Chapter-6

MATERIALS AND METHODOLOGY
6. Materials and Methodology

Types of study

1. Analytical

2. Experimental

Four Classical types of Sahachara are explained by different Nighantu granthas, later on their respective botanical sources are given by different Dravyaguna Scholars in their books based upon that four classical Variety Root samples were collected from three different localities as mentioned in the below table.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Type of Sahachara</th>
<th>Latin Name</th>
<th>Place of Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peeta Sahachara</td>
<td><em>Barleria prionitis</em> Linn.</td>
<td>Pune, Kolhapur, Nagpur</td>
</tr>
<tr>
<td>2</td>
<td>Rakta Sahachara</td>
<td><em>Barleria gibsoni</em> Dalz</td>
<td>Matheran, Junnar, Belgaum</td>
</tr>
<tr>
<td>3</td>
<td>Shweta Sahachara</td>
<td><em>Barleria cristata</em> Linn.</td>
<td>Pune, Sawantwadi, Mumbai</td>
</tr>
<tr>
<td>4</td>
<td>Neela Sahachara</td>
<td><em>Barleria strigosa</em> Willd.</td>
<td>Belgaum, Vegurla, Matheran</td>
</tr>
</tbody>
</table>

Table 1 - Type of Sahachara along with Latin name and place of collection.

In recent Dravyaguna books *Barleria cristata* Linn.sp. was advocated to be taken as Shweta and Rakta Sahachara both, but practically *Barleria cristata* Linn.is having white flowers and not red so after discussing with senior taxonomist, *Barleria gibsoni* Dalz.sp. has been selected as Rakta Sahachara because *B. gibsoni* Dalz. Sp. is having pinkish red flowers which are more appropriate as Rakta Sahachara. Sample codification has been done for each locality sample of Sahachara for better analytical study purpose.

As mentoined in ayurveda literature, collection period of root samples has been advised in *Grishma Ritu* (summer season) but for the authentication purpose Sahachara flowering samples were required for herbarium preparation, for this reason root samples were collected in flowering seasons instead of *Grishma Ritu*.
6.1 Pharmacognostical Material

- For field collection of the samples cutter, magnifying lens, mercuric chloride powder, press board, blotting paper were used.
- Digital Camera having Optical zoom 10x used to capture the images of different Species.
- For Microscopy, Research Trinocular microscope having micrometer arrangement with calibrated eyepiece micrometer was used.

6.2 Experimental Material

**Animals** – Adult healthy forty two Albino Rats of wistar strain, Digital Vernier calliper, Mice gavage needle, weighing machine, Pair of gloves, Aspirin, Carrageenin, Stop watch.

![Figure 1 - Inj. Carrageenan](image-url)
Figure 2 - Aspirin Strip

Aspirin Gastro-resistant Tablets IP 50mg
Company: Zydus Healthcare

Figure 3 - Digital vernier caliper

Methodical collection of information, documenting the result and its analysis is of paramount importance in any new study. Four classical types of Sahachara has been studied under following headings:

Pharmacognostical Study
Physico-chemical Study
Phyto-chemical Study
Animal Experimentation

6.3 Pharmacognostical Study

Four classical variety Root samples of Sahachara were collected from three different localities, in order to compare the pharmacognostical parameters of the each species with each other. These four types of Sahachara were collected from following three different regions as mentioned in Table.no 22. Three different localities were selected as per W.H.O. guidelines for single herbal drug research.
6.3.1 Dravya Sangraha Vidhi

For the field collection of root samples of Barleria varieties from different localities senior taxonomist’s, local villagers helped had been taken for the occurrence of that particular Sahachara type in that particular region. As mentioned above root samples of Barleria Sp. were collected and they were dried in shed. In dried state also mites, ants, flies, insects and also other microorganism contaminate the sample so necessary precautions were taken. Root samples had been weighed before cleaning and drying. Root samples had been given gentle wash with normal water. After drying with the help of air blower remaining dust removed. Sample were weighed after drying and kept in air tight containers for further analysis.

6.3.2 Herbarium Preparation

A pharmacognostical study of a plant enables the scientist for proper identification of a drug and removes adulteration to provide a basis for authentication of crude drug. By looking the high traditional use of crude drug, herbariums were prepared from collected Barleria samples as per following method.

Preparation of herbaria was carried out by certain methods. There are some steps as follows-

1) Selection of material
2) Pressing process
3) Technique of pressing
4) Drying of pressed material
5) Mounting
6) Identification and labeling
7) Protection of prepared herbaria sheet.

