CHAPTER – 3

MATERIAL & METHODS
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3.1 Collection and Identification of Plant material

Plant collection and Identification

The plant of *Oldenlandia corymbosa* was collected in the month of September from the Botanical garden of M.S.U, Vadodara, Gujarat. The plant of *Grangea maderaspatana* was collected in the month of December from Saputara, Gujarat. Both the plants were identified and authenticated by the taxonomist of Botanical Survey of India, Jodhpur and a voucher specimens were deposited at BSI, Jodhpur.

Both the plants were dried under shade separately. Air dried plant material was ground to #10 powder and the plant materials were then used for further investigations.

3.2. Assessment of quality of plant materials

The plant materials were assessed as per WHO guideline$^1,2$.

3.2.1 Determination of foreign matter

Both the plants were subjected to determination of any contamination by mould or insects and other animal contamination.

3.2.2 Macroscopic evaluation

Fresh plant parts of *Oldenlandia corymbosa* and *Grangea maderaspatana* were subjected to color, odor and taste, determination of shape, size, surface characteristics and appearance etc$^3$.

3.2.3 Microscopic evaluation

For microscopical examinations, free hand sections of the fresh leaf, stem and root of *Oldenlandia corymbosa* and *Grangea maderaspatana* were cut, cleared with chlora hydrate solution and water, and stained with a drop of hydrochloric acid and phloroglucinol. Photomicrographic images were taken by using Trino CXR camera$^4,5,6$. 

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3.2.3.1 Powder Microscopy
Dried powder of *Oldlandia corymbosa* and *Grangea maderaspatana* were treated with chloral hydrate and were stained with phloroglucinol: HCl (1:1). On slide, a drop of glycerin was placed and covered it with cover slip and observed under microscope.

3.2.3.2 Quantitative microscopy
Leaves of *Oldlandia corymbosa* and *Grangea maderaspatana* were subjected to quantitative microscopy for the following values using reported method.
- Stomatal index
- Palisade ratio
- Vein islet number
- Vein termination number

3.2.4 Proximate analysis
Proximate analysis of powdered plant material of *Oldlandia corymbosa* and *Grangea maderaspatana* was carried out using reported methods\(^1,2\).

Following determinations were done
- Loss on drying
- Total ash
  - Acid insoluble ash
  - Water soluble ash
- Extractive value
  - Alcohol soluble extractives
  - Water soluble extractives
- Foaming index

3.2.4.1 Loss on drying:
Placed about 2 gm of the air dried plant material, accurately weighed in a previously tarred flat weighing bottle. Dried the sample by heating in an oven at 100-105\(^\circ\) C for 5 hrs. Dried until two consecutive weighing was not differ by more than 5 mg, unless otherwise specified in the test procedure. Then calculate the loss of weight in mg/gm of the air dried material.
3.2.4.2 Determination of ash value:
The ash remaining following ignition of powder plant material was determined by three different methods which measures total ash, acid insoluble ash, water soluble ash.

3.2.4.2.1 Total ash
The total ash measures the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plant tissue itself and “non-physiological ash”, which is residue of the extraneous matter adhering to the plant surface.

About 4 g of the ground air-dried powdered material was accurately weighed, in previously ignited and tarred silica crucible. The material was spreaded in an even layer and ignited by gradually increasing the heating to 500-600 °C until it was white, indicating the absence of carbon. It was allowed to cool in a desiccator and weighed. Content of total ash was calculated in form of mg per gm of air-dried material.

3.2.4.2.2 Acid insoluble ash:
Acid insoluble ash measures the amount of silica present, especially as sand and siliceous earth.

In crucible containing the total ash, 25 ml of 2N hydrochloric acid was added, crucible was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot distilled water and this liquid was added to the crucible. The insoluble matter on an ashless filter paper was washed with hot water until the filtrate was neutral. Transfer the filter paper containing the insoluble matter to the original crucible, dry on a hot plate and ignite to constant weight. Allow the residue to cool in a suitable desiccator for 30 minutes and then weigh. Calculate the content of acid insoluble ash in mg per gm of air dried material.

