MATERIALS AND METHODS

3.1 Collection of Plants

Botanical survey was arranged in Parbhani district to collect medicinally important plant species of family Cucurbitaceae in flowering and fruiting period. The collected plants were identified with the help of floras Naik, (1979); Naik, et al., (1998), Singh and Kartekeyan (2001) and Yadav and Sardesai (2002). The plant specimens were authenticated by Botanical Survey of India, Pune. The voucher specimens in duplicate were deposited at Botany Research Centre, Dnyanopasak College Parbhani.

Five plants were selected for the confirmation of proclaimed ethno medicinal claims by using modern system like pharmacognosy, preliminary phytochemical screening, microbial assays etc for their validation.

<table>
<thead>
<tr>
<th>Name of the plant</th>
<th>BSI V. No</th>
<th>Place of collection</th>
<th>Collected by</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coccinia grandis</em> (L.) Voigt.</td>
<td>SARCOG2</td>
<td>Rani Sawargaon</td>
<td>Miss. S. R. Rodge</td>
</tr>
<tr>
<td><em>Lagenaria siceraria</em> (Molina) Standl.</td>
<td>SARLAS6</td>
<td>Pokharni</td>
<td>Miss. S. R. Rodge</td>
</tr>
<tr>
<td><em>Trichosanthes tricuspidata</em> Lour.</td>
<td>SARTT1</td>
<td>Landkwadi</td>
<td>Miss. S. R. Rodge</td>
</tr>
<tr>
<td><em>Diplocyclos palmatus</em> (L.) Jeffery</td>
<td>SARDIP3</td>
<td>Parbhani</td>
<td>Miss. S. R. Rodge</td>
</tr>
<tr>
<td><em>Cucumis setosus</em> Cogn.</td>
<td>SARCUS8</td>
<td>Sadgirwadi</td>
<td>Miss. S. R. Rodge</td>
</tr>
</tbody>
</table>

The collected plants were cleared from dust. The leaves, stem, flower, fruits, and seeds were separated and dried in shadow. The dried material was finely powdered, sieved through muslin cloth and stored for chemical and other physicochemical analysis. Fresh parts were preserved in FAA for anatomical studies.

3.2 Pharmacognostical Study

Pharmacognostic study was performed as per Rangari (2003) and Kokate et al., (2000) and anatomical studies as per Metcalfe and Chalk (1950).
3.2.1 Anatomical Study

The anatomy of leaves and stem was done by free hand sectioning method. The sections were stained by using double stained differential staining technique (Johanson, 1940) and mounted in DPX. Photographs of anatomical details were taken with help of digital camera.

3.2.2 Quantitative microscopy of leaves

The vein islet number, stomatal number, stomatal index and vein termination number, palisade ratio were calculated. The sample treated with 5% KOH solution used for microscopic study of leaf architecture, fresh/preserved material was cleared by boiling with chloral hydrate solution. Peels of upper and lower epidermis were taken separately by forceps for the study of stomatal number, stomatal complex and trichome complex.

a) Determination of stomatal number and stomatal index

Fresh leaf material was used for determination of stomatal number, stomatal index. The epidermis is peeled off, the peels were stained with 1% saffranin, mounted in glycerine and made semi permanent by ringing with DPX mountant. Stomatal index was calculated by formula as stomatal Index (SI) = S/ (E+S)× 100.

Where, ‘S’ is the number of stomata per unit area and ‘E’ is the number of epidermal cells in that area. The epidermal cell frequency (ECF) and stomatal frequency (SF) were calculated for leaf upper and lower surfaces. Stomatal indices were calculated in the hypostomatic leaves. (Salisbury 1927). Stomatal and epidermal cell frequencies were calculated for each square millimetre.

b) Determination of palisade ratio

Palisade ratio is the average number of palisade cells beneath an epidermal of cells of a leaf, the ratio (PR), was calculated as the average of palisade cells(P) beneath each epidermal cells (E) and determined as PR=P/E.

c) Determination of vein islet number

Small areas of the green tissues outlined by the veinlets are termed as vein islets or areoles. The vein islets number is defined as the average number of vein islet per mm of the leaf surface midway between midrib and margins. It was determined by counting the number of vein islets in an area of 439 mm of the central part of the leaf between the midrib and margin.
d) **Determination of vein let termination number**

A vein termination is ultimate free termination of veinlet. The number of veinlet terminations present within the square was counted and the average of number of veinlet termination number from the four adjoining squares was taken to get the value for one square mm.

**e) Trichome**

Peels of upper and lower epidermis of leaf were used for study of trichome; the peels were stained with 1% saffranin, mounted in glycerine and made semi-permanent by ringing with DPX mountant. The photographs of trichomes were taken with the help of digital camera.