1) Selection- The plant specimen collected was disease free, with all parts intact without any injuries or deformities. The plant was un-rooted; root was cleaned and gently washed. Plant twigs having leaves and flowers were collected.
2) **Pressing process**- After collection of the plant samples they were pressed immediately in the field condition. Witting of the plant material avoided.

3) **Technique of pressing** - Collected plant specimens were kept in newspapers. Sheets were arranged alternately by blotting paper sheets. These paper sheets were pressed. A wooden press was used. Spreading of plant material inside the sheets and weight on press was done carefully.

4) **Drying** - Blotting paper sheets were changed 2-3 times for proper soaking of moisture from the plant materials. Paper changing in the press was done carefully for 15-20 days by observing the condition of material.

5) **Mounting** - Good quality herbarium sheets were used for pasting or fixing material. Standard size herbarium sheets were used for mounting. Properly dried materials were fixed on the sheet by glue.

6) **Identification and labeling** - Labeling and identification was done. The identification information carries locality, botanical name, time of collection. The name of the collector is mentioned last.

7) **Protection** - Proper sanitation of storage condition was maintained. Mold, fungi, insects also create problem for herbaria sheet. Thoroughly dried and well ventilated warm conditions were maintained to save the samples from fungal infection.

6.3.3 **Authentication**

Authentication of the Root samples of *Barleria* species was carried out by botanical Survey of India, Regional Office Pune and Agharkar Research Institute, Pune. Voucher specimens were deposited in concerned institutes.
6.3.4 Microscopical Study

Freshly hand cut transverse sections of root were taken and then thoroughly washed with water and stained with 1% aqueous safranin and mounted on slide. The Anatomical Structures were observed under microscope. Images of these sections were taken.

6.4 Analytical Study and Standardization

Due to seasonal and geographical variation chemical composition of plants shows a great variation. These changes reflect in the bio-efficacy and credibility of drugs.

In the early times Vaidya’s were collecting the plants themselves and marketing the medicines as per requirement of the patients. Most of the time locally available plants were used for preparation.

This trend changed in 20th century. Now due to globalization, many herbal drugs, herbal preparations are imported into India and exported from India. This leads to many pharmaceutical development and increase demand of drug. At the same time due to industrialization there is depletion of forest, which is drug reserve. This position has led to short supply of genuine drugs, adulteration and substitute of drugs. Therefore, authentication and standardization of individual drugs with respective certain parameters which have been described in various pharmacopoeias and recently issued World Health Organization guidelines on the subject is an integral step before it is administered.

There are so many traditionally used drugs having good results in the management of the diseases but their analytical study and standardization procedures were not done before. To put forward them into this pharmaceutical world, there is need to analyze them as analytical parameters help in standardizing drug and gives us an idea of chemical constituents of plant and prove their efficacy according to modern standard parameters. Sahachara is one of the traditional drugs. Analytical study has been done as per following methods.

1) Organoleptic evaluation
2) Macroscopic and Microscopic evaluation
3) Physio-chemical study.
4) Phyto-chemical study
5) High Performance Liquid Chromatography.

6.4.1 Physio-chemical Study

The faulty collection or deterioration of the drug due to the incorrect or extended storage may alter the contact of the active constituents of the drug to a point where it is no longer acceptable. Drugs must therefore be submitted to evaluation procedures, which indicate acceptability by criteria other than their morphology. Following physico-chemical tests were carried out for the comparative analysis of four classical types of Sahachara.

1) Ash Value

Ash is a natural inorganic constituent of the entire herbal residue remaining after incineration.

Ash Value determination

1) The silica crucible was heated for about 10 min. on Bunsen burner.
2) The crucible was cooled and its weight was taken.
3) Accurately weighed 1gm of sample drug was took in the silica crucible and incinerated at 450°C temperature for 3 hours.
4) The crucible was transferred to desiccators for cooling.
5) The crucible was weighed accurately.

Formula = \( \frac{\text{Weight ash X 100}}{\text{Weight of powder}} = \% \text{ w/w} \)

i) Ash values -

Total ash :-

Method: 2 gm of ground air dried drug was taken in a previously weighed tarred silica crucible. It was incinerated in a muffle furnace at a high temperature not exceeding 450°C until the residue turned white, indicating absence of carbon. The carbon free residue was cooled in a desiccator and weighed. The percentage of ash was calculated with respect to the dried drug.


