwidth 6 mm.

**Chromatographic conditions:**
- Stationary phase: Precoated HPTLC plate of silica gel 60F_254
Materials and methods

- Mobile Phase: Toluene: Acetone: Acetic acid (100: 1: 0.1 v/v)
- Developing chamber: Twin trough chamber
- Saturation time: 30min.
- Separation technique: ascending
- Spotting volume: for calibration curve 8-16μl
  for sample 20μl
- Format (size of plate): 10*10 cm²
- Thickness of plate: 200μm
- Temperature: 25 ± 2° C
- Distance between bands 11.6 mm
- Distance developed: 7 cm.

Densitometric scanning:
- Mode: Absorbance
- Slit dimension: 6 x 0.4mm
- Lamp: Tungsten
- Wavelength: 523nm.

Standard solution and calibration curve of betulinic acid:
Accurately weighed 10mg of standard betulinic acid was taken in a 10ml of volumetric flask. Crystals were dissolved in methanol and the volume was adjusted to 10ml with methanol (1mg/ml). From this 1ml was diluted up to 10ml with methanol in a volumetric flask to give a final concentration of the standard solution (100μg/ml).
Graded concentration of standard solution (100μg/ml) in 8, 10, 12, 14 and 16 μl volume were applied on a precoated HPTLC silica gel 60 F₂₅₄ plate (E. Merck) using Linomat V sample applicator. The concentration of betulinic acid was 800, 1000, 1200, 1400 and 1600 ng/spot. The plate was developed in a mobile phase, toluene: acetone:
**Materials and methods**

cetic acid :: 100: 1: 0.1, derivatized by anisaldehyde sulphuric acid reagent followed by heating for 10 min and scanned at 523 nm. Data of peak height and peak area of each betulinic acid spot was recorded. The calibration curve was determined by plotting areas (Y axis) obtained against concentration of each peak (X axis) corresponding to the respective spot. (Table 24, Fig. 18-20)

**Sample solutions and estimation:**

10 mg of petrol ether extract (prepared as mentioned in 4.5) was taken in 10 ml volumetric flask and volume was adjusted to 10 ml with petrol ether (60-80 °) to get 1 mg/ml concentration.

20 μl of test sample was spotted along with standard solution of betulinic acid 8-16 μl on precoated HPTLC silica gel 60 F254 plate (E. Merck). The plate was developed in mobile phase and scanned at 523 nm. Peak area was noted and concentration was determined by comparing the area of standard solution from calibration curve. (Table 25)

**4.7.3 Isolation and identification of phytomarker for Euphorbia hirta:**

Plant of *E. hirta* is rich in flavanoids. Flavanoids are very good antioxidants and many of them like quercetin are having anti asthmatic effect too. So attempt was made to isolate flavanoid as a phytomarker.

**Procedure:** 250 g powder of *Euphorbia hirta* was extracted exhaustively by reflux with methanol. Methanolic extract was distilled off to get back maximum amount of methanol. Concentrated methanol extract was dried in petridish on waterbath. Dried extract was
dissolved in mixture of H₂O: methanol (80:20). Maximum solubility was obtained by warming the mixture on water bath. Filtrate was extracted with petrol-ether (60-80°) to remove non polar compounds. Aqueous phase was exhaustively extracted with solvent ether to separate semi-polar compound. All the ether phase was pooled and concentrated. Ether phase was kept in refrigeration for one day; yellow mass of crude material was separated. Mass was separated by filtration and further purification was done by preparative TLC using mobile phase Toluene: ethyl acetate: acetone: formic acid (5:4:4:0.5 v/v) and silica gel G as stationary phase. Procedure was same as 4.7.1. (page 87)

Isolated compound was confirmed by UV spectra, m.p. and hydrolysis pattern in which co-TLC of aglycone with standard quercetin using Toluene: ethyl acetate: acetone: formic acid (5: 4: 4: 0.5 v/v) and coTLC of aqueous hydrolyzed solution with standard rhamnose using precoated silica gel G F254 TLC plate as a stationary phase, Methanol: Chloroform: Acetone: concentrate ammonia (42: 16.5: 25: 16.5) as a mobile phase and anisaldehyde sulphuric acid as a spraying agent. (Fig. 21 & 22)

4.7.4 Estimation of quercitrin in aerial parts of _E. hirta_ by HPTLC method:

**Instrument:** Same as 4.7.2

**Reagent and other materials:**
Standard quercitrin (isolated), toluene, ethyl acetate, acetone, formic acid, distilled water, methanol (HPTLC grade) and silica gel 60F₂₅₄ precoated HPTLC plate (E-Merck)

**Spotting parameters:**
Materials and methods

- Start position: 10 mm from bottom edge
- Bandwidth 6 mm.