3.2.4.2.3 Water soluble ash:
In crucible containing the total ash, 25 ml of water was added and boiled gently for 5 min. Insoluble matter was collected on ashless filter paper and washed with hot water until the filtrate was neutral. Transfer the filter paper containing the insoluble matter to the original crucible, dry on a hot plate and ignite in a crucible for 15 min. at a temperature not exceeding 450 °C to constant weight. Allow the residue to cool in a suitable desiccator for 30 min. and then weigh. Weight of the residue was subtracted from weight of total ash. Calculate the content of water soluble ash in mg per gm of air dried material.
3.2.4.3 Extractive value

Extractive value determines the amount of active constituents extracted with solvents from given amount of herbal material. It is usually calculated as alcohol soluble extractive value and water soluble extractive value.

**Alcohol soluble extractive value:**
About 5.0 g of coarsely powdered air-dried material was weighed, in a glass-stoppered conical flask. Macerate with 100 ml of the alcohol for 6 hours, shaking frequently, and then allow standing for 18 hours. The content was filtered rapidly. Care was taken during filtration to avoid loss of solvent and 25 ml of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath. Dried the extract at 105°C for 6 hours, cooled in a desiccator for 30 minutes and weighed without delay. Calculated the content of extractable matter in mg per gm of air-dried material.

**Water soluble extractive value:**
About 5 g. of the air-dried drug was macerated with 100 ml of chloroform water in a closed flask for 24 hours shaken frequently during 6 hours and allowed to stand for 18 hours. The solution was filtered rapidly, 25 ml of filtrate was transferred to tarred flat bottom dish and evaporated to dryness on water bath. Extract was dried at 105 C for 6 hours, cooled in desiccator for 30 minutes and weighed without delay. Content of extractable matter was calculated in mg per g of air-dried material.

3.2.4.4 Determination of foaming index:
Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

**Procedure:**
Accurately 1 gm of the powdered plant was transferred to a 500 ml conical flask containing 100 ml of boiling distilled water. It was boiled moderately for 30 minutes. The filtrate was cooled and filtered in a 100 ml volumetric flask and sufficient distilled water was added through the filter to dilute to volume. The decoction was poured into 10 stoppered test-tubes (height 16cm, diameter 16mm) in successive portions of 1 ml, 2 ml, 3 ml, etc. up to 10 ml, and the volume of the liquid was adjusted in each tube with water to 10 ml. the tubes were
shaken in a lengthwise motion for 15 seconds, two shakes per second. Allowed to stand for 15 minutes and the height of the foam were measured.

**Foaming index: 1000 / a**

Where a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

### 3.2.4.5 Determination of Total Tannin content

About 2gm of powdered sample was extracted for 20hrs with Petroleum ether. The residue was boiled for 2 hrs with 300 ml of double distilled water. The solution was cooled and diluted up to 500ml and filter. From the filtrate, 25ml of infusion was taken in to conical flask and 20 ml indigo carmine solution was added and diluted with 750 ml of double distilled water. Then the solution was titrated with standard KMnO₄ solution, 1ml at a time until blue color changed to green. Then few drops were added at a time till solution became golden yellow in color.

Similarly the blank reading was taken by omitting the sample. Multiply the difference between two titration by the factor to obtain value of total tannins.

\[
\% \text{ Total Tannins} = (A-B) \times \text{Normality of KMnO}_4 \text{ solution} \times 0.004157 \times 1000 \\
\text{Weight of drug sample taken} \times 0.1
\]

### 3.2.5 Estimation of heavy metals

Elemental content of the plant material were estimated on atomic absorption spectrophotometer at Shree Dhanvantary Pharmaceutical Analysis & Research Centre, Kim.
3.3 Phytochemical screening

3.3.1 Preliminary phytoprofile

The powder of the air dried plant of *Oldenlandia corymbosa* and *Grangea maderaspatana* were extracted in soxhlet apparatus with solvents of increasing polarity as follows:

i) Petroleum ether  
ii) Chloroform  
iii) Ethyl acetate  
iv) Methanol  
v) Water  

Each time before extracting with the next solvent, the material was dried. All the extracts were concentrated by distilling the solvent and the extracts were dried on water bath. Then consistency, color, appearance of the extracts and their percentage yield were noted.

3.3.2 Establishment of qualitative phytoprofile of successive solvent extracts. (chemical tests):  

The extracts obtained from successive solvent extraction were then subjected to various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, phenolics and tannins, proteins and amino acids, saponins and phytosterols using reported methods.