**ii] Extractive values –**

**Determination of Alcohol Soluble Extractive:** Macerated 5 g of the air dried drug, coarsely powdered, with 100 ml of Methanol of the specified strength in a closed flask for twenty-four hours, shaking frequently during six hours and allowed to stand for eighteen hours. Filtered rapidly, taking precautions for loss of solvent, evaporate 25 ml of the filtrate to dryness in a cleaned dried evaporating dish, and dry at 105°C, to constant weight and weighed. Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

**Determination of Water Soluble Extractive:**

Macerated 5 g of the air dried drug, coarsely powdered, with 100 ml of chloroform-water of the specified strength in a closed flask for twenty-four hours, shaking frequently during six hours and allowed to stand for eighteen hours. Filtered rapidly, taking precautions for loss of solvent, evaporate 25 ml of the filtrate to dryness in a cleaned dried evaporating dish, and dry at 105°C, to constant weight and weighed. Calculated the percentage of water-soluble extractive with reference to the air-dried drug.

**Moisture Content**

The moisture content is necessary to prevent the destruction of crude drug either due to chemical change or due to microbial contamination, in case of moisture sensitive drugs definite moisture content is necessary. Excessive moisture is considered as an adulteration because of its added weight. Moisture content also determines the amount of volatile substance of any kind.

\[
\text{Formula} = \frac{\text{weight loss}}{\text{Weight of powder}} = \% \text{ W/w}
\]

10 gm of sample was accurately weighted in a tarred evaporating dish. It was dried 105°C in an oven for 3 hrs. and weighed. Then the drying and weighing procedure at the interval
of ½ an hour was continued until the difference between two successive weights were found to be constant approximately. Loss in weight is expressed as % of moisture.

**iii) pH Value**: For determination of pH, the instrument was started for half an hour before taking readings. The instrument was calibrated with standard buffer solution (6 pH tablets) after calibration the electrodes were dipped in sample extracts and readings were noted.

### 6.4.2 Phyto-chemical Study

Phyto-chemical study plays an important role in the standardization of the crude drugs. Phyto-chemical evaluation of a particular crude drug is mainly carried out for the following purpose.

1) To screen the plant for detecting the presence of the various groups of compounds.

2) To quantitatively estimate the various groups of compounds from the plant.

3) To isolate one or more constituents responsible for particular activity.

**Determination of Phyto-chemical Constituents**

The preliminary Phyto-chemical tests were conducted on root samples of *Barleria* species collected from field by using the two different extractive mediums as Aqueous and Methanol.

1) **Test for Alkaloids**:

   Hager’s Test - 2-3 ml extract added with Hager’s reagent.

   Observation -: The test is considered to be positive with the appearance of yellow precipitate.

2) **Test for Glycosides**:
Foam test – 2 to 3 ml of extract was shaken vigorously with distilled water in a test tube. Observation: Honeycomb like foam produced, persists for few minutes. It confirms the presence of saponin.

3) Test for Saponins-

Foam test – 2 to 3 ml of extract was shaken vigorously with distilled water in a test tube. Observation: Honeycomb like foam produced, persists for few minutes. It confirms the presence of saponin.

4) Test for Flavonoids-

To 2ml of aqueous extract, add lead acetate solution.
Observation: Yellow colored precipitate formation indicates the presence of Flavonoids.

5) Test for Phenols

Phenol test - When 0.5ml of FeCl₃ solution was added to 2ml of test extract, formation of an intense colour indicates the presence of Phenols.

6) Test for Tannins and Phenolic Compound:

Add few drops of 5% FeCl₃ solution to 2-3 ml of aqueous extract.
Observation: Deep blue black color precipitate formation indicates presence of Tannins and phenolic compound

As all four classical variety samples collected from three different localities were more or less similar according to Pharma-cognostic, Physico-chemical and Phyto-chemical parameters. As Kwatha kalpana was used for animal experimentation, only one sample having highest water soluble extractive value among three locality samples of each Sahachara type was taken for further HPLC analysis and animal experimentation purpose. In Barleria prionitis Linn. root samples collected from three different localities, Pune region sample was having highest water soluble extractive value as compare to other two samples. Similarly, in Barleria gibsoni Dalz. group Matheran
sample, in *Barleria cristata* Linn. group Sawantwadi sample and in *Barleria strigosa* group Matheran sample had highest water soluble extractive values as compared to other two locality samples.

6.4.3 High Performance Liquid Chromatography (HPLC) analysis

Following HPLC method was preferred for analysis.