Chromatographic conditions:
- Stationary phase: Precoated HPTLC plate of silica gel 60F_{254}
- Mobile Phase: Toluene: Ethyl Acetate: Acetone: Formic acid. (5:4:4:0.5v/v)
- Developing chamber: Twin trough chamber
- Saturation time: 30 min.
- Separation technique: ascending
- Spotting volume: for calibration curve 30-150μl
  : for sample 50μl
- Format (size of plate): 10*10 cm²
- Thickness of plate: 200μm
- Temperature: 25 ± 2°C
- Distance between bands 11.6 mm
- Distance developed: 7 cm.

Densitometric scanning:
- Mode: Absorbance
- Slit dimension: 6 x 0.4mm
- Lamp: Deuterium
- Wavelength: 254nm.

Standard solution and calibration curve of quercitrin:
Accurately weighed 10mg of standard quercitrin was taken in a 10ml of volumetric flask. Crystals were dissolved in methanol and the volume was adjusted to 10ml with methanol (1mg/ml). From this 1ml
was diluted up to 10ml with methanol in a volumetric flask to give a final concentration of the standard solution (100µg/ml).

Graded concentration of standard solution (100µg/ml) in 30, 60, 90, 120 and 150 µl volume were applied on a precoated HPTLC silica gel 60 F<sub>254</sub> plate (E. Merck) using Linomat V sample applicator. The concentration of quercitrin was 3000, 6000, 9000, 12000 and 15000 ng/spot. The plate was developed in a mobile phase, Toluene: Ethyl Acetate: Formic acid :: 5: 4: 4: 0.5 and scanned at 254 nm. Data of peak height and peak area of each quercitrin spot was recorded. The calibration curve was determined by plotting areas (Y axis) obtained against concentration of each peak (X axis) corresponding to the respective spot. (Table 31, Fig. 23-25)

**Sample solutions and estimation:**

10mg of methanol extract (prepared as mentioned in 4.5) was taken in 10 ml volumetric flask and volume was adjusted to 10ml with methanol to get 1mg/ml concentration.

50µl of test sample was spotted along with standard solution of quercitrin 30-150µl on precoated HPTLC silica gel 60 F<sub>254</sub> plate (E. Merck). The plate was developed in mobile phase and scanned at 254nm. Peak area was noted and concentration was determined by comparing the area of standard solution from calibration curve. (Table 32)

**4.7.5 Isolation of Volatile oil from aerial parts of Sphaeranthus indicus:**

500gm powder of aerial parts of *Sphaeranthus indicus* and sufficient quantity of water were taken in round bottom flask of Clavenger's apparatus. Assembly was set up and heat was applied for 5-6hrs. The
oil collected in side tube was separated from water. Percent w/v of volatile oils is calculated and it was stored in well closed container.

**4.7.6 Fingerprinting of oil of *Sphaeranthus indicus* by GCMS method**

Fingerprinting of oil of *Sphaeranthus indicus* was established by GCMS analysis.

**Specifications:**

Instrument used was Gas Chromatograph auto system XL with Turbo Mass (Perkin Elmer). Column: PE- 5ms 30m in length, Carrier gas: Helium, Flowrate: 1ml/min, Split ratio: 1:50, Ionization energy: 70eV and Mass range: 50-600m/z.