1. Alkaloids:
   a. Dragendorff’s test: To the extract add Dragendorff’s reagent, reddish brown precipitate indicates the presence of alkaloids.
   b. Mayer’s test: To the extract add Mayer’s reagent, cream colored precipitate indicates the presence of alkaloids.
   c. Wagner’s test: To the extract add Wagner’s reagent, reddish brown precipitate indicates the presence of alkaloids.
   d. Hager’s test: To the extract add Hager’s reagent, yellow precipitate indicates presence of alkaloids.
   e. Tannic acid test: To the extract add tannic acid solution, buff colored precipitate indicates presence of alkaloids.
2. **Amino acids:**
   a. **Millon's test:** To the extract add about 2 ml of Millon’s reagent, white precipitate indicates the presence of amino acids.
   b. **Ninhydrin test:** To the extract add Ninhydrin solution, boil, violet color indicates the presence of amino acids.

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

4. **Flavanoids:**
   a. **Shinoda test:** To the extract add few magnesium turnings and concentrated hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.
   b. **Alkaline reagent test:** To the extract add few drops of sodium hydroxide solution, intense yellow color is formed which turns to colorless on addition of few drops of dilute acid indicates presence of flavonoids.
   c. **Zinc hydrochloride test:** To the extract add a mixture of zinc dust and concentrated hydrochloric acid. It gives red color after few minutes.

5. **Glycosides**

   **General test:**

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

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

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

Chemical test for specific glycosides:

i. Anthraquinone glycosides
   a) Borntrager’s test: Boil the test material with 1 ml of sulphuric acid in a test tube for 5 minutes. Filter while hot. Cool the filtrate and shake with equal volume of dichloromethane or chloroform. Separate the lower layer of dichloromethane or chloroform and shake it with half of its volume of ammonia. A rose pink to red color is produced in the ammonical layer.
   b) Modified Borntrager’s test: Boil the test material with 2 ml of sulphuric acid. Treat it with 2 ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes, shake it with equal volume of chloroform and continue the test as above. As some plants contain anthraceneaglycone in reduced form, if ferric chloride is used during extraction; oxidation to anthraquinones takes place, which shows response to Borntrager’s test.

ii. Cardiac glycosides:
   a) Kedde’s test: Extract the drug with chloroform, evaporate to dryness. Add one drop of 90% alcohol and 2 drops of 2 % 3, 5-dinitro benzoic acid in 90% alcohol. Make alkaline with 20% sodium hydroxide solution, purple color is produced. The color reaction with 3, 5-dinito benzoic acid depends on the presence of α, β- unsaturated lactones in the aglycone.
   b) Killer- Killiani test: Extract the drug with chloroform and evaporate it to dryness. Add 0.4 ml of glacial acetic acid containing trace amount of ferric chloride. Transfer to a small
test tube; add carefully 0.5 ml of concentrated sulphuric acid by the side of test tube. Acetic acid layer shows blue color.

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

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

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

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

iv. **Saponin glycosides:**
   a. **Froth formation test:** Place 2 ml solution of drug in water in a test tube, shake well, stable froth (foam) is formed.
   b. **Haemolysis test:** Add 0.2 ml of extract to 0.2 ml of blood in normal saline and mix well. Centrifuge and note the red supernatant compare with control tube containing 0.2 ml of 10% blood in normal saline diluted with 0.2 ml of normal saline.

6. **Phenolic compounds (Tannins):**
   a. **Ferric chloride test:** Treat the extract with ferric chloride solution, blue color appears if hydrolysable tannins are present and green color appears if condensed tannins are present.
   b. **Phenazone test:** Add about 0.5 gm of sodium acid phosphate to 5 ml of extract warm it and filter. To the filtrate add 2 % phenazone solution, bulky precipitate is formed, which is often colored.
   c. **Gelatin test:** To the extract add 1 % gelatin solution containing 10% sodium chloride. Precipitate is formed.

7. **Proteins:**
   a. **Biuret test:** To the extract (2 ml) add Biuret reagent (2 ml), violet color indicates presence of proteins.
b. **Xanthoproteic test:** To the 5 ml of extract, add 1 ml of concentrated nitric acid and boil, yellow precipitate is formed. After cooling it, add 40 % sodium hydroxide solution, orange color is formed.