### 3.2.3 Stem vessels

The slices of stem were macerated by using Jeffery's fluid. It is a mixture of 10% nitric acid and 10% potassium dichromate solution in equal proportion. The vessel elements were stained with aqueous 1% saffranin for 30 minutes. There after excess stain is removed by washing with water then mounted in glycerine and ringing with DPX. The photographs were taken using digital camera.

### 3.3 Preliminary phytochemical screening

**Extraction Method**

The dried material was finely powdered, sieved through muslin cloth and used for chemical analysis. The extraction of plant drug was done through soxhlet apparatus. Qualitative phytochemical analysis was performed by using plant extract according to Johansen (1940), Gurr (1965), Harbone (1984), Edeoga *et al.*, (2005) and Daniel (1991).

1. **Tests for alkaloid**

   a) **Dragendroff’s Test**

   2g of sample was stirred with few drops of hydrochloric acid and tested with Dragendroff’s reagent; appearance of orange precipitation indicates the presence of alkaloids.

   b) **Wagner’s reagent Test**

   2g of sample was stirred with few drops of hydrochloric acid and tested with Wagner’s reagent appearance of reddish brown precipitation indicates presence of alkaloids.
c) Meyer’s reagent Test

2g of sample was stirred with few drops of hydrochloric acid and treated with a few drops of Meyer’s reagent and observed for the appearance of turbidity or yellow precipitation.

2. Test for flavonoids
a) Alkaline Reagent Test

Extract was treated with few drops of sodium hydroxide solution a yellow colour appears, which becomes colourless on addition of dilute HCl acid, indicates presence of flavonoids.

b) Lead acetate test

Extract was treated with few drops of lead acetate solution formation of yellow coloured precipitation indicates the presence of flavonoids.

c) NaoH test

Water extract sample was reduced in water bath. The residue was treated with dilute NaoH along with dilute HCl, solubility and colouration was noted. A yellow coloured precipitation was appeared and it turns colourless with the addition of dilute HCl confirms the flavonoids.

d) Shinda’s test

A sample was dissolved in alcohol with a piece of magnesium, then addition of conc. hydrochloric acid was added drop-wise and heated. Appearance of pink, crimson red, green to blue colour shows the presence of flavonoids.

3. Test for Triterpenoids
a) Liebermann’s Test

Treat the sample with few drops of acetic anhydride, boil and cool, then add conc. Sulphuric acid from one side of the test tube, formation of deep red coloured indicates presence of triterpenoids.
b) Salkowski Test

Treat the sample with few drops of conc. sulphuric acid formation of yellow coloured lower layer indicates presence of triterpenoids.

4. Cardiac Glycoside Test
Keller-Killani Test

50gm of sample was dissolve in 2ml of chloroform and sulphuric acid, formation of brown ring at interphase appears which confirms the presence of cardiac glycoside.

5. Test for Steroids

2ml of acetic anhydride was added to 0.5gm extract with 2ml sulphuric acid formation of violet to blue or green colour indicates presence of steroids.

6. Test for Saponins

Foam Test

Small amount of extract was shaken with little amount of water, if foam produced and persists for ten minutes it indicates the presence of saponins.

7. Test for Tannins

a) Ferric chloride Test

A small quantity of the sample is taken along with water and few drops of 5% ferric chloride solution. The colour changes to blue shows presence of tannins.

3.4 Detection of inorganic constituents

In freshly prepared ash of plant with 50%v/v HCl kept for 1-2 hours and the filtrate then used for chemical tests for detection of inorganic constituents.

1. Test for Calcium

10ml filtrate, one drop dil. NH₄OH with saturated ammonium oxalate solution gives white precipitate of Calcium oxalate. The precipitate is soluble in HCl but insoluble in acetic acid which confirms the presence of Calcium.

2. Test for Magnesium

White precipitate of calcium oxalate obtained by above method was filtered and the filtrate was heated and cooled. In this filtrate, solution of sodium phosphate
and dilute ammonia were added which gives white crystalline precipitate.

3. **Test for Sodium**

In 10ml of ash extract 2ml of potassium pyroanthololate was added the formation of white precipitate indicates presence of Sodium.

4. **Test for potassium**

In 2-3.ml of extract solution few drops of sodium cobalt nitrate solution was added. The formations of yellow precipitate of potassium cobalt nitrate confirm the presence of Potassium.

5. **Test for Iron**

5ml extract solution with few drops of 2% potassium ferrocyanide gives dark blue colouration which indicates the presence of Iron.

3.5 **Physico chemical analysis**

a) **Determination of moisture of crude drug**

About 1.5 g powdered drug was weighed in thin porcelain dish (I w) and dried in oven at 100\(^\circ\)c to 105\(^\circ\)c cooled in desiccators and weighed. The dry weight was taken (D W). Moisture content was determined by the formula:

\[
\text{Moisture contents} = \frac{(I\ W - D\ W)}{D\ W} \times 100.
\]

b) **Determination of Ash values of Crude drug**

Ash values are used to determine quality and purity of a drug. Ash contains inorganic radicals like phosphorus, carbonates and silicates of sodium, potassium, magnesium, calcium etc. Sometimes inorganic variables like calcium, oxalate, silica, carbonate contents of the crude drug affects total ash value, such variables are then removed by treating with acid, as they are soluble in HCl, and then acid insoluble ash value is determined.

c) **Determination of total ash value**

10 gm powdered drug incinerated in silica crucible over the burner. The charred material was heated up to 500 -600 \(^\circ\)c for six hours in furnace. The residue remaining after incineration of sample is of known as ash. The crucible is cooled in desiccators. The percentage of total ash content was calculated with reference to the air dried sample of the dried drug.
Where, $X =$ weight of empty petridish, $Y =$ weight of used crude drug, 
$Z =$ weight of ash along with petridish

Weight of total ash $(A) =$ weight of ash along with petridish $[Z]-$Weight of empty petridish $[X]$  
$A = [Z - X]$ gm

`$y$ gm of the crude drug gives $[Z – X]$ gm of the ash
100gm of the crude drug gives
100$X \ [Z – X]$ gm of the sample = 100 $[Z – X] \ \%$.

**d) Determination of Acid – Insoluble Ash**

The ash was boiled for five minutes with 25ml of dilute hydrochloric acid. Insoluble matter was collected in the crucible or on an ash less filter paper and washed with hot water, ignited and weighed. Percentage of acid insoluble ash was calculated with reference to the air dried drug by formula given as follows.

$X =$ weight of empty petridish.
$Y =$ weight of used crude drug.
$Z =$ weight of ash along with petridish.

Weight of total ash $[A] =$ weight of ash along with petridish $[Z] –$Weight of empty Petridish $[X]$

`$y$ gm of the crude drug gives $[Z – X]$ gm of the ash.
100gm of the crude drug gives
100$X \ [Z – X]$ gm of the ash.

`$a$ gm =weight of residue [acid insoluble ash].

`$y$ gm of the air dried gives a gm of acid insoluble ash

Acid insoluble ash value of the sample = 100 $x \ a / y \ \%$.

**e) Determination of water soluble ash**

The ash was boiled for five minutes with 25ml distilled water. Insoluble matter was collected on filter paper and washed with hot water ignited and weighed. Weight of the insoluble matter was subtracted form the weight of ash. The difference in weight represents the water soluble

Percentage of water soluble ash was calculated with reference to the air dried drug. It is determines in a similar way to acid insoluble ash, using 25ml of water, in place of dilute hydrochloric acid.
f) Determination of extractive values

Extractive values of drug plant material are useful for the evaluation of a crude drug. It gives idea about the nature of the chemical constituents present in the crude drug. It is also useful for the estimation of specific constituents, soluble in the particular solvent used for extraction.

g) Determination of water soluble executive

5g of coarsely powdered drug was macerated with 100 ml of distilled water in conical flask of 250 for 24 hours with frequent shaking solution was filtered and 25ml of filtrate was evaporated in a weighed, thin porcelain dish. Evaporate to dryness on water bath and complete the drying in and oven at 100’c cooled in desiccators and weighed. The percentage of extractive was calculated with reference to the air dried drug.

Calculations

25ml of water extract gives – X gm of residue.
100ml of water extract gives= 4x g of residue.
Since, 5gm of air – dried drug gives = 4 xg of water soluble residue.
Therefore 100g of air dried gives – 80 x g of water soluble residue.
Water soluble extractive value of the sample = 80 X%.

h) Determination of alcohol soluble extractive

5gm of coarsely powdered drug was macerate with 100ml. of alcohol in closed flask of 25mlfor 24h with frequent shaking. It was filtered rapidly taking precautions against loss of alcohol. 25ml of filtrate was then evaporated in a weighted, thin porcelain dish and then dried at 100°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried drug.

Calculation

25ml of alcoholic extract gives = X g of residue.
100ml. of alcoholic extract gives = 4 x gm of residue.
Since, 5gm of air dried drug gives = 4 x g of alcohol. [90%] Soluble residue.
Therefore, 100g of air - dried drug
gives = 80 X g. of alcohol soluble residue.
Alcohol soluble extractive value of the sample = 80X%..
3.6 Determination of minerals by Atomic Absorption Spectroscopy

Trichloroacetic acid digestion: 0.5g ash was weighed and digested in mixture of nitric acid and perchloric acid (5:1). After digestion few drops of concentrated HCl was added. The solution was heated gently and filtered. The entire filtrate diluted with deionised water. Dilute filtrate solution was used for analysis of minerals by Atomic absorption spectrometer. It was performed at Department of Soil Science, Vasantrao Naik, Marathwada Agriculture University, Parbhani.

Mineral in % = Reading x 1/ Wt of Sample x1/ aliquot x Volume of extract /10000

Volume of extract: 50ml
Wt of sample: 0.5g
Aliquot: 10ml