Fragmentation of peaks obtained was done and results were noted. *(Fig. 26-28)*

**4.7.7 Isolation and identification of phytomarker from oil of *Sphaeranthus indicus*:**

Literature survey reveals that volatile of *Sphaeranthus indicus* is rich in many terpenoids like α-terpinene, citral, geraniol, geranyl acetate, β-ionone, Sphaeranthene, Ocimene, Cinnamaldehyde, β-Caryophyllene etc. Out of these terpenoids β- Caryophyllene is present in good amount on TLC and its presence was confirmed by GCMS analysis. β- Caryophyllene was reported as a constituent having antiasthmatic activity, therefore an attempt was made to isolate the same.

β- Caryophyllene was isolated by preparative TLC using Petroleum ether: Carbon tetrachloride :: 75: 25 as mobile phase and compared with standard β- caryophyllene (Sigma). Isolated compound was confirmed by b. p. and Co-TLC of standard and isolated compound using Petroleum ether: Carbon tetrachloride :: 75: 25 as mobile phase
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and anisaldehyde sulphuric acid as spraying reagent. Its identity was further confirmed by overlain gas chromatogram of standard and isolated β-caryophyllene. (Fig. 29 & 30)

4.7.8 Quantitation of β-caryophyllene by Gas chromatography in oil of Sphaeranthus indicus:
Standard solutions of β-caryophyllene was prepared in petrol ether (60-80) AR grade in the concentration range 250, 500, 750, 1000, 1250 ppm. Sample was prepared by diluting 0.1 ml oil up to 10 ml petrol ether (60-80) AR grade.

Instrument: Instrument used was Gas chromatograph auto system XL Perkin Elmer, column BP-5 (Capillary column), carrier gas Hydrogen, Carrier flow 3 PSI, Temperature 100° C for 5 min., rate 10° C per minute up to 280° C hold for 20 min.; injector temperature 230° C, Detector Flame ionization detector, detector temperature 280° C. Using microsyringe, standard solutions of β-carophyllene in the concentration range 250-1250 ppm and sample were injected separately and area was obtained. Calibration curve was generated and concentration of β-carophyllene in the sample was determined. (Table 38, 39 Fig. 31-33)

4.7.8 Validation of analytical methods: 282-286
1. Linearity:
The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of an analyte in sample within a given range. The range of the analytical method is the interval between the upper limit and lower limit of an analyte.
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The range of concentration of the standard compound was determined for the linearity. The obtained test results must be in direct proportion to the concentration of analyte in the sample calibration curve for the analyte. The results were expressed in terms of correlation coefficient of the linear regression analysis.

2. Precision:

Precision is measure of either the degree of reproducibility or repeatability of the analytical method. It is expressed as standard deviation (SD) or relative standard deviation (RSD) (coefficient of variance). It provides an indication of random error; result should be expressed as relative standard deviation or coefficient of variance.

**Interday and intraday precision:**

Variation of results within the same day is called intraday variation. Variation of results within day is called interday variation. The intraday precision was determined by analyzing quercitrin, betulinic acid and caryophyllene for five times on the same day.

The interday precision was determined by quercitrin, betulinic acid and caryophyllene daily for five days.

**Repeatability:**

Repeatability can be defined as the precision of the procedure when repeated by same analyst under same operating conditions (same reagent, equipments, settings and laboratory) over a short interval of time.

It is normally expected that at least six replicates be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD value are important for showing degree of
variation expected when the analytical procedure is repeated several
time in a standard situation.

Repeatability of measurement of peak area: (RSD < 1% based on
seven times measurement of the same spot).

All other conditions were as per the proposed method. Area of peak
was measured seven times without changing the plate position.

Repeatability of sample application (RSD < 3% based on application
of equal volume of seven spots)

3. Accuracy:
The accuracy of an analytical method may be defined as the closeness
of the test results obtained by the method to the true value. It is the
measure of the exactness of the analytical method developed.

Accuracy may often express as percent recovery by the assay of a
known amount of analyte added.

Accuracy may be determined by applying the method to samples or
mixtures of excipients to which known amount of analyte have been
added above and below the normal level expected in the samples.

Accuracy is then calculated from the test results as the percentage of
the analyte recovered by the assay. Dosage form assays commonly
provide accuracy within 3-5% of the true value. The accuracy of an
analysis was determined by calculated systemic error involved.

Adding known concentration of marker compound to the pre
quantified sample solution and estimating the quantity of marker
compound using the proposed methods ascertained accuracy of the
above method.

The ICH documents recommend that accuracy should be assessed
using a minimum of nine determinations over a minimum of three
Materials and methods

concentration levels, covering the specified range (i.e. three concentration and three replicated of each concentration.)

4. **Limit of detection and limit of quantitation:**

**Limit of detection:**

The limit of detection is parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below certain level.

The determination of the limit of the detection of instrumental procedures is carried out by determining the signal- to-noise ratio by comparing test results from the samples with known concentration of analyte can be reliably detected. A signal- to-noise ratio of 2:1 or 3:1 is generally accepted.

The signal- to-noise is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by the taking the concentration of the peak of interest divided by three times the signal- to-noise ratio.

For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (S_a) which may be related to LOD and the slope of the calibration curve, b, by

\[ \text{LOD} = 3S_a / b \]

**Limit of quantitation:**
Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determined the lowest level at which acceptable degrees of accuracy and precision are attainable. The final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the limit of quantitation is approximately twice the limit of detection.

5. **Selectivity and Specificity:**

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix.

If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific.

Hence one basic difference in the selectivity and specificity is that, the former is restricted to qualitative detection of the components of a
sample, the later means quantitative measurement of one or more analytes.

Selectivity may be expressed in terms the bias of the assay results obtained when the procedure is applied to the analyte in presence of expected levels of other components, compared to the results obtained on the same analyte without added substances. When the other components are all known and available, selectivity may be determined by comparing the test results obtained on the analyte with and without the addition of the potentially interfering materials. When such components are either unidentified or unavailable, a measure of selectivity can often be obtained by determining the recovery of a standard addition of pure analyte to a material containing a constant level of the other components.

Validation parameters for betulinic acid Table 26-30
Validation parameters for quercitrin Table 33-37
Validation parameters for β-caryophyllene Table 40-43
4.8 PHARMACOLOGICAL ACTIVITY:

ANIMALS

Albino rats (150-200g), mice (20 to 30g) and Guinea pigs (300-600g) of either sex were used for various experiments.

SELECTION OF ANIMALS

All animals were housed at ambient temperature (22±1°C), relative humidity (55±5%) and 12h/12h light dark cycle. Animals had free access to standard pellet diet and water given ad libitum. The protocol 365/01/ab/CPCSEA was approved by the institutional Animal Ethics 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 before carried out the project. Animals were divided into different groups for different models. The distribution of animals in the groups, the sequence of trials and the treatment allotted to each group were randomized throughout the experiments. Antiasthmatic activity of alcoholic extract of all the three drugs was evaluated by both in-vitro and in-vivo models.

4.8.1 IN-VITRO METHOD:

Material and methods:

Powder of each of bark of Albizzia lebbeck, aerial parts of Euphorbia hirta and Sphaeranthus indicus was exhaustively extracted with alcohol (95%) by reflux separately and alcohol was recovered. Each alcoholic extract was dried under vacuum. Each extract was suspended in distilled water to get suitable concentration and used.
Study on albino rat mesenteric mast cell degranulation by compound 48/80.

Method described by Norton (1954) was slightly modified for the study.\textsuperscript{287}

Albino rat was sacrificed by cutting the neck blood vessel after a sharp blow of steel rod on head. The pieces of mesentery were collected in petri dish containing Ringer Locke’s solution and then subjected to the following treatment schedules.

Table 8 Treatment schedule:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Petri dish no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer Locke solution (vehicle control)</td>
<td>1</td>
</tr>
<tr>
<td>Ringer Locke solution (positive control)</td>
<td>2</td>
</tr>
<tr>
<td>Dexamethasone (10\mu g/ml)</td>
<td>3</td>
</tr>
<tr>
<td>Alcoholic extract of \textit{E. hirta} a) 100\mu g/ml</td>
<td>4</td>
</tr>
<tr>
<td>b) 250\mu g/ml</td>
<td>5</td>
</tr>
<tr>
<td>c) 500\mu g/ml</td>
<td>6</td>
</tr>
<tr>
<td>Alcoholic extract of \textit{A. lebbek} a) 100\mu g/ml</td>
<td>7</td>
</tr>
<tr>
<td>b) 250\mu g/ml</td>
<td>8</td>
</tr>
<tr>
<td>c) 500\mu g/ml</td>
<td>9</td>
</tr>
<tr>
<td>Alcoholic extract of \textit{S. indicus} a) 100\mu g/ml</td>
<td>10</td>
</tr>
<tr>
<td>b) 250\mu g/ml</td>
<td>11</td>
</tr>
<tr>
<td>c) 500\mu g/ml</td>
<td>12</td>
</tr>
</tbody>
</table>

Each petri dish except Petri dish 1 was incubated for 10 min at 37°C and then to each petri dish 0.1ml of compound 48/80 having concentration 10\mu g/ml was added and again incubated for 10 min at 37°C. After that all pieces were transferred to 4\% formaldehyde solution containing 0.1\% toluidine blue and kept aside for 20 to 25mins. After staining and fixation of mast cells pieces were transferred through acetone and xylene two times and mounted on slides. All the pieces were examined under microscope with 450x magnification. Minimum of 100 cells were counted and percentage of
intact and disrupted mast cells were determined. Percent protection
form degranulation of mast cells by the alcoholic extracts was
determined. (Table 36, Fig. 33)

4.8.2 IN-VIVO METHOD:
Heterologus Passive cutaneous anaphylaxis model: 288
Six albino rats were injected with each of egg albumin and Bordetella
pertussis vaccine (0.1ml, i.p.) on 1st, 3rd and 5th day. After 21 days of
the immunization, blood was collected by cardiac puncture under light
ether anesthesia. Serum was separated by centrifugation at 3000 rpm
for 15 min. and stored below –20°C until used for further experiments.
Mice were selected randomly and divided in six Groups, each
consisting of six animals:
Group 1: Control group
Group 2: Anti-ovalbumin sensitized
Group 3: Dexamethasone (5 mg /kg, P.O.)
Group 4: Alcoholic extract of Euphorbia hirta (250mg/kg, P.O.)
Group 5: Alcoholic extract of Albizia lebbek (250mg/kg, P.O.)
Group 6: Alcoholic extract of Sphaeranthus indicus (250mg/kg, P.O.)

The anti-ovalbumin antisera was injected intradermally on the clipped
dorsal skin. After 48 hrs of injection, 1 ml of 0.5% Evans blue solution
containing 20mg of egg albumin was injected intravenously through
tail vein. 24 hrs after injection of Evans blue dye solution, mice were
sacrificed and definite area of skin was removed and transferred to the
solution of 70% acetone for 24 hrs to extract out the Evans blue dye
and its intensity was measured colorimetrically at 574 nm.
Drug/extracts were administered to mice according to their group for 3 consecutive days from the day of passive sensitization. As promising anti asthmatic activity was obtained in above two models, therefore it was thought worthwhile to develop a formulation combining these three drugs in different proportions according to their therapeutic activity. *(Fig. 34 & 35)*

**Formulation:** Churna from these three drugs bark of *A. Lebbeck*, aerial parts of *E. hirta & S. indicus* in proportion of 4: 6: 1 was formulated and its dried alcoholic extract in the form of suspension was used as test formulation to exploit further promising anti-asthmatic activity.

**4.8.3 Egg albumin induced bronchospasm in Guinea pigs.**

In this model, egg albumin was used as sensitizer. Guinea pigs, weighing around 400 gm were selected and divided into four groups consisting each of six animals. On day zero, the animals were examined for the following parameters.

a) Serum pCO₂ and pO₂  
b) Serum bicarbonate level  
c) Respiratory volumes  
d) Eosinophils and macrophages count in the broncho alveolar fluid  
e) Histopathological changes in the lung tissues.  
f) Measurement of oxidant and antioxidant parameters.

The following schedule of treatment was administered to the animals:

- **Group I:** Normal Control  
- **Group II:** Positive control (EA sensitized)  
- **Group III:** Dexamethasone 5 mg/kg, P.O. treated
Group IV: Formulation treated (300 mg/kg, P.O.)