8. **Steroids and Triterpenoids:**
   a. **Libermann-Burchard test:** Treat the extract with few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid from the side of test tube, brown ring is formed at the junction. The layer turns green which shows presence of steroids and formation of deep red color indicates presence of triterpenoids.
   b. **Salkowski test:** Treat the extract with few drops of concentrated sulphuric acid red color at lower layer indicates the presence of steroids and formation of yellow colored lower layer indicates presence of triterpenoids.
   c. **Sulfur powder test:** Add small amount of sulfur powder to the extract, it sinks at the bottom.

3.3.3 **TLC of successive solvent extracts**
The various extracts obtained in the successive solvent extraction were then subjected to thin layer chromatographic studies using reported methods to confirm the presence of various phytoconstituents. These results were compared with the results obtained in qualitative tests$^{7,8}$.

3.3.4 **HPTLC Fingerprinting**
HPTLC fingerprinting of chloroform and methanol extracts of *O. corymbosa* and *G. maderaspatana* was performed for oleanolic acid and ursolic acid$^{9,10}$.

a) **Sample preparation:**
Accurately weighed 20 mg of each extracts individually into volumetric flask and 10 mL methanol was added to it. Dissolved it and filtered it with whatman filter paper no. 1 and used for HPTLC profiling.
b) **Standard preparation:**
Accurately weighed 10 mg of each standard individually into volumetric flask and 10 mL methanol was added to it. Dissolved it and filtered it with whatman filter paper no. 1 and used for HPTLC profiling.

### Chromatographic Conditions:

<table>
<thead>
<tr>
<th>Application Mode</th>
<th>CAMAG Linomat 5 - Applicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application of sample</td>
<td>Automatic device “CAMAG LINOMAT – 5”</td>
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<tr>
<td>Stationary Phase</td>
<td>MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets (10 x 10 cm)</td>
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<tr>
<td>Application Volume</td>
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<tr>
<td>Mobile Phase</td>
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<tr>
<td>Development Mode</td>
<td>CAMAG TLC Twin Trough Chamber</td>
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<tr>
<td>Spray reagent</td>
<td>Anisaldehyde sulphuric acid reagent</td>
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<tr>
<td>Derivatization mode</td>
<td>CAMAG – Dip tank for about 1 minute</td>
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<tr>
<td>Visualization</td>
<td>@ 510 nm after derivatization</td>
</tr>
</tbody>
</table>

### 3.3.5 Quantification of Gallic acid by HPLC

Estimation of Gallic acid in methanol extracts of *O. corymbosa* and *G. maderaspatana* was performed by HPLC\(^1\).

a) **Sample preparation:**
Accurately weighed 1 mg of each extracts individually into volumetric flask and 10 mL methanol was added to it. Dissolved it and filtered it with whatman filter paper no. 1 and used for HPLC profiling.

b) **Standard preparation:**
Accurately weighed 1 mg of Gallic acid was transferred into volumetric flask and 10 mL methanol was added to it. Dissolved it and filtered it with whatman filter paper no. 1. From stock solution, different concentrations (10-70 µg/ml) were prepared and used for HPLC profiling.
Chromatographic Conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
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<tr>
<td>Stationary Phase</td>
<td>Phenomenex Luna C18 (4.6 x 250mm, 5μ particle size)</td>
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<tr>
<td>Mobile Phase</td>
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<td>Wavelength</td>
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<td>Flow Rate</td>
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<td>Total Run time</td>
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<tr>
<td>Mode of Operation</td>
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</table>

4. Psychopharmacological studies

4.1 Acute toxicity study\(^{12,13}\)
Acute toxicity study was performed for chloroform and methanol extracts of *O. corymbosa* and *G. maderaspatana* according to the acute toxic classic method as per guidelines prescribed by OECD (OECD, 1996). Acute toxicity test aims at establishing the therapeutic index, i.e. the ratio between pharmacological effective dose and lethal dose on the same strain and species (LD50/ED50). Greater the index, safer the compound.