Standard Working Conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>C&lt;sub&gt;18&lt;/sub&gt; (150 x 4.6mm)</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Potassium dihydrogen phosphate buffer: Methanol (40:60)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>28°C</td>
</tr>
<tr>
<td>Pressure</td>
<td>Minimum 0; Maximum 5000 psi</td>
</tr>
<tr>
<td>Detection</td>
<td>UV at 210 nm.</td>
</tr>
</tbody>
</table>

**Sample Preparation:**

250mg of fine powder sample was accurately weighed in a 25ml volumetric flask. This was then dissolved in a mixture of (50:50) Methanol: Water and the final volume adjusted to 25ml. These flasks were sonicated for 30minutes. The contents were then filtered and used for injection. Initially the mobile phase was allowed to run to stabilize the column. 20µl of the solutions were injected. The corresponding areas were noted.

6.5 Experimental Methodology

**Study Center:** National Toxicological Center, Pune.

**Preparation of animals -**

- Animals :- Wistar strain male
- Body weight:-Adult albino rats having average body weight 150-200 gms.
- At the commencement of the study, the weight variation of the animals was minimal and not exceeded ±20% of the mean weight for each sex.
- The temperature of the experimental animal room was maintained up to 22<sup>0</sup>C (±3<sup>0</sup>C)
• The relative humidity of the room was at least 30% & preferably not exceeded 70% other than during room cleaning.
• Lighting of the room was artificial, the sequence being 12 hours light & dark cycle.
• Balanced commercially available animal feed was provided to animals.
• Potable drinking water was being given *ad libitum*.
• The animals were randomly selected, marked to permit individual identification.
• Animals were kept in the cages at least five days prior to the start of the test to allow for acclimatization to the laboratory conditions.
• Animals were fasted prior to dosing overnight, however drinking water was given *ad libitum*.
• After the dose administration the feed was given after 3-4 hours.

**Acute Inflammatory Model**

The apparatus used for the measurement of rat paw volume was developed by Buttle et al and modified by Sing and Ghosh (Method of Winter et al) Carrageenan induced hind paw edema method was followed. Standard and Test drugs were administered 1 hr prior to induction of edema. Edema was measured at 0, 30, 60 and 120, 240 min. & 24 hr. intervals.

**Drug Preparation**

Kwatha of four varieties of Sahachara was prepared as per the guidelines mentioned in Sharangadhar Samhita. The drug was administered orally in the form of freshly prepared decoction with 1 part of drug and 16 parts of water reduced to 1/8 part as per the text Sharangadhara Samhita.

**Selection of the Animals**

Male albino rats with a body weight between 150 - 200 gms, bred in the animal house at National Toxicological Centre, Pune were used as experimental purpose. The rats were selected and grouped into seven groups of six rats each. The animals were administered the drug as per the following table.
<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Details of the group</th>
<th>Dose Administration Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A- Normal Control group</td>
<td>Distilled water as required</td>
</tr>
<tr>
<td>2</td>
<td>B- Disease Control Group</td>
<td>Inj. carrageenan (0.05 ml of 1% sol.)</td>
</tr>
<tr>
<td>3</td>
<td>C- Standard Control group</td>
<td>Standard drug Aspirin (100 mg/kg Body wt.)</td>
</tr>
<tr>
<td>4</td>
<td>D- Test group - <em>Peeta Sahachara moola kwatha</em></td>
<td>As per the animal dose calculation formula.</td>
</tr>
<tr>
<td>5</td>
<td>E- Test group - <em>Rakta Sahachara moola kwatha</em></td>
<td>As per the animal dose calculation formula.</td>
</tr>
<tr>
<td>6</td>
<td>F- Test group - <em>Shweta Sahachara moola kwatha</em></td>
<td>As per the animal dose calculation formula.</td>
</tr>
<tr>
<td>7</td>
<td>G- Test group - <em>Neela Sahachara moola kwatha</em></td>
<td>As per the animal dose calculation formula.</td>
</tr>
</tbody>
</table>

Table 2 - Experimental groups.

**Dosage:**

Each Animal dose was calculated by using Conversion formula by Paget and Branes (1964).

\[
\text{Total Clinical Dose} \times 0.018 \\
= 80 \text{ ml} \times 0.018 \\
= 1.44 \text{ ml}
\]

The volume of the injected paw was measured by Digital Vernier Caliper immediately after carrageenan injection and again at the interval of 0, 30, 60, 120, 240 min and 24 hours readings of the paw volumes were noted.