Each group consisted of six animals.
The guinea pigs of all the groups except Group I were sensitized with egg albumin (0.1 ml, 10% w/v). The animals of Group III and IV, were dosed once daily for fifteen days with standard and test formulation respectively. Two hours after the last dose of drug administration on day fifteen all the animals except Group I animals were challenged with egg albumin (0.5 ml, 2% w/v, i.p.) through saphenous vein. After 3 hours of the challenge of the egg albumin or just prior to death of animals, whichever was earlier the above given parameters were checked.

4.8.3.1 Serum pCO₂ and pO₂:
Serum pCO₂ was measured by using instrument Capnograph. Serum pO₂ was measured by using pulse oximeter.

4.8.3.2 MEASUREMENT OF SERUM BICARBONATE LEVEL:
The method used in present study to measure serum bicarbonate level was slightly modified, from that described by Godkar, 1996.²⁹⁰

Specimen collection:
About 2-3 ml of blood was collected after 1 hr of the i.v. injection of egg-albumin. The serum was separated from blood by minimum exposure to air and it was stored in a sealed tube till bicarbonate level was estimated.

Procedure:
10 ml of 1.0 gm/dl saline was pipetted out in 100 ml beaker. To this 0.1 ml of test sample was added. 2 drops of phenol red indicator was added and mixed well. Drop by drop NaOH (0.01N) was added till the end point was achieved (7.35 PH, yellow to pink). This volume of
.. Materials and methods ..

NaOH (0.01N) required was noted and considered as control reading (as X ml).

In another act, 9.0 ml of 1 gm/dl saline was pipetted out in another 100 ml beaker; 1 ml HCl (0.01N) was added. The above experiment was repeated and volume of NaOH (0.01N) required was noted (as Y ml).

Now, \( R = Y \text{ ml} - X \text{ ml} \)

**Calculation:**

Serum bicarbonate level m.Eq/ltr = \((1 - R) \times 100\)

4.8.3.3 MEASUREMENT OF TIDAL VOLUME:
The method used in the present study was similar to that described by Khandpur, 1996 was used.\(^{291}\)

**Definition:**

It is volume of air inspiated or expired per breath during normal breath.

The measurement of tidal volume was done with the help of “Respiratory volume transducer” that was used with “Strain gage coupler” and student physiograph.

The physiograph was calibrated with the help of 0.02 cc and 0.1 cc volume air supplier.

The tidal volume was measured in terms of height of response obtained. Then, the height was converted in to volume.

4.8.3.4 MEASUREMENT OF RESPIRATORY RATE:
The method described by Apps 1992 and Fabbri 1997 was used.\(^{292,293}\)

**Definition:** It is a no. of breath per unit time.

Unit: Breath per min. (BPM).

The respiratory rate was measured visually.
4.8.3.5 MEASUREMENT OF AIRFLOW RATE:
The method used in the present study was similar to that described by Guyton and Hall, 2006.\textsuperscript{294}

**Definition:**
It is a volume of air inspired or expired per unit time during normal breathing.
Unit: Volume per unit time (ml/min).

**Calculation:**
Air flow rate = Respiratory rate × Tidal volume

4.8.3.6 BAL FLUID STUDY: DIFFERENTIAL LEUCOCYTES COUNT
After 3 hours of challenge with egg albumin or just prior to death of animal, the tracheobronchial tree was lavaged with 10ml of saline by inserting cannula and the bronchoalveolar lavage fluid was collected. Centrifugation was done at 2000 rpm for 5 min. and the pellet was resuspended in 0.5 ml saline. 0.2ml of Geimsa stain in buffered saline (6.8 pH) was added to it. After 5 min of adding Geimsa stain the numbers of each type of leucocytes in 50μl BAL fluid was determined under the light microscope at 450x magnification. The result obtained was compared with unsensitized (Group I) and untreated egg albumin sensitized (Group II) animals.

4.8.3.7 HISTOPATHOLOGY OF LUNGS:
It included fixation of the tissue with formalin, embedding in paraffin blocks, sectioning with microtome (0.7 μ thickness) and finally staining by Haemotoxylin and Eosin stain technique.

**Principle:**
Materials and methods

Haemotoxylin stains nucleus light blue which turns red in presence of acid. The cell differentiation is achieved by treating the tissue with acid solution the counter staining is performed by using eosin which imparts pink color to cytoplasm.

4.8.3.8 ESTIMATION OF FREE RADICAL GENERATION:

Preparation of tissue homogenate:
Lung was dissected out and immediately stored at -20°C. The one gram of tissue was homogenized with 10 ml phosphate buffer solution. Then prepared homogenates were centrifuged at 10000 rpm for 10 minutes and supernatant used for determination of oxidant-antioxidant parameters. Piece of tissue stored in 10% formalin for microscopic studies.

Malondialdehyde (MDA) level:
Method described by Ohkawa et al., 1979 was used.²⁺

Procedure:
Briefly 1.0 ml sample was mixed with 0.2ml 4 % (w/v) sodium dodecyl sulphate, 1.5ml 20% acetic acid in 0.27M hydrochloric acid (pH 3.5) and 1.5ml 0.8% thiobarbituric acid (TBA). The mixture was heated in a hot water bath at 85°C for 1hr. The intensity of pink color developed was read against a reagent blank at 532nm following centrifugation at 1200 g for 10 min. The amount of malondialdehyde was calculated using molar extinction coefficient 1.56x10⁵ M⁻¹ Cm⁻¹ and reported as nmoles of MDA/mg protein.

PREVENTIVE ANTIOXIDANTS:

Superoxide dismutase (SOD):
Material and methods

Method described by Misra et al., 1972 was used.\textsuperscript{296}

**Procedure:**

0.1ml of sample was mixed with 0.1ml EDTA (1\times10^{-4}M), 0.5ml of carbonate buffer (pH 9.7) and 1.0ml of epinephrine (3\times10^{-3}M). The optical density of formed adrenochrome was read at 480nm for 3 min. at interval of 30 sec and results were expressed as U/min/mg of protein.

**Catalase activity:**

Method described by Aebi, 1974 was used.\textsuperscript{297}

**Procedure:**

50\mu l of sample was added to buffered substrate (50mM phosphate buffer pH 9.7 containing 30mM H\textsubscript{2}O\textsubscript{2}) to make volume 3.0ml. The decrease in absorbance was read at 240nm for 2.5 min. at interval of 15sec. The results were expressed as mean change of absorbance per 15sec. The activity was calculated using extinction coefficient of H\textsubscript{2}O\textsubscript{2}, 0.041/\mu mole/cm\textsuperscript{2}. Results were expressed as \mu moles of H\textsubscript{2}O\textsubscript{2} utilized/min/mg/protein.

**CHAIN BREAKING ANTIOXIDANTS:**

**Reduced glutathione (GSH):**

Method described by Beutler et al., 1963 was used.\textsuperscript{298}

**Procedure:**

2.0ml sample was mixed with 10\% chilled TCA. The mixture was kept in ice-bath for 30min. and centrifuged at 1000g for 10min. at 4°C. 0.5ml supernatant was mixed with 2.0ml 0.3M disodium hydrogen phosphate and 0.25ml 5, 5'-dithiobis-2-nitrobenzoic acid (40mg/100ml in 1\%sodium citrate) was added just before measuring
Materials and methods

the absorbance at 412nm. Standard curve of GSH was prepared using standard glutathione. Results were expressed as ng of GSH/mg of protein.

Protein estimation:
Method described by Lowry et al., 1951 was used.299

Procedure:
100mg of tissue was weighed and homogenized in 5.0ml of distilled water. 0.2ml of homogenate was added to 4.0ml of solution-C (solution-A: 2 g of sodium hydroxide, 10g of sodium carbonate, 0.1g of sodium-potassium tartrate in 500 ml of distilled water, solution-B: 0.5g of cuprous sulphate in 100ml of distilled water, solution-C: 10ml of solution-A and 0.2ml of solution-B) and 0.6ml distilled water was added and allowed to stand for 15min at 37°C. Folin-phenol reagent 0.4ml was added and incubated for 30min. Absorbance was read at 540nm. Amount of protein in 100mg of tissue was calculated from the graph of standard albumin.

4.8.3.9 STATISTICAL ANALYSIS:
Results are presented as mean ± SEM. Statistical differences between the means of the various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey test. Data were considered statistically significant at P value < 0.05.

All the chemicals used for experiments were of analytical grade.