2000 mg/kg of extract was administered as per OECD guidelines per orally to 6 mice. Effects were observed on behavior for 72 hours. Mice were examined for behavioral effects 45 minutes post administration of the extracts. No change in behavior or any abnormality in behavior was observed and no mortality was seen. Thus it was concluded that chloroform and methanol extract of *O. corym osa* and *G. maderaspatana* was nontoxic up to 2000 mg/kg doses. Then 1/5\(^{th}\) and 1/10\(^{th}\) of the administered dose was selected for future studies as per OECD guidelines.

Experimental Animals

**Animals:**
Swiss albino male mice weighing 25-30 gms, were used for all sets of experiments in groups of six animals. They were maintained at controlled room temperature (25±2°C) on 12 hour light/dark cycle and allowed free access to food and water. The experiments were performed
after the experimental protocols approved by the Institutional Animal Ethics Committee of Babaria Institute of Pharmacy and care of animals was taken as per CPCSEA guidelines. Animals were divided into control group, standard group and extracts treated group. Each group consists of 6 animals.

**Treatment:**
Animals were divided into six (I-VI) groups for the assessment of both the plant extracts. Group I was a negative control; Group II was positive control; Groups III to IV received chloroform extract of *O. corymosa* at doses of 200 and 400 mg/kg p.o respectively. Group V to VI received methanolic extract of *O. corymosa* at doses of 200 and 400 mg/kg. p.o respectively.

For assessment of psychopharmacological activity of *Grangea maderaspatana* extracts, the animals were divided into six (I-VI) groups. Group I was a negative control; Group II was positive control; Groups III to IV received chloroform extract of *G. maderasatana* at dose of 200 and 400 mg/kg p.o respectively. Groups V to VI received methanol extract of *G. maderasatana* at dose of 200 and 400 mg/kg p.o respectively.

**Psychopharmacological activity was screened by:**

a) Antidepressant activity was performed using forced swim test model.

b) Anxiolytic activity was done using Elevated plus maze model.

c) Exploratory behavior pattern was studied by Head dip test method.

d) CNS inhibitory activity was performed using Actophotometer.

**4.2 Forced Swimming Test**
The apparatus consisted of an opaque Plexiglas cylinder (50 cm high × 20 cm wide) filled with water at room temperature, to a depth of 30 cm. During the 6 min swimming test, immobility behavior was observed, defined as when the animal made no further attempts to escape except for the movements necessary to keep its head above the water. Reduction in immobility is considered as a behavioral profile consistent with an antidepressant like action\textsuperscript{14,15}. 
4.3 Elevated Plus Maze
This apparatus consists of two open arms (50×10 cm) crossed with two closed arms (50×10×40 cm). The arm was connected together with a central square (10×10 cm). The apparatus was elevated to a height of 70 cm in a dimly illuminated room. Each mouse was placed individually at the center of the elevated maze, 45 minutes post administration of the extracts and the standard. The number of entries in the open and closed arm of the elevated maze during a period of 5 minutes and the duration of stay in the open and closed arm were noted\textsuperscript{16,17}. After each test, the maze was carefully cleaned up with a wet tissue paper (10% ethanol solution). Entry into the arms was defined as the point when the animal places all four paws in the arm. Subsequently, the percentage of open arm entries (100 × open/total entries) and the percentage of time spent in the open arms (100 × open/open + enclosed) were calculated for each animal\textsuperscript{18,19}.

4.4 Head dip test method
Exploratory behavior of mice in a novel environment was measured using a hole-board test (locally constructed). This method is used for measuring the response of the rat to an unfamiliar environment. The apparatus consisted of a grey cardboard box (50×50×50 cm) with 18 equidistant holes 3 cm in diameter in the floor. 30 minutes after proposed treatment with std/samples, head-dipping behaviors were checked for 20 minutes\textsuperscript{20}.

4.5 CNS Inhibitory Activity- Actophotometer
The actophotometer was switched on and the animals were placed individually in the activity cage for 10 min. Standard, test and vehicle were injected in each animal of proposed groups and after 30 min. each animal was tested for 10 min. The locomotor activity after treatment was noted\textsuperscript{20}.

Statistical Analysis
Results are represented as Mean ± SEM. The test extract, standard and control were analyzed with the help of one-way analysis of variance (ANOVA) followed by Dunnett’s Test. P values < 0.05 were considered as statistically significant.
References:


